13.4.00

INCREASING THE THERMOSTABILITY OF BARLEY (1→3,1→4)-β-GLUCANASES

Richard John Stewart

BSc (Hons)

December 1999

A thesis submitted in fulfillment of the requirements

for the degree of Doctor of Philosophy

Department of Plant Science

Faculty of Agricultural and Natural Resource Sciences

The University of Adelaide, Waite Campus

Glen Osmond 5064

South Australia

STATEMENT OF AUTHORSHIP

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis presented by me for another degree or diploma. No other person's work has been used without due acknowledgment in the main text of this thesis. This thesis has not been submitted for the award of any other degree or diploma in any other tertiary institution. I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Richard John Stewart

December 1999

PUBLICATIONS

Stewart, R.J., Garrett, T.P.J., Varghese, J.N., Høj, P.B., and Fincher, G.B. (1996) 'Protein engineering of enzymes for improved malting quality of barley'. *Proceedings of the VII International Barley Genetics Symposium, Saskatoon, Canada.*

Stewart, R.J., Qureshi, J., Burton, R.A., Garrett, T.P.J., Varghese, J.N., Høj, P.B., and Fincher, G.B. (1997) 'Transient expression of a thermostable barley (1→3,1→4)-β-glucanase in barley immature embryos'. *Proceedings of the 8th Australian Barley Technical Symposium, Gold Coast, Australia.*

Stewart, R.J., Colman, P., Varghese, J.N., Garrett, T.P.J., Høj, P.B., Chen, L., and Fincher, G.B. (1995) 'The use of site-directed mutagenesis to enhance the thermostability of barley (1→3,1→4)-β-glucanases'. *Proceedings of the 45th Australian Cereal Chemistry Conference, Adelaide, Australia.*

Patent

Chen, L., Colman, P.M., Fincher, G.B., Garrett, T.P.J., Høj, P.B., **Stewart, R.J.,** and Varghese, J.N. '(1→3,1→4)-β-Glucanases of enhanced thermostability'. International patent application number PCT/AU/00377. Lodged 6th July, 1994; Granted in Australia, 1998.

ACKNOWLEDGMENTS

I am particularly indebted to Professor Geoff Fincher for his invaluable guidance, inspiration and perseverance. I also extend my sincerest gratitude to Professor Peter Høj who has offered unconditional support and friendship throughout my candidature.

My sincere thanks are due to the current and past members of the Fincher Laboratory for their constant support and tolerance. In particular, I would like to acknowledge the trio of Andrew Harvey, Rob Lee, and Ron Osmond for providing friendship and moral support, whether at work or at play. Many thanks are also extended to Drs Mitali Banik, Rachel Burton, David Gibeaut, Maria Hrmova and Peilin Xu for their invaluable technical advice and support during this work. Special thanks are also due to Rohan Singh and Dr Javed Qureshi of the barley transformation group for their technical assistance and advice. I would also like extend a general 'thank you' to the staff and students of the Department of Plant Science for providing a pleasant working environment.

The invaluable assistance of Drs Jose Varghese and Tom Garrett (Biomolecular Research Institute) with various molecular modelling aspects of this work is gratefully acknowledged. The ongoing support of Professor Peter Colman from the BRI is also much appreciated. The financial support of the Grains Research and Development Corporation is also acknowledged.

Finally, this thesis would not have been completed without the uncompromising support of my family Rhondda, Gavin, and Greg, who all helped in their own special way, and Jane, who provided me with great encouragement and inspiration through good times and bad. Finally, thanks to Bob Dylan for providing four decades of creative lyrics and music to relax and inspire the mind.

ABBREVIATIONS

The universal one-letter and three-letter codes for amino acids were used throughout this thesis. Standard abbreviations are used without definition and non-standard abbreviations are defined below:

Å	Angstrom
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
COOH-terminus	Carboxy-terminus
^{o}C	Degrees centigrade
dATP	2` Deoxyadenosine 5`-triphosphate
dCTP	2` Deoxycytidine 5`-triphosphate
dGTP	2` Deoxyguanosine 5`-triphosphate
dTTP	2` Deoxythymidine 5`-triphosphate
Da	Dalton
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DNase	Deoxyribose endonuclease
DP	Degree of polymerisation
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticulum
GA_3	Gibberellic acid
HPLC	High performance liquid chromatography
IPTG	Isopropylthio-β-D-galactoside
kb	Kilo bases

kDa	Kilo Daltons
LB	Luria-Bertani
mA	Milliampere
M_r	Molecular mass
mRNA	Messenger RNA
NH_2-terminus	Amino-terminus
Ni-NTA	Nickel-nitro-tri-acetic-acid
Ori	Origin of replication
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pI	Isoelectric point
PMSF	Phenyl methyl sulphonyl fluoride
PR	Pathogenesis related
rms	Root mean square
RNA	Ribonucleic acid
RNAse	Ribonucleic endonuclease
RT-PCR	Reverse transcriptase-PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSC	Sodium saline citrate
T_m	Melting temperature
Tris	Tris (Hydroxymethyl) aminomethane
TPBS	Phosphate-buffered saline with Tween 80
UTR	untranslated region
X-gal	5-Bromo-4-chloro-3-indoyl β-D-galactopyranoside

ABSTRACT

Germinated barley provides the raw material for the malting and brewing industries. In the germinated grain, hydrolytic enzymes are secreted from the aleurone and scutellum into the starchy endosperm where they participate in the breakdown of the starch and protein reserves, and release fermentable products which are subsequently utilised in the brewing process. However, the starchy endosperm cell walls represent a physical barrier to the free diffusion of the starch and protein hydrolases to their substrates, and degradation of the starchy endosperm cell walls is therefore an important prerequisite for the efficient mobilisation of endospermic reserves. The starchy endosperm cell walls are composed mainly of (1→3,1→4)-β-glucans, and the enzymes primarily responsible for the initial degradation of these polysaccharides are the barley (1→3,1→4)-β-glucan endohydrolases.

Because of their propensity to form solutions of high viscosity, undegraded (1→3,1→4)-β-glucans in the malt can cause serious filtration problems during brewing and can also contribute to the formation of precipitates and hazes in the beer. Barley (1→3,1→4)-β-glucanases are rapidly inactivated at the elevated temperatures used during the kilning and mashing processes. Thus, if the thermostability of barley (1→3,1→4)-β-glucanases could be increased, more of the enzyme would survive the mashing and kilning processes and high molecular weight (1→3,1→4)-β-glucans would be more extensively degraded. In this study, two approaches were used in attempts to increase the thermostability of barley (1→3,1→4)-β-glucanases. The first approach involved comparing the structure of barley (1→3,1→4)-β-glucanase isoenzyme EII with that of a related barley enzyme, (1→3)-β-glucanase isoenzyme GII. Barley (1→3)-β-glucanase isoenzyme GII is a PR protein which plays a role in plant defence, and is significantly more thermostable than barley (1→3,1→4)-β-glucanase isoenzyme EII. A three-dimensional structural comparison of the two enzymes reveals that their different substrate specificities are due to a limited number of amino acid substitutions in their substrate binding grooves. Thus, an attempt was made

to produce a more heat-stable (1→3,1→4)-β-glucanase by changing the specificity of barley (1→3)-β-glucanase isoenzyme GII to that of a (1→3,1→4)-β-glucanase. Four single amino acid substitutions were introduced into the substrate binding groove of barley (1→3)-β-glucanase isoenzyme GII using site-directed mutagenesis. These mutations decreased the specific activity of the enzyme but did not change its substrate specificity.

The second approach to producing a thermostable barley (1→3,1→4)-β-glucanase again involved site-directed mutagenesis, but in this case a number of rational amino acid substitutions were introduced into barley (1→3,1→4)-β-glucanase isoenzyme EII itself. The amino acid substitutions were based on a detailed structural analysis of barley (1→3,1→4)-β-glucanase isoenzyme EII, together with our knowledge of the factors which affect protein stability. Three of the resulting mutant enzymes showed increased thermostability. The largest increase in stability was observed when the histidine at position 300 was changed to a proline (mutant H300P), which effectively decreases the entropy of the unfolded state of the enzyme. The three amino acid substitutions which increased the thermostability of barley (1→3,1→4)-β-glucanase iosenzyme EII were all located in the COOH-terminus loop of the enzyme. Thus, it was proposed that this loop represents a particularly unstable region of the enzyme, and could be involved in the initiation of unfolding of the enzyme at elevated temperatures. The NH_2- and COOH-termini of barley (1→3,1→4)-β-glucanase isoenzyme EII are in close proximity, which allowed the termini to be fused in an attempt to stabilise the COOH-terminal loop region. However, the resulting mutant enzyme was inactive.

Finally, the gene encoding the thermostable barley (1→3,1→4)-β-glucanase mutant H300P was stably integrated into the genome of barley callus cells. To date, attempts to regenerate vegetative tissues from the transgenic callus have been unsuccessful. In addition, the cDNA encoding thermostable mutant enzyme was transiently expressed in barley immature embryos under the control of the constitutive rice actin promoter. The transiently expressed mutant enzyme was active, which indicated that it was expressed and processed correctly in barley cells.

TABLE OF CONTENTS

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Anatomy and Composition of the Barley Grain 1

1.2 Barley Cell Wall Structure and Composition 3

1.2.1 Cell Wall Constituents 4

1.2.2 (1→3,1→4)-β-Glucan 4

1.2.3 Arabinoxylan 5

1.3 Reserve Mobilisation in the Germinated Barley Grain 6

1.3.1 Endosperm Cell Wall Degradation 7

1.3.2 Hydrolysis of Starch and Protein Reserves 10

1.4 Barley (1→3)-β-Glucanases 12

1.5 Barley (1→3,1→4)-β-Glucans in Industry 14

1.5.1 The Malting Process 14

1.5.2 Brewing 17

1.5.3 The Stockfeed Industry 20

1.6 Overcoming the Problems Associated with (1→3,1→4)-β-Glucans 21

1.6.1 Using Selective Breeding to Reduce (1→3,1→4)-β-Glucan Levels 22

1.6.2 Reducing the Synthesis of (1→3,1→4)-β-Glucans 22

1.6.3 Increasing (1→3,1→4)-β-Glucanase Gene Expression 23

1.6.4 Thermostable (1→3,1→4)-β-Glucanases 24

1.6.5 Increasing the Thermostability of Barley (1→3,1→4)-β-Glucanases 24

1.7 Aims of this Study 26

CHAPTER TWO: HETEROLOGOUS EXPRESSION OF BARLEY (1→3)- AND (1→3,1→4)-β-GLUCANASES

2.1 Introduction 29

2.1.1 Utilising E. coli *for the Expression of Barley β-Glucanases* 33

2.1.2 The pET Expression System 34

2.2 Materials and Methods 36

2.2.1 Materials 36

2.2.2 Construction of Expression Vectors 36

2.2.3 Expression of Recombinant Fusion Proteins 41

2.2.4 Purification of Recombinant Fusion Proteins 41

2.2.5 Removal of the Polyhistidine Tag with Thrombin 42

2.2.6 SDS-PAGE 43

2.2.7 (1→3)- and (1→3,1→4)-β-Glucanase Activity 43

2.3 Results and Discussion 45

2.3.1 Construction of Expression Plasmids 45

2.3.2 Expression and Purification of Recombinant Fusion Proteins 45

2.3.3 (1→3,1→4)- and (1→3)-β-Glucanase Activity and Kinetics 47

2.3.4 Removal of the Polyhistidine Tag with Thrombin 48

2.4 Summary and Conclusions 50

CHAPTER THREE: ALTERING THE SUBSTRATE SPECIFICITY OF BARLEY (1→3)-β-GLUCANASE ISOENZYME GII

3.1 Introduction 51

3.2 Materials and Methods 54

3.2.1 Materials 54

3.2.2 Analysing the Substrate-binding Grooves of Barley β-Glucanases 54

3.2.3 Site-Directed Mutagenesis 54

3.2.4 Expression and Purification of (1→3)-β-Glucanase Fusion Proteins 56

3.2.5 (1→3)- and (1→3,1→4)-β-Glucanase Activity 57

3.3 Results and Discussion 58

3.3.1 Proposed Amino Acid Substitutions for Changing Substrate Specificity 58

3.3.2 Site-Directed Mutagenesis 59

3.3.3 Production of Recombinant Wildtype and Mutant (1→3)-β-Glucanases 59

3.3.4 Substrate Specificity of Mutant (1→3)-β-Glucanases 60

3.4 Summary and Conclusions 61

CHAPTER FOUR: USING PROTEIN ENGINEERING TO INCREASE THE THERMOSTABILITY OF BARLEY (1→3,1→4)-β-GLUCANASE ISOENZYME EII

4.1 Introduction 63

4.1.1 Factors Affecting Protein Stability 63

4.1.2 The Intrinsic Thermostability of Plant (1→3)-β-Glucanases 67

4.1.3 Enhancing the Thermostability of Barley (1→3,1→4)-β-Glucanases 68

4.2 Materials and Methods 70

4.2.1 Materials 70

4.2.2 Amino Acid Substitutions for Enhancing Thermostability 70

4.2.3 Rationalising Amino Acid Substitutions 71

4.2.4 Site-Directed Mutagenesis 73

4.2.5 Expression and Purification of Mutant (1→3,1→4)-β-Glucanases 75

4.2.6 Thermostability, pH Stability, and Kinetic Parameters 75

4.2.7 Simulated Mashing at 65°C 75

4.3 Results and Discussion 77

4.3.1 Introduction of Mutations using Site-Directed Mutagenesis 77

4.3.2 Expression and Purification of Mutant (1→3,1→4)-β-Glucanases 77

4.3.3 Thermostability of Mutant (1→3,1→4)-β-Glucanases 78

4.3.4 Clustering of Amino Acid Substitutions which Increase Thermostability 81

4.3.5 Performance of Mutant H300P during Simulated Mashing at 65°C 82

4.4 Summary and Conclusions 84

CHAPTER FIVE: CIRCULAR PERMUTATION OF BARLEY (1→3,1→4)-β-GLUCANASE ISOENZYME EII

5.1 Introduction 85

5.2 Materials and Methods 87

5.2.1 Materials 87

5.2.2 Designing a Circular Permutation 87

5.2.3 Creating a Circular Permutation at the cDNA Level 88

5.2.4 Expression and Purification of (1→3,1→4)-β-Glucanase NC-F 89

5.2.5 Western Blot Analysis of (1→3,1→4)-β-Glucanase NC-F 90

5.2.6 HPLC Purification and NH_2-Terminal Protein Sequencing of NC-F 90

5.3 Results and Discussion 92

5.3.1 Creating a Circular Permutation 92

5.3.2 Expression and Purification of (1→3,1→4)-β-Glucanase NC-F 92

5.3.3 Western Blot Analysis of Recombinant (1→3,1→4)-β-Glucanase NC-F 93

5.3.4 HPLC Purification and Protein Sequencing of NC-F 93

5.4 Summary and Conclusions 95

CHAPTER 6: TRANSFORMATION OF BARLEY WITH THE GENE ENCODING (1→3,1→4)-β-GLUCANASE MUTANT H300P

6.1 Introduction 96

6.1.1 Cereal Transformation 96

6.1.2 DNA Delivery Methods 96

6.1.3 Reporter Genes and Selectable Markers 99

6.1.4 Plant Tissue Culture and Regeneration 100

6.1.5 *Producing Transgenic Barley for Industry* 101

6.2 Materials and Methods 103

6.2.1 Materials 103

6.2.2 Plasmid pE2HPg: The Gene Construct 103

6.2.3 Plasmid pE2HPc: The cDNA Construct 104

6.2.4 *Plasmid pCAM/HPg: The* Agrobacterium *Construct* 105

6.2.5 *Microprojectile Bombardment* 105

6.2.6 Tissue Culture and Selection 107

6.2.7 Genomic DNA Isolation and Southern Blot Analysis 107

6.2.8 PCR Analysis of Transgenic Tissue 109

6.2.9 Analysis of Transient Expression using RT-PCR 110

6.2.10 Western Blot Analysis 111

6.2.11 Viscometric Determination of (1→3,1→4)-β-Glucanase Activity 111

6.3 Results and Discussion 113

6.3.1 Preparation of DNA Constructs for Barley Transformation 113

6.3.2 Stable Transformation of Barley Cells with Plasmid pE2/HPg 114

6.3.3 Transient Expression of Plasmid pE2/HPc in Immature Embryos 116

6.3.4 Agrobacterium-*mediated Transformation* 119

6.4 Summary and Conclusions 121

CHAPTER SEVEN: SUMMARY AND FUTURE DIRECTIONS

7.1 Summary of Work Described in this Thesis 122

7.2 Further Enhancing the Thermostability of Mutant H300P 124

7.3 Improving Barley Transformation 127

7.4 Transgenic Barley: The Final Product 131

APPENDIX 1 132

REFERENCES 133

CHAPTER ONE:

GENERAL INTRODUCTION

The overall aim of the work described in this thesis was to increase the thermostability of barley (1→3,1→4)-β-glucanases. These enzymes are involved in endosperm cell wall degradation during reserve mobilisation in the germinated grain, where they catalyse the hydrolysis of (1→3,1→4)-β-glucan, a major cell wall constituent. Both (1→3,1→4)-β-glucans and the enzymes which hydrolyse them are particularly important in the malting, brewing, and stockfeed industries. Because of their tendency to form aqueous solutions of high viscosity, the (1→3,1→4)-β-glucans are generally considered troublesome in these industrial processes. The production of high levels of (1→3,1→4)-β-glucanases in the germinated grain is desirable because their action in depolymerising (1→3,1→4)-β-glucans leads to a significant reduction in solution viscosity. However, industrial processes are normally undertaken at elevated temperatures, and the barley (1→3,1→4)-β-glucanases are rapidly inactivated at these temperatures. For this reason, increasing the thermostability of barley (1→3,1→4)-β-glucanases would be considered advantageous for commercial applications.

In this General Introduction, the major constituents of the barley grain are described and an overview of the germination process is presented, with an emphasis on cell wall degradation. The roles of barley (1→3,1→4)-β-glucans and (1→3,1→4)-β-glucanases are discussed in an industrial context, and several approaches for increasing (1→3,1→4)-β-glucanase activity during the industrial processing of barley grain are proposed.

1.1 Anatomy and Composition of the Barley Grain

A knowledge of the anatomy and composition of the barley (*Hordeum vulgare* L.) grain is important for understanding the processes that occur during germination. The two major structural components of the mature barley grain are the embryo and the endosperm (Figure 1.1). These components are surrounded by the testa (seed coat) and the pericarp, which are remnants of the integuments and the ovary wall, respectively (Figure 1.1). The husk represents the outermost layer of the grain, and originates from the two flowering glumes, the palea and lemma (Figure 1.1; Briggs, 1992; Bewley and Black, 1994).

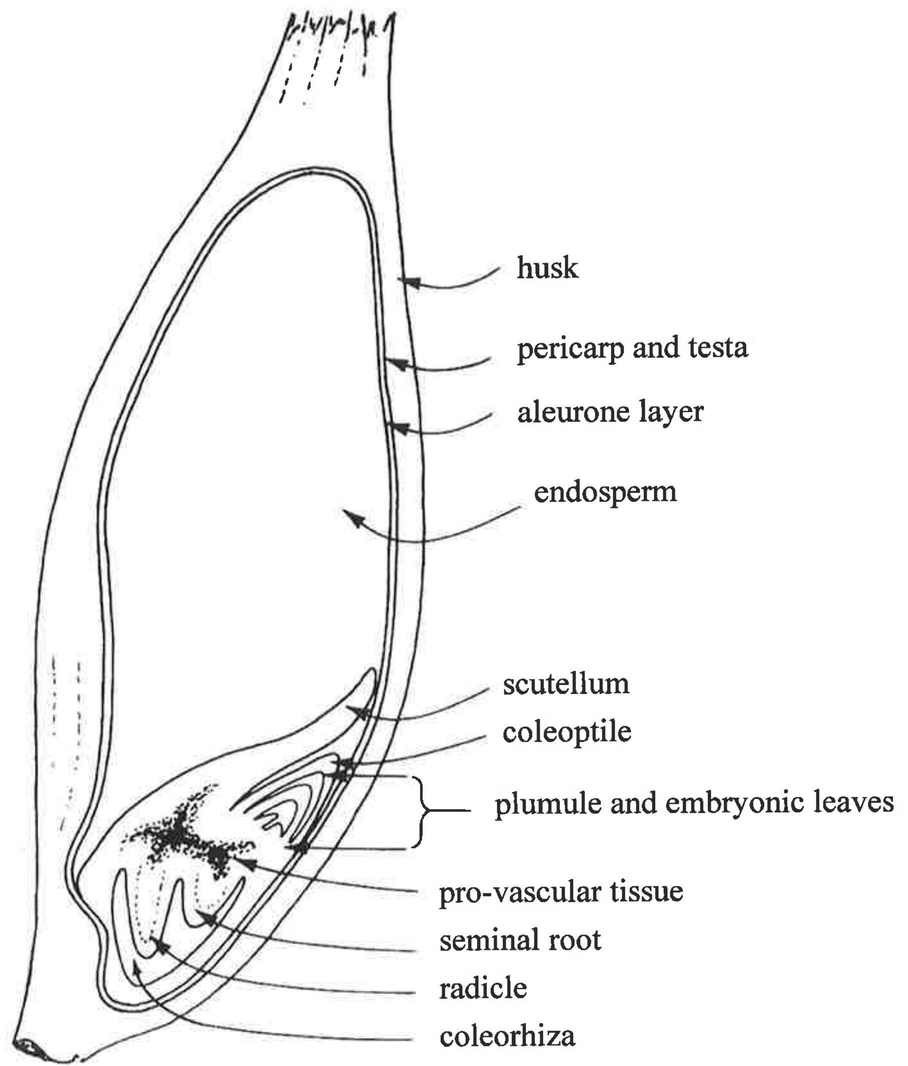


Figure 1.1 Diagram of a barley grain (adapted from Salisbury and Ross, 1978).

The embryo is located at the basal end of the grain, and consists of the embryonic axis and the scutellum (Figure 1.1). The embryonic axis consists of the radicle (embryonic root) and plumule (embryonic shoot) which are protected by the coleorhiza and coleoptile, respectively (Figure 1.1; Briggs, 1992; Bewley and Black, 1994). In the mature grain, the embryo contains small amounts of sugars such as sucrose and raffinose, and is therefore supplied with an immediate energy source to support its growth before reserve mobilisation in the remainder of the grain has commenced (Bewley and Black, 1994). Following the initiation of radicle elongation, seedling growth is supported exclusively by products released during reserve mobilisation in the starchy endosperm. This reliance on stored reserves diminishes as the shoots emerge from the soil and become autotrophic (Briggs, 1992).

The scutellum is the other major component of the embryo. It is a shield-shaped tissue composed of thin-walled parenchyma cells and an epithelial layer. The scutellar epithelium is a single layer of elongated cells which lies at the interface between the embryo and endosperm (Figure 1.1; Briggs, 1992). In cereals, the scutellar epithelial cells contain protein and lipid bodies (Vance and Huang, 1988), phosphate-rich phytins, and vitamins (Nieuwdorp, 1963; Smart and O'Brien, 1979a, 1979b; Aisien *et al.*, 1986). The proteins, lipids, and phytins contained within the scutellar epithelium are used for endoplasmic reticulum (ER) proliferation and enzyme synthesis during the early stages of germination (Fincher, 1989). The scutellar epithelial cells therefore play a major role both in the early secretion of hydrolytic enzymes into the starchy endosperm, and during the subsequent translocation of endosperm degradation products to the embryo.

The endosperm is the second major tissue of the grain, and encompasses both the aleurone layer and the starchy endosperm (Figure 1.1). The aleurone layer is a thin layer of cells surrounding the starchy endosperm, and in the mature grain, represents the only living tissue outside the embryo (Briggs, 1992). During grain development, the aleurone cells develop specialised protein bodies referred to as aleurone grains, which become

interspersed in the cytoplasm (Morrison *et al.*, 1975). The protein matrix within the aleurone grains has phytin and niacytin inclusions (Fulcher *et al.*, 1977; Bacic and Stone, 1981a). Although the aleurone cells do not normally contain starch, they do contain soluble carbohydrates such as sucrose, raffinose, and stachyose (Briggs *et al.*, 1981). The broad range of storage molecules found in the aleurone cells provides essential amino acids and energy required for the *de novo* synthesis of hydrolytic enzymes following the initiation of germination (Fulcher *et al.*, 1972). These hydrolytic enzymes are involved in cell wall breakdown and in the hydrolysis of starch, protein, nucleic acids and other reserves in the starchy endosperm (see Section 1.3).

The starchy endosperm occupies the bulk of the barley grain and represents the major nutrient store which is mobilised following germination. In the mature grain, the starchy endosperm cells are non-living, and their cytoplasm is packed with starch granules embedded in a protein matrix (McFadden *et al.*, 1988; Briggs, 1992; Bewley and Black, 1994). The starchy endosperm consists of approximately 9% (w/w) protein which is mainly comprised of hordeins, glutenins and globulins, with small amounts of albumins and free amino acids (Brandt, 1976; Briggs, 1992).

1.2 Cell Wall Structure and Composition

In barley, the cell walls of the aleurone and starchy endosperm have specialised polysaccharide components which are a direct reflection of their function in the grain. In contrast to the cell walls of vegetative tissues, which are predominantly composed of cellulose (up to 65% by weight) to provide structural support, the cell walls of the aleurone layer and starchy endosperm contain less than 2% cellulose by weight (Fincher, 1975; Bacic and Stone, 1981a, b). The aleurone and starchy endosperm cell walls are composed mainly of (1→3,1→4)-β-glucan and arabinoxylan. The structures of the polysaccharides themselves are well defined, but their exact molecular organisation in the wall has not been elucidated. Several models for the organisation of plant cell wall polymers have been proposed (Fincher and Stone, 1986; Albersheim *et al.*, 1994; Gibeaut and Carpita, 1994;

Fry, 1995; Carpita, 1996). In this section, discussion is focused mainly on the polysaccharide composition of cell walls from the starchy endosperm and aleurone of barley.

1.2.1 Cell Wall Constituents

Barley aleurone cell walls contain approximately 70% arabinoxylan and 25% (1→3,1→4)-β-glucan by weight, together with minor components such as cellulose, glucomannan and (1→3)-β-glucan (Bacic and Stone, 1981b; Fincher and Stone, 1986). Phenolic acids such as ferulic acid and *p*-coumaric acid are covalently bound to the arabinoxylan component of aleurone cell walls (Fulcher *et al.*, 1972; Bacic and Stone, 1981a, b). The aleurone cell walls consist of two separate layers, neither of which has any apparent secondary thickening or lignification (Taiz and Jones, 1973; Bacic and Stone, 1981a, b). Adjacent aleurone cells are connected by relatively large plasmodesmata (Taiz and Jones, 1973). The distinct lack of wall thickening in the aleurone cells (Smart and O'Brien, 1979a, b) facilitates the secretion of hydrolytic enzymes into the starchy endosperm during reserve mobilisation (Fincher and Stone, 1986).

In contrast to those of the aleurone, the starchy endosperm cell walls in barley are composed of approximately 75% (1→3,1→4)-β-glucan, 20% arabinoxylan, and other minor constituents including glucomannan, cellulose, and some phenolic compounds (Fincher, 1975; Ballance and Manners, 1978). The walls of the starchy endosperm are non-lignified, contain little or no pectin, and are thinner than the walls of the aleurone cells (Fincher and Stone, 1986). The absence of thickening renders the endosperm cell walls more amenable to rapid hydrolysis during reserve mobilisation. The structures of the two major polysaccharide components of the endosperm cell walls, (1→3,1→4)-β-glucan and arabinoxylan, are described below.

1.2.2 (1→3,1→4)-β-Glucans

Barley (1→3,1→4)-β-glucans constitute a class of polysaccharides in which individual members differ in size, structure, and solubility (Bacic and Stone, 1981b; Woodward and

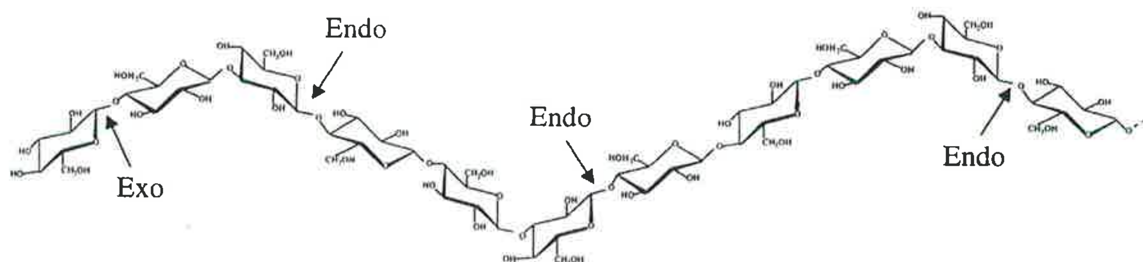
Fincher, 1983). The polysaccharides are comprised of linear chains of about 30% (1→3)- and 70% (1→4)-β-linked glucosyl residues (Parrish *et al.*, 1960), with a degree of polymerisation (DP) of between 200 and 1200 residues (Figure 1.2). The (1→4)-β-linkages typically occur as cellotriosyl and cellotetraosyl residues connected by single (1→3)-β-linkages (Woodward and Fincher, 1983). Approximately 10% by weight of the water-soluble barley (1→3,1→4)-β-glucan is made up of blocks of contiguous (1→4)-β-linkages which range from 4 to 14 residues in length (Figure 1.2).

The irregular distribution of (1→3)-β-linkages along the (1→3,1→4)-β-glucans molecule prevents aggregation and precipitation of the polysaccharide in aqueous solutions (Figure 1.2; Staudte *et al.*, 1983; Woodward and Fincher, 1983). However, (1→3,1→4)-β-glucans have a tendency to form solutions of high viscosity because of their highly asymmetrical structure and relatively high DP values. For this reason, residual (1→3,1→4)-β-glucans cause significant problems in the malting, brewing, and stockfeed industries (see Section 1.5).

1.2.3 Arabinoxylans

Cereal arabinoxylans represent a family of polysaccharide molecules which consist of a linear (1→4)-β-xylopyranosyl backbone substituted with α-L-arabinofuranosyl residues (Viëtor *et al.*, 1992). In arabinoxylans from barley aleurone and starchy endosperm cell walls, xylosyl residues of the backbone are substituted with single α-L-arabinofuranosyl residues at C(O)2 and/or C(O)3 positions. Barley arabinoxylans are often associated with phenolic acids such as ferulic acid, which are ester linked to the C(O)5 position of the arabinosyl residues (Fincher, 1976; Bacic and Stone, 1981b; Gubler *et al.*, 1985). The ratios of xylosyl to arabinosyl residues in barley cell wall arabinoxylans vary between approximately 1:1 to 2:1; this implies that the xylan backbone is highly substituted (Fincher 1975; Bacic and Stone, 1981a, b; Ballance *et al.*, 1986). As a consequence of their asymmetric structure and relatively high DP, aqueous solutions of arabinoxylans are also highly viscous (Andrewartha *et al.*, 1979; Viëtor *et al.*, 1993).

A)



B)

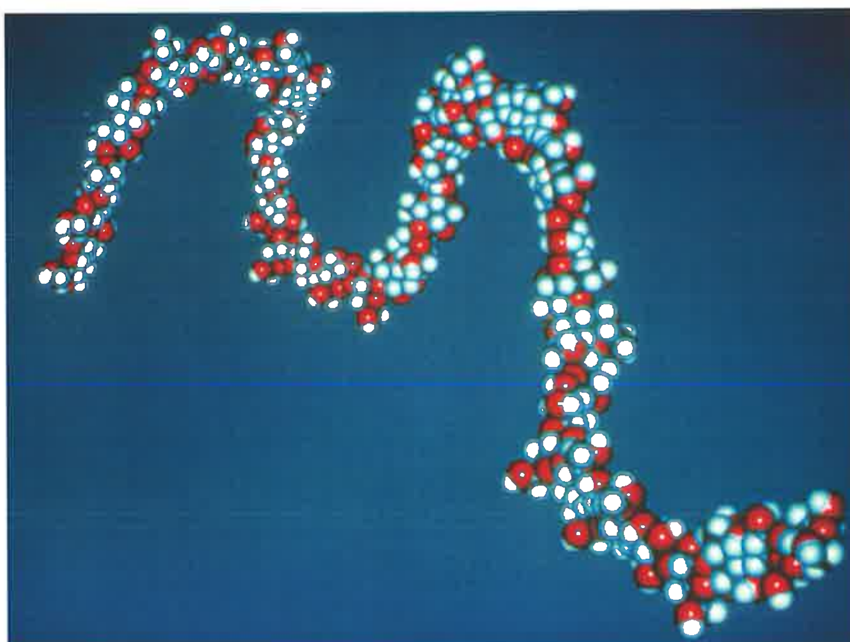


Figure 1.2 Molecular structure of barley (1→3,1→4)-β-glucan. A) (1→4)-β-Linkages constitute the linear portions of the polysaccharide chain, whereas (1→3)-β-linkages are found at the 'kinks' in the molecule. The non-reducing end of the polysaccharide chain is at the left, and arrows indicate the linkages which are hydrolysed by β-glucan exohydrolases (Exo) and (1→3,1→4)-β-glucan endohydrolases (Endo; adapted from Gibeaut and Carpita, 1994). B) A space-filling model incorporating the major structural features of barley (1→3,1→4)-β-glucan (Buliga *et al.*, 1986). Carbons, hydrogens, and oxygens are shown in black, white, and red, respectively.

1.3 Reserve Mobilization in the Germinated Barley Grain

The uptake of water by the mature barley grain signals the onset of germination, which is accompanied by an increase in metabolic activity in the living tissues of the grain (Briggs, 1992). Following the completion of germination, gibberellic acid (GA_3) diffuses from the embryo to the scutellum and aleurone layer, where it stimulates the *de novo* synthesis of a number of hydrolytic enzymes (Paleg, 1960; Yomo, 1960; Radley, 1967; Chrispeels and Varner, 1967). Patterns of endosperm dissolution indicate that hydrolytic enzyme production and secretion is stimulated initially in the scutellar epithelium and subsequently in the aleurone layer, as the front of GA_3 diffusion moves from the proximal to the distal end of the grain (Paleg, 1960; Gibbons *et al.*, 1979; Gibbons, 1981; McFadden *et al.*, 1988). Although the site of GA_3 synthesis has not been unequivocally determined, it is likely to occur in the embryonic axis or scutellum (MacLeod and Palmer, 1967; Radley 1967; Fincher, 1989).

The hydrolytic enzymes which are secreted from the aleurone and scutellum into the starchy endosperm include protein hydrolases (Mikola, 1983, 1987; Hammerton and Ho, 1986; Zhang and Jones, 1996, 1999), starch-degrading enzymes (Chrispeels and Varner, 1967), and cell-wall degrading enzymes such as $(1\rightarrow3,1\rightarrow4)$-β-glucanases (Mundy and Fincher, 1986; Stuart *et al.*, 1986; McFadden *et al.*, 1988; Slakeski *et al.*, 1990; Slakeski and Fincher, 1992a, b) and endoxylanases (Taiz and Honigman, 1976; Banik *et al.*, 1996). Other enzymes which are synthesised in the aleurone in response to GA_3 include $(1\rightarrow3)$-β-glucanases (Taiz and Jones, 1970; Bennet and Chrispeels, 1972; Xu *et al.*, 1992) and nucleases (Bennet and Chrispeels, 1972; Brown and Ho, 1986). Limit dextrinase is also synthesised *de novo* in the aleurone layer, but recent evidence suggests that it may not be secreted into the starchy endosperm (Burton *et al.*, 1999a).

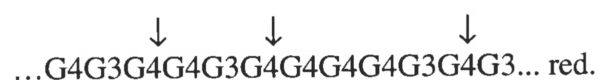
Following their secretion from the aleurone and scutellum, the hydrolases diffuse throughout the starchy endosperm where they participate in the breakdown of the starch and protein reserves stored in the starchy endosperm cells. Because the hydrolytic enzymes

are too large to pass through the intact walls of the starchy endosperm cells, the breakdown of these walls is a critical event which must precede starch and protein mobilization. The enzymes which are involved in the degradation of the major polysaccharide components of the cell walls in the starchy endosperm are discussed in the following section.

1.3.1 Endosperm Cell Wall Degradation

(i) (1→3,1→4)-β-Glucanases

The enzymes primarily responsible for hydrolysing the (1→3,1→4)-β-glucan component of the endosperm cell walls are the (1→3,1→4)-β-glucan endohydrolases (EC 3.2.1.73), commonly referred to as (1→3,1→4)-β-glucanases. Barley (1→3,1→4)-β-glucanases will hydrolyse (1→4)-β-linkages of (1→3,1→4)-β-glucans only where the corresponding glucosyl residue towards the non-reducing end of the molecule is substituted at the C(O)3 position. The action of barley (1→3,1→4)-β-glucanases is represented diagrammatically below;



where 'G' represents β-D-glucosyl residues; '3' and '4' represent (1→3)-β- and (1→4)-β-linkages, respectively. Vertical arrows show hydrolysis positions and 'red.' refers to the reducing end of the molecule. Hydrolysis products are predominantly 3-O-β-cellobiosyl-D-glucose ($G4G3G_{red}$) and 3-O-β-cellotriosyl-D-glucose ($G4G4G3G_{red}$) (Woodward and Fincher, 1982b).

Two (1→3,1→4)-β-glucanases have been purified from germinated barley grain (Woodward and Fincher, 1982a) and designated isoenzymes EI and EII. The two isoenzymes share identical substrate specificities and have similar kinetic properties (Woodward and Fincher, 1982b). However, they can be clearly distinguished on the basis of their isoelectric points, and carbohydrate content (Woodward and Fincher, 1982a; Doan and Fincher, 1992). Isoenzyme EI has an isoelectric point of 8.5 and no associated

carbohydrate, whereas isoenzyme EII has an isoelectric point of greater than 10.0 and has 3.6% (w/w) associated carbohydrate (Doan and Fincher, 1992). The two enzymes have an amino acid positional identity of 92% and cDNA sequences show that they both contain a signal peptide of the type which would direct their secretion from the aleurone and scutellar cells during reserve mobilisation. Expression of barley (1→3,1→4)-β-glucanase isoenzyme EII is almost exclusively confined to the aleurone layer of germinated grain and is enhanced by GA_3 (Slakeski *et al.*, 1990). In contrast, (1→3,1→4)-β-glucanase isoenzyme EI is expressed in young leaves and roots, and in both the scutellum and the aleurone layer of the germinated grain (Slakeski *et al.*, 1990; Slakeski and Fincher, 1992b).

(ii) β-Glucan exohydrolases and β-glucosidases

The oligosaccharides released from the cell wall by the action of (1→3,1→4)-β-glucanases are subsequently hydrolysed by a different class of enzymes in the germinated grain, to release glucose. The two most likely candidates for the hydrolysis of the oligosaccharides are the β-glucan exohydrolases and the β-glucosidases. Two β-glucosidases which have been purified from extracts of germinated barley (Simos *et al.*, 1994; Leah *et al.*, 1995; Hrmova *et al.*, 1996) are able to hydrolyse (1→3,1→4)-β-oligoglucosides (Leah *et al.*, 1995; Hrmova *et al.*, 1998a). Although we cannot yet assign an unequivocal function to barley β-glucosidases, it is likely that they play a role in the degradation of (1→3,1→4)-β-oligoglucosides in the germinated grain. β-Glucan exohydrolases have also been detected in germinated barley grain and in young barley seedlings (Hrmova *et al.*, 1996; Kotake *et al.*, 1997; Hrmova and Fincher, 1998). These enzymes remove single glucose residues from the non-reducing ends of polymeric cereal (1→3,1→4)-β-glucans, and are also capable of hydrolysing the tri- and tetrasaccharides resulting from the action of (1→3,1→4)-β-glucanases on (1→3,1→4)-β-glucan. The broad specificity and ability of β-glucan exohydrolases to hydrolyse polysaccharide substrates distinguishes them from the β-glucosidases (Labrador and Nevins, 1989; Hrmova *et al.*, 1996, 1998a; Hrmova and Fincher, 1998). The β-glucan exohydrolases are less likely to play a role in the hydrolysis of (1→3,1→4)-β-oligoglucosides in the germinated grain, because they hydrolyse (1→4)-

β-glucosyl linkages considerably more slowly than (1→3)-β-glucosyl linkages, and their corresponding mRNAs are most abundant in elongating coleoptiles (Harvey *et al.*, 1999).

(iii) Arabinoxylan-degrading enzymes

Arabinoxylans are degraded by the concerted action of (1→4)-β-D-xylan endohydrolases (endoxylanases, EC 3.2.1.8), α-L-arabinofuranosidases (EC 3.2.1.55), (1→4)-β-D-xylan exohydrolases (exoxylanases, EC 3.2.1.37), and possibly β-D-xylopyranosidases (xylosidases, EC 3.2.1.37; Preece and McDougall, 1958; Taiz and Honigman, 1976; Dashek and Chrispeels, 1977; Banik *et al.*, 1996). Endoxylanases hydrolyse (1→4)-β-linkages between unsubstituted xylosyl residues, releasing low molecular weight oligosaccharides that contain xylose and/or arabinose residues and have DP values of 2 or 3 (Preece and McDougall, 1958; Dekker and Richards, 1976; Dekker, 1989). In barley, three endoxylanases have been purified from 5-day germinated grain (Slade *et al.*, 1989), and endoxylanase activity has been detected in the media surrounding GA_3-treated aleurone layers (Benjavongkulchai and Spencer, 1986). However, the barley endoxylanases have not been extensively characterised because of their low abundance in the germinated grain and their inherent lack of stability.

Two other enzymes which may be involved in the degradation of barley arabinoxylan are the exoxylanases and xylosidases. In general, exoxylanases release xylose from the non-reducing end of polysaccharide substrates. Xylosidases release xylose groups from the non-reducing ends of relatively short β-oligoxylosides, and their ability to hydrolyse aryl- and alkyl-β-xylosides distinguishes them from the exoxylanases (Taiz and Honigman, 1976; Dashek and Chrispeels, 1977). Another class of enzyme, the α-L-arabinofuranosidases, hydrolyse α-L-arabinofuranosyl residues from substituted xylosyl residues of arabinoxylan (Kaji, 1984). Efforts to purify and characterize xylosidases and α-L-arabinofuranosidases in more detail are currently underway (R.C. Lee, M. Hrmova and G.B. Fincher, unpublished data).

1.3.2 Hydrolysis of the Starch and Protein Reserves

Degradation of the starchy endosperm cell walls allows starch and protein hydrolases released from the aleurone or scutellum access to their substrates, which are packaged in the starchy endosperm cells. Starch and protein reserves are hydrolysed into low molecular weight products, which are subsequently translocated to the embryo, *via* the scutellum, where they support early seedling growth (Bewley and Black, 1994). The enzymes responsible for degrading the starch and protein reserves are briefly discussed below.

(i) Starch hydrolysis

The major storage reserves in the barley starchy endosperm consist of starch granules surrounded by a protein matrix. Starch granules are composed predominantly of amylopectin and amylose, which in barley comprise approximately 70% and 30% of starch granules, respectively (MacGregor and Fincher, 1993). Amylose is a linear (1→4)-α-glucan polymer, and amylopectin is a branched (1→4,1→6)-α-glucan polymer. In the barley endosperm, starch is converted to glucose by the combined action of α- and β-amylase, limit dextrinase, and α-glucosidase (MacGregor and Fincher, 1993).

Amylose and amylopectin are initially hydrolysed by the action of α-amylases (EC 3.2.1.1; (1→4)-α-D-glucan glucanohydrolase), which are endohydrolases that hydrolyse internal (1→4)-α-glucosidic linkages. In barley, α-amylases are classified into two main groups, designated as low pI and high pI groups (Svensson *et al.*, 1985; Jacobsen and Chandler, 1987; MacGregor *et al.*, 1988). Linear (1→4)-α-oligoglucosides can be hydrolysed by α-amylase to release oligosaccharides with an average DP values of between 2 and 7 (MacGregor and Fincher, 1993), but α-amylase cannot hydrolyse the (1→6)-α-linkages of amylopectin or branched (1→4,1→6)-α-oligoglucosides. Thus, the digestion of starch by α-amylase results in the production of glucose, maltose and (1→4,1→6)-α-oligoglucosides (MacGregor and Fincher, 1993).

(1→4)-α-D-Glucan maltohydrolase (β-amylase, EC 3.2.1.2) releases maltose units from the non-reducing termini of linear (1→4)-α-glucans but cannot bypass the (1→6)-linkages of (1→4,1→6)-α-oligoglucosides or amylopectin (Maeda *et al.*, 1978). β-Amylase is not synthesised *de novo* in the barley aleurone layer or scutellum following germination (Hardie, 1975), but is found in ungerminated barley grain in both a 'bound' and a 'free' form. The bound form of the enzyme is believed to be attached by disulphide bonds to endospermic proteins such as glutenin, and is associated with the surface of starch granules (Laurière *et al.*, 1986; Hara-Nishimura *et al.*, 1986). The free form is found in the protein matrix of the starchy endosperm (Daussant and Corvazier, 1970; Laurière *et al.*, 1986; Hara-Nishimura *et al.*, 1986; Shen-Miller *et al.*, 1991). There is also evidence for the existence of a third 'latent' form of the enzyme (Evans *et al.*, 1997).

The branched (1→4,1→6)-α-oligoglucosides resulting from the digestion of starch by α-amylase are often referred to as 'limit dextrins'. Although limit dextrins can theoretically be hydrolysed by α-glucosidase, this hydrolysis would probably occur at a relatively slow rate. Barley limit dextrinase (EC 3.2.1.41) belongs to a group of so-called 'debranching enzymes', and can hydrolyse the (1→6)-α-linkages of limit-dextrins to release linear (1→4)-α-oligoglucosides (Lee *et al.*, 1971; MacGregor *et al.*, 1994; Burton *et al.*, 1999a). These linear products can subsequently be converted to glucose and maltose by the action of α-amylase and β-amylase. Limit dextrinase production is induced by GA_3 in the aleurone of germinated barley (MacGregor *et al.*, 1994), but there is some doubt as to how it is secreted from these cells (Burton *et al.*, 1999a).

The maltose and other low molecular weight maltodextrins which result from the action of α- and β-amylases on starch are converted to glucose by the action of α-glucosidase (EC 3.2.1.20). This enzyme is found in ungerminated cereal grains and is also synthesised in the scutellum and aleurone layer in the germinated grain (Jørgensen, 1965; Hardie, 1975; MacGregor and Lenoir, 1987). The glucose which results from the action of barley α-

glucosidase is absorbed by the scutellum and rapidly converted to sucrose for translocation to the developing seedling (Bewley and Black, 1994).

(ii) Storage protein hydrolysis

In barley grain, approximately 65% of the total reserve proteins are located in the starchy endosperm (Mikola and Kolehmainen, 1972). The storage proteins contained in the starchy endosperm are rich in amino acids such as glutamine, proline and asparagine, and the degradation of these proteins provides essential nitrogen for seedling growth (Brandt, 1976). Several exo- and endopeptidases are responsible for the degradation of protein reserves in the barley grain. These peptidases are found in ungerminated grain, and are also synthesised *de novo* in the scutellum and aleurone layer of germinated grain (Sundblom and Mikola, 1972; Hammerton and Ho, 1986). Barley carboxypeptidases, the major exopeptidases involved in the degradation of endosperm proteins, have been extensively characterised (Mikola and Mikola, 1980; Breddam *et al.*, 1983; Mikola, 1983, 1987; Breddam and Sorensen, 1987). Endopeptidases have been detected in both the resting and germinated barley grain (Hammerton and Ho, 1986; Tormakangas *et al.*, 1994; Zhang and Jones, 1996, 1999). The amino acids and small peptides (normally 2-3 amino acids) resulting from the degradation of endosperm storage proteins by exo- and endopeptidases are absorbed by the scutellum, where the peptides are converted to amino acids for transport to the developing seedling (Sopanen, 1979; Higgins and Payne, 1981; Mikola, 1987).

1.4 Barley (1→3)-β-Glucanases

In Chapter 3 of this thesis, attempts to alter the substrate specificity of a barley (1→3)-β-glucanase to that of a (1→3,1→4)-β-glucanase using site-directed mutagenesis are described. This required detailed knowledge of the three-dimensional structures and substrate specificities of both barley (1→3,1→4)-β-glucanases (see Section 1.3.1) and barley (1→3)-β-glucanases. Barley (1→3)-β-glucanases are discussed below, with particular emphasis on their role in the germinated grain.

In higher plants, (1→3)-β-glucanases (EC 3.2.1.39) are involved in many developmental processes including pollen formation, dormancy, senescence, fruit ripening, and the removal of wound callose (Simmons *et al.*, 1992; Stone and Clarke, 1993; Hennig *et al.*, 1993). The (1→3)-β-glucanases have also been implicated in the protection of plants against invasion from microbial pathogens, and therefore have been classified among the numerous 'Pathogenesis-Related' (PR) proteins (Boller, 1987; Van Loon *et al.*, 1985; Mauch *et al.*, 1988; Leah *et al.*, 1991; Stintzi *et al.*, 1993).

Barley (1→3)-β-glucanases hydrolyse (1→3)-β-glucosyl linkages only where several contiguous (1→3)-β-linked glucosyl residues are present, and consequently will not hydrolyse the single (1→3)-β-linkages present in barley (1→3,1→4)-β-glucans (Fincher, 1989; Høj and Fincher, 1995). Although barley (1→3)-β-glucanases are expressed at relatively high levels in the scutellum and aleurone layer of the germinating grain (Ballance *et al.*, 1976), their function is difficult to assign unequivocally. Endogenous (1→3)-β-glucan in the grain is restricted to small deposits of callose which are dispersed in the intercellular space throughout the endosperm of ungerminated grain. However, the relatively low abundance of this polysaccharide cannot account for the high levels of (1→3)-β-glucanases expressed during germination (Fulcher *et al.*, 1977; Bacic and Stone, 1981a, b; Hrmova and Fincher, 1993).

One explanation for the apparent dilemma of high (1→3)-β-glucanase activity and low substrate abundance in the barley grain is that the (1→3)-β-glucanases play a pre-emptive role in protecting the grain from pathogen attack. Indeed, the mature barley grain represents a rich source of nutrients for soil-borne microbial pathogens, and therefore requires protection following the initiation of germination (Fincher, 1989). Furthermore, it has been demonstrated *in vitro* that barley (1→3)-β-glucanases can hydrolyse the (1→3)- and (1→3,1→6)-β-glucans commonly found in fungal cell walls. This results in the destruction of the hyphal tip and arrests fungal growth (Mauch *et al.*, 1988; Leah *et al.*, 1991).

There is *in vivo* evidence that transcription of barley (1→3)-β-glucanase genes is up-regulated in response to pathogen attack. Infection of barley tissues with fungal pathogens can lead to a significant increase in the production of (1→3)-β-glucanases. However, the extent of this induction appears to be limited to particular pathogens, and certain barley varieties are more responsive than others (Xu *et al.,* 1992; Malehorn *et al.*, 1993). When infected with *Rhynchosporium secalis*, the causal agent for 'barley leaf scald', young seedlings of resistant backcross lines of barley (cv. Clipper) synthesise (1→3)-β-glucanases more rapidly and to higher levels than susceptible lines (Roulin *et al.*, 1997). Furthermore, it appears that (1→3)-β-glucanase isoenzymes GI and GII are preferentially synthesised during this infection. When mature barley leaves (cv. Clipper) are infected with *Erysiphe graminis* ('Powdery Mildew'), synthesis of (1→3)-β-glucanase isoenzyme GII is dramatically increased (Xu *et al.*, 1992). Thus, there is compelling evidence from both *in vitro* and *in vivo* studies to suggest that barley (1→3)-β-glucanases indeed play a role in defence against the invasion of pathogens, although the exact nature and extent of their involvement is yet to determined.

1.5 Barley (1→3,1→4)-β-Glucans in Industry

Barley grain is used in the preparation of stockfeed and malted barley provides the major raw material for the production of alcoholic beverages, predominantly beer. The efficient production of beer from germinated barley grain is reliant on the rapid and extensive degradation of starchy endosperm cell walls (see Section 1.3.1). The performance of barley cell wall-degrading enzymes is therefore particularly important during industrial processing. In this section, barley (1→3,1→4)-β-glucans and (1→3,1→4)-β-glucanases are discussed in an industrial context.

1.5.1 The Malting Process

i) Steeping

During malting, barley grain is germinated under conditions which favour the extensive and uniform modification of the starchy endosperm, while minimizing vegetative growth.

The first step in the malting process involves soaking mature, ungerminated barley grain in water for a period of up to three days. During this process, which is known as 'steeping', water is taken up by the grain predominantly *via* the micropyle, and this leads to a rise in metabolic activity that signals the beginning of germination. Steeping is generally performed in large metal vessels which are specially designed to facilitate aeration of the grain and the gradual draining and replenishment of water (Bamforth and Barclay, 1993).

During steeping, the synthesis of hydrolytic enzymes is initiated in the grain, but conditions must be carefully controlled to ensure a subsequent uniform modification of the starchy endosperm and to minimise malting losses through rootlet growth (chitting). In order to meet these requirements, steeping is normally performed at approximately 15°C (Bamforth and Barclay, 1993). Although (1→3,1→4)-β-glucanases and other hydrolytic enzymes are synthesised during steeping, little degradation of (1→3,1→4)-β-glucan occurs (Morgan *et al.*, 1983). The moisture content of the grain reaches 38-45% under typical steeping conditions (Bamforth and Barclay, 1993).

ii) Germination

During the germination phase of the malting process, steeped grain is germinated under controlled conditions by laying the grain out on large beds for up to 8 days, with regular turning to ensure adequate aeration. This allows rapid mobilization of the protein and starch reserves in the endosperm (see Section 1.3.2). It is very important to avoid excess embryo growth during this stage, because the retention of endosperm breakdown products for fermentation must be maximised. Germination is normally performed in humidified air at temperatures between 14°C and 20°C, and GA_3 is often added at this stage to enhance the production of hydrolytic enzymes and to ensure uniform modification of the endosperm (Bamforth and Barclay, 1993).

The (1→3,1→4)-β-glucan content of the starchy endosperm cell walls and the ability of the grain to synthesise high levels of (1→3,1→4)-β-glucanase are critical factors in the success

during the early stages of the malting process (Loi *et al.*, 1987; Stuart *et al.*, 1988; Henry, 1986, 1988). Indeed, there is a high correlation between the important malt quality parameter, malt extract, and the ability of the barley grain to rapidly produce and sustain high levels of (1→3,1→4)-β-glucanases (Stuart *et al.*, 1988). Rapid endosperm cell wall degradation leads to a higher rate of endosperm reserve mobilization, and consequently more fermentable products can eventually be extracted from the malt. Finally, (1→3,1→4)-β-glucanase activity in the grain is probably sufficient to completely hydrolyse cell wall (1→3,1→4)-β-glucans under normal physiological conditions, but because the time period for germination in the malting process must be restricted to minimise embryo growth, residual high molecular weight (1→3,1→4)-β-glucans may remain.

iii) Kilning

Following germination, the modified malt is kiln-dried to arrest endosperm modification and to produce a friable product for subsequent storage and milling. Drying is effected in deep kilns in which air flow rates and temperature can be accurately controlled. Typical kilning regimes start at 50°C to 60°C and increase progressively to a maximum temperature of between 75°C and 110°C, depending on the style of beer being produced (Bamforth and Barclay, 1993). The kilning process is normally completed in less than 24 hours. Chemical reactions that take place during kilning result in the production of a variety of compounds which enhance the flavour and/or colour characteristics of the malt (Bamforth and Barclay, 1993; Bamforth, 1998). The moisture content of the grain drops to less than 5% during kilning (Kunze, 1996). Because of the gradual increases in temperature and the simultaneous reduction in moisture content experienced during the kilning process, the activities of hydrolytic enzymes synthesised during malting are preserved to various extents. For example, approximately 30% of (1→3,1→4)-β-glucanase activity survives the kilning process, and this activity can be entirely attributed to isoenzyme EII (Figure 1.3; Loi *et al.*, 1987).

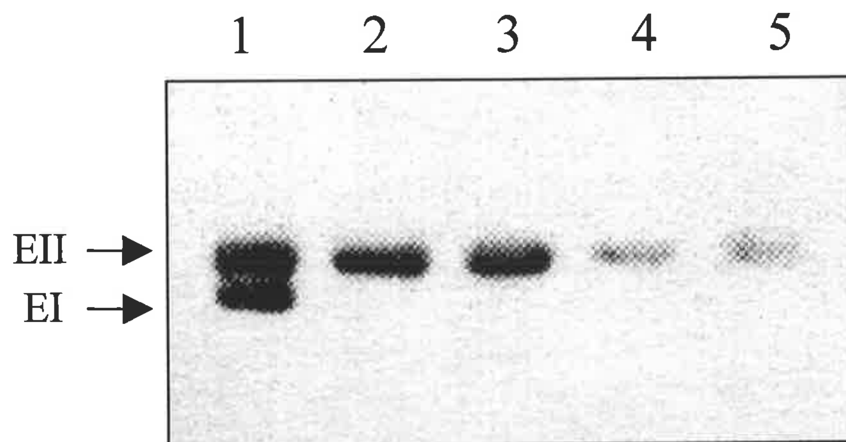


Figure 1.3 Western blot analysis showing the loss of barley (1→3,1→4)-β-glucanase isoenzymes EI and EII during commercial kilning (adapted from Loi *et al.*, 1987). Proteins from grain extracts were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with radiolabelled polyclonal antibodies raised against isoenzyme EI. Lane 1, green malt extract; 2, extract of malt kilned at 60-65°C for 10 h; 3, extract of malt after an additional 4 h at 65-75°C; 4, extract of malt after an additional 2 h at 75-80°C; 5, finished malt extract.

1.5.2 The Brewing Process

The overall objective of the brewing process is to extract high levels of fermentable sugars and other nutrients from malted barley grain. Yeast converts the fermentable sugars to ethanol, and the fermented extract is matured and filtered to produce beer. In this section, the main steps involved in the brewing processes will be discussed, again with an emphasis on the degradation of cell wall (1→3,1→4)-β-glucans.

(i) Mashing

Kiln-dried malt is milled in order to expose a large surface area of the starchy endosperm without destroying the husk. This facilitates the subsequent extraction of fermentable products. For an infusion-style mash, the milled 'grist' is subjected to extraction in hot water at temperatures of approximately 65°C (Bamforth and Barclay, 1993). The temperature is gradually ramped up to 78°C, and fermentable sugars and other low molecular weight products are thereby extracted from the grain. The resulting sweet liquid extract is called 'wort'. Starchy adjuncts such as wheat flour, gelatinised rice, maize, or barley may be added to the mash as an additional source of carbohydrate. However, these adjuncts may also contain high molecular weight (1→3,1→4)-β-glucan, and their addition to the mash must be carefully monitored to ensure that the mash viscosity is not significantly increased. Exogenous fungal or bacterial β-glucan hydrolases can also be added to the mash to facilitate the hydrolysis of (1→3,1→4)-β-glucans, thereby reducing total wort viscosity (Bamforth, 1994). Most Australian breweries use either a fungal (1→4)-β-glucanase (Quest International Ltd, Bussum, Netherlands) or a *Bacillus subtilis* lichenase (Deltagen Ltd, Wicklow, Ireland) to aid the breakdown of residual (1→3,1→4)-β-glucans in the mash.

One of the most important processes during mashing is the hydrolysis of starch to fermentable sugars. Starch becomes gelatinised at temperatures of between 60°C and 70°C in the presence of excess water (Colonna and Mercier, 1985). Starch is hydrolysed much efficiently in this gelatinised form, and mashing is typically performed at 65°C to facilitate

the complete degradation of the starch component of the endosperm. However, at this temperature cell wall-degrading enzymes such as (1→3,1→4)-β-glucanases are relatively unstable. Indeed, after only 10 minutes of mashing at 65°C all (1→3,1→4)-β-glucanase activity is lost, and the residual (1→3,1→4)-β-glucans which remain unhydrolysed or partially hydrolysed at this stage will therefore contribute to wort viscosity (Brunswick *et al.*, 1988; Loi *et al.*, 1987) and can subsequently cause difficulties in the process.

(ii) Lautering and boiling

At the conclusion of mashing, the wort is usually filtered through the bed of spent grain in a process known as 'lautering'. Wort which is highly viscous because of the presence of excess high molecular weight (1→3,1→4)-β-glucans and heteroxylans will slow the lautering rate considerably (Bathgate and Palmer, 1975; Palmer and Bathgate, 1976; Bamforth and Barclay, 1993). The (1→3,1→4)-β-glucans can interact with small starch granules and proteins to form a gel layer on the top of the grain bed, which has reduced porosity and can therefore slow lautering (Lewis and Young, 1995; Kunze, 1996).

Filtered wort is boiled in a 'kettle' for up to two hours with hop (*Humulus lupulus*) extracts, which contribute to the many aromas and the bitter flavours of the beer (Hughes and Simpson, 1993). During boiling, extracts from cane sugar or modified wheat starch may be added to the wort as an additional source of fermentable sugars and flavour (Briggs *et al.*, 1981; Lewis and Young, 1995). The boiling step destroys remaining enzyme activity and sterilises the wort. Boiled wort is typically separated from the solid phase by sedimentation, and subsequently cooled and oxygenated in preparation for fermentation (Bamforth and Barclay, 1993).

(iii) Fermentation

Brewer's yeast (commonly *Saccharomyces cerevisiae* or *S. uvarum*) is added to the cooled, oxygenated wort and fermentation is allowed to proceed at temperatures of between 12°C and 20°C. During this process, fermentable sugars are converted to ethanol and carbon dioxide, and flavoured compounds such as esters are also produced (Briggs *et al.*, 1981).

The characteristics of particular types of beer are highly dependent on the strain of yeast used and on the duration of fermentation, which typically lasts from 3 to 7 days (Lewis and Young, 1995). The enzymes which are synthesised by brewer's yeast are not able to hydrolyse barley (1→3,1→4)-β-glucan, and (1→3,1→4)-β-glucans present in the wort will therefore remain essentially unchanged during fermentation.

Filtration of the fermentation products is essential to separate yeast and other insoluble materials from the beer. Using relatively high pressures, the fermentation mixture is forced through fine filters which typically consist of either diatomaceous earth or perlite (Patterson, 1995). Residual high molecular weight (1→3,1→4)-β-glucans can cause filters to become blocked, which requires time-consuming and costly filter maintenance, and considerably slows the filtration rate (Bamforth and Barclay, 1993; Stewart *et al.*, 1998). Furthermore, the presence of residual (1→3,1→4)-β-glucans in the filtrate can lead to the formation of hazes and precipitates in the mature beer, particularly under conditions of high ethanol concentrations and/or low temperatures (Bamforth, 1985; Bamforth and Barclay, 1993).

Residual heteroxylans also contribute to filtration problems during brewing (Ducroo and Frelon, 1989; Viëtor *et al.*, 1991, 1993) because they too form solutions of high viscosity (Andrewartha *et al.*, 1979; Viëtor *et al.*, 1991, 1993). Furthermore, there is evidence to suggest that arabinoxylans contribute to haze in commercial beer (Coote and Kirsop, 1976). Following germination, the enzymes which depolymerise arabinoxylan generally develop later than the (1→3,1→4)-β-glucanases (Slade *et al.*, 1989), and one would not expect them to be present at high levels during most commercial malting processes. Indeed, the contribution of residual arabinoxylans to wort viscosity and beer precipitates and hazes may have been underestimated (Stewart *et al.*, 1998).

1.5.3 The Stockfeed Industry

Barley is widely used as a source of livestock feed. The starch and protein reserves stored in the starchy endosperm and aleurone cells of the barley grain provide substrates for the digestive enzymes of monogastric animals, which in turn hydrolyse these compounds to provide a source of energy and nitrogen. However, because monogastric animals do not produce enzymes capable of hydrolysing the polysaccharide component of cell walls, the endosperm and aleurone walls therefore remain essentially intact during the passage of barley feed through the upper alimentary tract (Fincher and Stone, 1986; Bhatty, 1993b). Microbial enzymes present in the lower digestive tract account for a significant amount of barley cell wall degradation (Bertrand *et al.*, 1981; Chen *et al.*, 1981).

In relation to the poultry industry, the presence of high levels of (1→3,1→4)-β-glucan in barley feed preparations leads to a rise in the viscosity of chicken gut contents, impairing the efficient action of digestive enzymes and resulting in poor nutrient uptake and slower growth rates (Burnett, 1966; Hesselman and Åman, 1986; Bhatty, 1993b). Furthermore, high levels of (1→3,1→4)-β-glucan and arabinoxylan in the chicken gut cause an increase in the 'stickiness' of the faeces, which causes significant problems with hygiene during the collection of eggs and handling of carcasses (Burnett, 1966; Hesselman and Thomke, 1982; Fincher and Stone, 1986).

In the pig and poultry stockfeed industries, barley grain is routinely pre-treated in an attempt to disrupt the starchy endosperm and aleurone cell walls. Grain can be either treated with water to encourage the production of cell wall-degrading enzymes, or supplemented with exogenous fungal or bacterial hydrolytic enzymes to facilitate cell wall hydrolysis. The dietary value of barley-based stockfeed preparations is increased significantly if fungal (1→4)-β-glucanases (cellulases) are added exogenously to the grain preparations during pre-treatment (White *et al.*, 1981; Hesselman *et al.*, 1982; Newman and Newman, 1987; Campbell and Bedford, 1992). Increasing levels of enzyme activity in the grain in this way results in enhanced degradation of the endosperm and aleurone cell

walls, effectively reducing the viscosity of the gut contents, while also allowing the animal's digestive enzymes more efficient access to the starch reserves in the grain (Hesselman and Åman, 1986). Both the water and the enzyme treatments result in a significant reduction in the incidence of problems associated with viscous gut contents (Bhatty, 1993b).

Physical methods such as grinding, de-hulling, extrusion, and pelleting are also used to facilitate efficient access of hydrolytic enzymes in the animals's digestive tract to the starch and protein reserves in the grain (Graham *et al.*, 1989; Bhatty, 1993a; Plavnik and Sklan, 1995; Hancock *et al.*, 1997). A major disadvantage associated with pelleting and extrusion methods, which involve temperatures of up to 115°C (Edwards, 1999), is the inactivation of endogenous cell wall-degrading enzymes in the grain. This can lead to relatively high levels of residual (1→3,1→4)-β-glucans and other detrimental non-starchy polysaccharides in the feed.

In summary, the addition of enzymes capable of hydrolysing (1→3,1→4)-β-glucan during the pre-treatment of barley grain in the stockfeed industry is beneficial in reducing the problems associated with the viscosity of wall (1→3,1→4)-β-glucans. Furthermore, the thermostability of both exogenous and endogenous enzymes is an important consideration when elevated temperatures are used in conjunction with germination or water treatments. Approaches which may be used to increase the quantity and/or thermostability of endogenous barley (1→3,1→4)-β-glucanases for the stockfeed industry, and for the malting and brewing industries, are discussed below in Section 1.6.

1.6 Overcoming the Problems Associated with (1→3,1→4)-β-Glucans

In a normal physiological context, endogenous levels of (1→3,1→4)-β-glucanase present in germinated grain are likely to be high enough to completely, or at least extensively, hydrolyse (1→3,1→4)-β-glucan during endosperm cell wall degradation. However, their relative heat instability means that barley (1→3,1→4)-β-glucanases are rapidly inactivated

at the temperatures encountered during industrial processes such as kilning, mashing and steam-pelleting. Several different approaches can be taken to overcome the (1→3,1→4)-β-glucan problem during the commercial processing of barley grain. These approaches involve either decreasing levels of (1→3,1→4)-β-glucans present in the mature grain, or increasing (1→3,1→4)-β-glucanase activity following germination, and are discussed below in Sections 1.6.1 to 1.6.5.

1.6.1 Using Selective Breeding to Reduce (1→3,1→4)-β-Glucan Levels

One method of overcoming the problems associated with residual (1→3,1→4)-β-glucans in industry is to select barley varieties with a low (1→3,1→4)-β-glucan content or varieties which produce relatively high levels of (1→3,1→4)-β-glucanase activity in the grain following germination (Briggs *et al.*, 1981; Aastrup, 1983; Henry, 1986; Lance *et al.*, 1986; Pitz, 1990). The importance of these two parameters in barley breeding programs is emphasised by the extensive efforts to map the quantitative trait loci (QTLs) that control (1→3,1→4)-β-glucan content and (1→3,1→4)-β-glucanase activity (Loi *et al.*, 1988; Han *et al.*, 1995). The total (1→3,1→4)-β-glucan content of barley varies considerably, and is influenced by genetic, varietal, environmental, and agronomic factors (Aastrup, 1983; Lance *et al.*, 1986; Pitz, 1990; Bhatty, 1993b; Dunn *et al.*, 1996). The breeding of low (1→3,1→4)-β-glucan varieties is restricted by the fact that there is probably a minimum (1→3,1→4)-β-glucan content which can be tolerated before the structural and functional integrity of the endosperm cell walls is compromised. The selection of malting barley varieties with high levels of (1→3,1→4)-β-glucanase activity following germination is also complicated by the observation that some varieties with high levels of (1→3,1→4)-β-glucanase activity also contains relatively high levels of (1→3,1→4)-β-glucan (Kenn *et al.*, 1993).

1.6.2 Reducing the Synthesis of (1→3,1→4)-β-Glucans

Reducing the synthesis of (1→3,1→4)-β-glucan in the barley starchy endosperm cell wall during grain development may help alleviate the industrial problems associated with the polysaccharides. Three cDNA clones encoding putative barley β-glucan synthases have

recently been isolated and sequenced (Burton *et al.*, 1999b). This presents us with an opportunity to use antisense (Kumria *et al.*, 1998) or viral-induced gene silencing technology (Baulcombe, 1999) to inhibit (1→3,1→4)-β-glucan synthesis during grain development. Because (1→3,1→4)-β-glucan is an important structural component of cell walls in most barley tissues, this approach would require the use of a promoter which is specifically expressed during grain development. Promoters which regulate the genes involved in starch or storage protein biosynthesis would be likely candidates. The viability of this particular approach will require further characterisation of the enzymes involved in β-glucan synthesis, and may be limited by the ability of cell walls with reduced (1→3,1→4)-β-glucan content to function normally.

1.6.3 Increasing (1→3,1→4)-β-Glucanase Gene Expression

Total (1→3,1→4)-β-glucanase activity in the barley grain could be increased by placing the two endogenous barley (1→3,1→4)-β-glucanase genes under the control of a more powerful promoter. For example, if a barley α-amylase gene promoter (Skriver *et al.*, 1991; Gubler and Jacobsen, 1992) were used to control the transcription of the barley (1→3,1→4)-β-glucanase genes, we would expect the genes to be expressed with similar spatial and temporal patterns to those of endogenous barley (1→3,1→4)-β-glucanase genes. However, the α-amylase gene promoters are generally 'stronger' than the barley (1→3,1→4)-β-glucanase gene promoters, and we would therefore expect levels of (1→3,1→4)-β-glucanase activity in the germinated grain to increase. Indeed, transient expression and secretion of barley (1→3,1→4)-β-glucanase isoenzyme EII was obtained when the corresponding gene was controlled by a low pI α-amylase gene promoter from barley (Phillipson, 1993).

Another approach to enhancing (1→3,1→4)-β-glucanase activity in the germinated grain could involve increasing the number of (1→3,1→4)-β-glucanase genes present in the barley genome. A potential disadvantage of increasing levels of (1→3,1→4)-β-glucanase activity in the germinated grain, whether this is achieved by using a more powerful

promoter or by increasing gene copy number, is that it may lead to a metabolic imbalance in the cells of the aleurone and scutellum. For example, the nutrients and energy required for the synthesis of the extra barley (1→3,1→4)-β-glucanases may lead to a reduction in the synthesis of other key hydrolytic enzymes which, in turn, may be detrimental to malt quality.

1.6.4 Using Thermostable (1→3,1→4)-β-Glucanases

Thermostable fungal or bacterial (1→3,1→4)-β-glucanases or cellulases are often used as additives in industry to help alleviate the problems associated with incomplete (1→3,1→4)-β-glucan degradation (Stone and Clarke, 1993; Bamforth, 1994). An extremely thermostable (1→3,1→4)-β-glucanase has been engineered by intragenic recombination of gene segments from *Bacillus macerans* and *Bacillus amyloquefaciens* (Olsen *et al.*, 1991). The gene encoding this hybrid enzyme has been expressed in barley protoplasts (Phillipson, 1993) and in transgenic barley (Jensen *et al.*, 1996, 1998). Barley has also been transformed with a heat-stable (1→4)-β-glucanase from *Trichoderma reesei* (Mannonen *et al.*, 1993; Aspegren *et al.*, 1995).

1.6.5 Increasing the Thermostability of Barley (1→3,1→4)-β-Glucanases

Perhaps a more practical approach to increasing (1→3,1→4)-β-glucanase activity in germinated barley grain would be to produce a barley (1→3,1→4)-β-glucanase with enhanced thermostability. If transgenic barley which expresses a thermostable barley (1→3,1→4)-β-glucanase in the germinating grain could be produced, it would alleviate the need to use exogenous enzyme supplements, which would be beneficial to industry in terms of both processing time and cost. This approach would also help alleviate the technical problems which are often associated with expressing heterologous genes in barley. Finally, from an ethical point of view, the use of barley grain that expresses an altered barley (1→3,1→4)-β-glucanase gene might be more acceptable to the consumer than the use of barley which has been genetically modified using microbial genes.

Engineering heat stability into barley (1→3,1→4)-β-glucanases requires changing the primary structure of the enzymes, which can be facilitated at the DNA level using random mutagenesis or site-directed mutagenesis. Random mutagenesis has been successfully used to increase the heat stability of a barley β-amylase (Okada *et al.*, 1995), and a xylanase from *Bacillus pumilus* (Arase *et al.*, 1993). This technique involves the introduction of a large number (greater than 10,000) of essentially random mutations into a gene or cDNA using chemical mutagens or error-prone PCR. The mutant DNA sequences are expressed in a heterologous cell expression system, and the resulting mutant enzymes are screened for increased heat stability. Random mutagenesis provides a useful method for engineering protein stability, and it does not require a detailed knowledge of the protein's three-dimensional structure. However, random mutagenesis relies on the availability of an efficient method for screening a large number of enzymes, and such a method has not been developed for barley (1→3,1→4)-β-glucanases.

Site-directed mutagenesis provides an alternative method for engineering barley (1→3,1→4)-β-glucanases with increased heat stability. Using site-directed mutagenesis, *specific* amino acid substitutions can be introduced into the enzymes using specially-designed DNA oligonucleotides to effect mutations at the DNA level. The rational prediction of amino acid substitutions which will lead to increased protein stability requires a detailed knowledge of the protein's secondary and tertiary structure. The three-dimensional structure of barley (1→3,1→4)-β-glucanase isoenzyme EII has been solved to high resolution using x-ray crystallography (Varghese *et al.*, 1994), and protein engineering of this enzyme by site-directed mutagenesis should therefore be possible. The polypeptide backbone of barley (1→3,1→4)-β-glucanase isoenzyme EII adopts an $(\beta/\alpha)_8$ barrel structure (Varghese *et al.*, 1994), which consists of eight major α-helices positioned at the surface of the enzyme, and 8 major β-strands arranged on the interior of the enzyme to form a 'TIM-barrel' shape (Figure 4.1; Banner *et al.*, 1975; Branden and Tooze, 1991). The putative substrate-binding site is represented by a deep groove approximately 40Å in length, which traverses the entire enzyme and lies perpendicular to the central axis of the

barrel motif. The putative catalytic amino acids are positioned near the centre of the groove (Varghese *et al.*, 1994; Chen *et al.*, 1995; Henrissat *et al.*, 1995; Jenkins *et al.*, 1995).

In addition to three-dimensional structural information, increasing the thermostability of barley (1→3,1→4)-β-glucanases using site-directed mutagenesis requires the availability of the full length gene or cDNA encoding the enzymes, and a heterologous system for the rapid expression and purification of mutant enzymes. The full-length cDNA and gene encoding (1→3,1→4)-β-glucanase isoenzyme EII have been characterised, and methods for the heterologous expression and rapid purification of the enzyme have been developed (Chapter 2; Doan and Fincher, 1992; Chen *et al.*, 1995). Thus, the basic materials and experimental tools required to increase the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII by rational protein engineering are available.

1.7 Aims of this Study

The principal aim of the experiments described in this thesis was to use protein engineering to increase the thermostability of barley (1→3,1→4)-β-glucanases. As previously mentioned, protein engineering requires the development of a heterologous expression system to facilitate the rapid purification and evaluation of mutant enzymes. The heterologous expression of barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII in *E. coli*, and their subsequent purification and characterisation, are described in Chapter 2.

Two approaches were used in an attempt to engineer a thermostable barley (1→3,1→4)-β-glucanase. The first approach involved changing the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII to allow it to hydrolyse a (1→3,1→4)-β-glucan, by identifying and altering those amino acid residues which might be involved in substrate binding. If successful, this approach would effectively result in the generation of a barley (1→3,1→4)-β-glucanase which retains the superior thermostability characteristics of barley (1→3)-β-

glucanase isoenzyme GII. Attempts to change substrate specificity in this way are described in Chapter 3. The second approach to increasing the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII again involved using three-dimensional structural information, but in this case it was used in conjunction with our knowledge of the factors that affect protein stability. This approach involved the introduction of specific amino acid substitutions into (1→3,1→4)-β-glucanase isoenzyme EII, and the analysis of the resulting mutant enzymes for enhanced thermostability, and is presented in Chapter 4.

Attempts to increase the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII using protein engineering were successful. Three mutant enzymes showed increased thermostability relative to their wildtype (1→3,1→4)-β-glucanase isoenzyme EII counterpart. The three thermostable (1→3,1→4)-β-glucanase mutants contained amino acid substitutions which were located near a loop structure in the COOH-terminal region. Based on this observation, it was evident that the COOH-terminal loop may represent a 'weak link' in the enzyme's structure in terms of stability. It was therefore proposed that, if the COOH- and NH_2-termini of barley (1→3,1→4)-β-glucanase isoenzyme EII were fused, the COOH-terminal loop region may be stabilized, thereby increasing the overall thermostability of the enzyme. Attempts to fuse the termini of (1→3,1→4)-β-glucanase isoenzyme EII to create a 'circular permutation' of the enzyme are described in Chapter 5.

The performance of the most thermostable (1→3,1→4)-β-glucanase mutant was further tested under simulated mashing conditions at 65°C. Under these conditions the mutant enzyme was significantly more thermostable than its wildtype counterpart. These results, which are presented with the thermostability work in Chapter 4, were sufficiently encouraging to suggest that the performance of the thermostable (1→3,1→4)-β-glucanase mutant may be of benefit to industry. Thus, DNA encoding the thermostable (1→3,1→4)-β-glucanase was introduced into barley cells using the microprojectile bombardment transformation procedure. Transient expression of the thermostable mutant (1→3,1→4)-β-glucanase in barley immature embryos and the stable transformation of the corresponding

gene in barley callus cells are described in Chapter 6. Finally, a summary of the work presented in this thesis and possible future directions for the barley (1→3,1→4)-β-glucanase thermostability project are discussed in Chapter 7.

CHAPTER TWO:

HETEROLOGOUS EXPRESSION OF BARLEY (1→3)- AND (1→3,1→4)-β-GLUCANASES

2.1 Introduction

Evaluation of attempts to introduce amino acid substitutions into barley β-glucanases in order to produce a more thermostable (1→3,1→4)-β-glucanase, required a rapid and efficient method for the expression and purification of mutant enzymes, and the development of a simple assay to screen them for enhanced stability. Choosing the appropriate expression system depends on many factors, including the size of the protein being expressed, the requirements for post-translational modification, the desired levels of protein yield and purity, and time and cost considerations. There is a diverse range of host cells available for the heterologous expression of plant proteins including yeast, insect, bacterial, and plant cells. The advantages and disadvantages associated with each type of heterologous host are briefly discussed below.

(i) Yeast cells

Yeast (*Saccharomyces cerevisiae*) cells are typically eukaryotic in terms of their biological complexity and therefore represent an ideal system in which to express foreign eukaryotic genes (reviewed by Romanos *et al.*, 1992). Yeast cells have a well-defined secretory pathway, are able to recognise the signal sequences of most eukaryotic proteins, and they can consequently process and secrete the proteins in an active form (Overbeeke *et al.*, 1989; Romanos *et al.*, 1992). In addition, yeast cells contain the necessary cellular 'machinery' to perform post-translational modifications such as glycosylation and phosphorylation. Plant proteins which have been successfully expressed in yeast cells include a wheat α-amylase (Rothstein *et al.*, 1984; Gatenby *et al.*, 1986), barley α-amylases (Søgaard and Svendsen, 1990), and barley (1→3,1→4)-β-glucanases (Jackson *et al.*, 1986; Thomsen *et al.*, 1988; Olsen and Thomsen, 1991).

There are several disadvantages associated with the use of yeast cells for the expression of foreign proteins. The maintenance of yeast cell cultures and the manipulation of yeast expression vectors is often time-consuming and difficult, and yeast cell transformation frequencies can be low (Romanos *et al.*, 1992). Furthermore, the glycosylation recognition

sequence is the same in yeast and plant proteins, but the structures of oligosaccharides synthesised in the glycosylation process are different. For example, it was found that a barley (1→3,1→4)-β-glucanase is glycosylated with different sugars when expressed in yeast (Olsen *et al.*, 1991; Olsen and Thompson, 1991; Meldgaard and Svendsen, 1994).

(ii) Insect cells

Insect cells provide an alternative host for the heterologous expression of plant proteins (Summers and Smith, 1987; Doan and Fincher, 1992; Korth and Levings, 1993; Frommer and Ninneman, 1995). The most commonly used system for expressing recombinant proteins in insect cells utilises the infection of cultured *Spodoptera frugiperda* (sf9) cells with the baculovirus *Autographa californica* (AcMNPV). A DNA vector containing the foreign gene of interest is introduced into the insect cells, and the gene is transcribed *via* a strong viral polyhedrin promoter which directs a very high level of transcription late in the infection process. Because of the relatively large size of the viral genome however, the vector must first be co-transfected with viral DNA into the insect cells before integrating into the viral genome by *in vivo* homologous recombination (Summers and Smith, 1987).

Traditionally, the cloning, transfection, and recombination procedures involved with the insect-baculovirus system have been technically difficult and time-consuming (Summers and Smith, 1987), but recent advances have been made in baculovirus expression vector technology. These advances include improved methods for recombinant virus selection and further developments in virion display vectors (Possee, 1997). It has not always been possible to produce glycoproteins with complex *N*-linked glycans using the insect-baculovirus expression system, but the cell glycosylation pathway of the host cells can now be modified to alter the structure of glycans which are added to the recombinant protein (Possee, 1997; Jarvis *et al.*, 1998).

(iii) Plant cells

Plant cells contain the necessary cellular machinery for the correct translation and post-translational modification of plant proteins. Plant cell and protoplast cultures have been

successfully used for the transient expression of several plant genes (Heim *et al.*, 1995; Frommer and Ninnemann, 1995). The use of plant cells for expression studies is restricted due to its dependence on cell transformation frequencies. Furthermore, it is often difficult to purify expressed proteins to homogeneity from crude plant cell extracts. Despite recent interest in the specific use of plants for the production of antibodies (Ma and Hein, 1995; Whitelam and Cockburn, 1996; Conrad and Fiedler, 1998), plant cells are not extensively utilised for the heterologous expression of eukaryotic proteins.

(iv) Bacterial cells

The simple prokaryote *E. coli* represents the most popular system for the heterologous expression of plant genes (reviewed by Gold, 1990; Hockney, 1994). High transformation frequencies can be achieved and *E. coli* cells have a relatively short cell replication cycle, which makes them ideal for *in vitro* manipulation. Furthermore, there is a diverse range of DNA plasmids available which contain different antibiotic resistance genes for the efficient selection of transformed cells. There are strong promoters which can be used to control the expression of foreign genes in *E. coli*, and it is therefore possible to produce milligram quantities of protein from a relatively small culture volume (Gold, 1990; Hockney, 1994).

The heterologous expression of a foreign protein in *E. coli* generally involves ligating the corresponding gene or cDNA sequence into an approporiate expression vector. Expression plasmids normally contain a promoter sequence upstream of the foreign DNA insert, and the promoter can be activated in the presence of a particular inducing compound. Expression plasmids often contain an additional DNA sequence which encodes a polypeptide 'tag' which is inserted in-frame at the 5' or 3' end of the foreign DNA insert. The resulting translation product is therefore a fusion-protein consisting of the foreign protein fused to the polypeptide tag at its NH_2- or COOH-terminus. The fusion protein can be purified, usually in a single affinity chromatography step, using the unique properties of the polypeptide tag. Where necessary, the polypeptide tag can be removed by cleavage

with a specific protease such as Factor Xa or thrombin (Resenberg *et al.*, 1987; Studier *et al.*, 1990).

Although *E. coli* remains the most widely used host for the heterologous expression of eukaryotic proteins, it does have some disadvantages. Firstly, bacterial cells do not perform post-translational modifications in the same way as eukaryotic cells. For example, eukaryotic proteins are not glycosylated when expressed in *E. coli* and this can affect the stability and/or folding of the protein (Doan and Fincher, 1992). Furthermore, eukaryotic proteins that contain signal peptides or transit peptides which are normally associated with secretion or organelle targeting will not be processed correctly in the *E. coli* cell. The major disadvantage associated with using *E. coli* as a heterologous host is that foreign proteins often do not adopt the correct three-dimensional conformation. Instead, they form insoluble aggregates or 'inclusion bodies' in the cytosol (Marston, 1986; Schein and Noteborn, 1988; Schein, 1989). Indeed, when barley xylanase isoenzyme X-I was overexpressed in *E. coli*, 100% of the enzyme aggregated as inclusion bodies (Banik, 1996), regardless of the expression conditions.

In some cases, inclusion body formation can be significantly reduced by altering expression conditions such as the cell growth temperature (Shein and Noteborn, 1988). However, in the majority of cases, the aggregated protein must be completely denatured by dissolving it in a strong denaturing agent such as 8 M urea or 6 M guanidine hydrochloride, and subsequently refolded under specific conditions (Rudolph and Lilie, 1996). Ironically, the formation of insoluble inclusion bodies in bacteria provides one of the most efficient methods of producing recombinant proteins in high yields, as long as the denatured proteins can be successfully refolded (Marston, 1986; Hockney, 1994; Rudolph and Lilie, 1996). Additives such as molecular chaperones can be used to assist the *in vitro* refolding of denatured proteins, or to ensure the correct folding of proteins during expression (Ellis, 1991; Rudolph and Lilie, 1996).

2.1.1 *Utilising* E. coli *for the Expression of Barley β-Glucanases*

For this study, *E. coli* was chosen as the preferred host for the expression of barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII. This decision was based on the fact that *E. coli* is a very well-characterised system which is efficient in terms of cost and time, and because the two barley β-glucanases have been previously expressed in *E. coli* (Xu *et al.*, 1994; Chen *et al.*, 1995). Although barley (1→3,1→4)-β-glucanase isoenzyme EII has been successfully expressed in insect cells, this system proved to be both time-consuming and inefficient (Doan and Fincher, 1992). Barley cells would provide the ideal host for the expression of recombinant barley β-glucanases, but they were not considered for this purpose because barley transformation is still not routine (see Chapter 6). Furthermore, protocols for the purification of barley (1→3)- and (1→3,1→4)-β-glucanases from crude protein extracts of barley are complicated and time-consuming (Woodward and Fincher, 1982a; Hrmova and Fincher, 1993), and this would significantly hamper the rapid, precise evaluation of the mutant enzymes.

As mentioned above, barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII have been previously expressed in *E. coli*, using the pMAL system (Xu *et al.*, 1994; Chen *et al.*, 1995). The pMAL vector involves the fusion of the gene sequence encoding a maltose-binding protein (MBP) to the 5' end of the gene of interest and, following expression, the resulting fusion protein can be purified by affinity chromatography on a maltose column (di Guan *et al.*, 1988). In addition, a Factor Xa protease recognition site is situated between the MBP and the NH_2-terminus of the protein of interest, and the MBP portion of the fusion protein can be removed using Factor Xa. In the case of barley (1→3)-β-glucanase isoenzyme GII however, cleavage of the MBP was very inefficient (Chen *et al.*, 1995). Activity assays were therefore performed using the purified fusion protein itself, and this consisted of the 33 kDa barley (1→3)-β-glucanase isoenzyme GII fused to the 40 kDa MBP. When the three-dimensional structure for barley (1→3)-β-glucanase isoenzyme GII was later solved (Varghese *et al.*, 1994), it was

apparent that the inability of Factor Xa to cleave the MBP was probably because the NH_2-terminus of the enzyme lies buried in its interior core. Thus, while the protein remains in a folded state, steric hindrance would deny access of the Factor Xa protease to its cleavage recognition site (Chen *et al.*, 1995).

2.1.2 *The pET Vector Expression System*

Because of the potential problems associated with expressing barley (1→3)-β-glucanases in *E. coli* using the pMAL system (Xu *et al.*, 1994; Chen *et al.*, 1995), it was decided to use a different expression system based on the pET (**p**lasmid for the **E**xpression of **T**7 polymerase) vector (Studier, 1990). The pET system utilises a short polyhistidine tag to facilitate protein purification (Resenberg *et al.*, 1987; Studier *et al.*, 1990). A modified version of the pET3a expression vector, designated pET3a-HT (Dr. Helen Healy, personal communication), consists of a *NdeI/Sac*II cloning site into which the gene or cDNA to be expressed is ligated (Figure 2.1). The *Nde*I site incorporates an 'ATG' start codon (CAT**ATG**) at the 5' end of the DNA insertion site. The *Sac*II site is positioned at the 3' end of the DNA insertion site, and forms part of a thrombin cleavage recognition site (Figures 2.1).

The pET3a-HT vector also contains a DNA sequence encoding six consecutive histidine residues at the 3' end of the DNA insert (Figure 2.1). Expression therefore results in the production of a fusion protein consisting of the recombinant protein fused at its COOH-terminus to a polyhistidine 'tag'. The polyhistidine tag has a very high affinity for a commercially available nickel-based resin called Ni-NTA (**N**ickel-**N**itro-**T**ri-**A**cetic acid), and the fusion protein can therefore be purified by affinity chromatography (Resenberg *et al.*, 1987; Studier *et al.*, 1990). The thrombin recognition site (LVPRGS) which directly precedes the polyhistidine tag facilitates the removal of the tag following purification of the fusion protein.

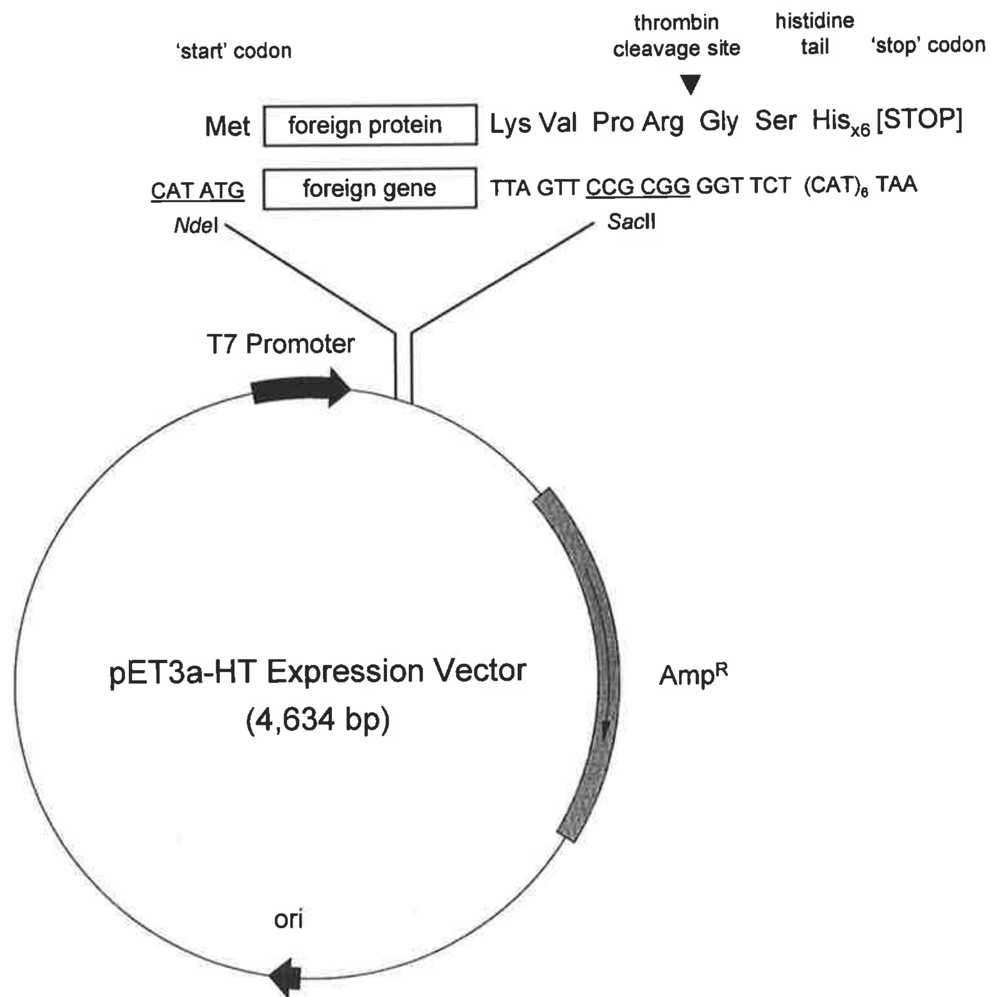


Figure 2.1 The pET3a-HT expression vector. The foreign gene of interest is ligated into the *Nde*I and *Sac*II sites (underlined) of plasmid pET3a-HT. The strong T7 RNA polymerase promoter drives the constitutive expression of a fusion protein consisting of the foreign protein fused at its COOH-terminus to a thrombin cleavage site and a polyhistidine tail. The expressed fusion protein can be purified by one-step affinity chromatography using a nickel resin which has a high affinity for the polyhistidine tail. The vector also contains the gene for ampicillin resistance (Amp^R) and the *E. coli* origin of replication (ori).

For expression, the pET3a-HT vector containing the gene/cDNA of interest is transformed into *E. coli* strain BL21 (DE3) pLysS. The genome of *E. coli* BL21 (DE3) contains the T7 RNA polymerase gene under the regulation of the *lac* operon and expression of the gene can therefore be induced with IPTG. Furthermore, the *E. coli* strain BL21 (DE3) is deficient in the *Ion* and omPT proteases which may otherwise degrade the expressed protein during cell lysis (Grodberg and Dunn, 1988). Another plasmid, designated pLysS, is also present in the expression host cells and contains the gene encoding bacterial lysozyme under the control of the *tet* promoter. Lysozyme is a natural inhibitor of T7 RNA polymerase and therefore minimises expression of the recombinant protein prior to induction. Lysozyme also has the ability to break down the peptidoglycan component of the host cell wall (Inouye *et al.*, 1973), and thereby assists in cell lysis following expression.

Using the pET system, expression is initiated with the addition of IPTG, which induces the production of T7 RNA polymerase and subsequently initiates transcription of the foreign gene/cDNA. After several hours of expression, cells containing the expressed protein are harvested and lysed. The resulting cell lysate is passed through a column containing Ni-NTA resin, and the bound fusion protein is removed from the column by competitive elution with imidazole, a structural analogue of histidine. The pET expression system can result in the production of several milligrams of pure recombinant protein per litre of cell culture (Resenberg *et al.*, 1987; Studier *et al.*, 1990). The expression and purification of barley (1→3)-β-glucanase isoenzyme GII and barley (1→3,1→4)-β-glucanase isoenzyme EII in *E. coli*, using the pET system, are described in this chapter.

2.2 Materials and Methods

2.2.1 *Materials*

The pET3a-HT vector was generously provided by Dr. Helen Healy, Department of Plant Science, University of Adelaide, South Australia. *E. coli* strain BL21 (DE3) pLysS was from Novagen (Madison, WI, USA) and *E. coli* XL1-Blue cells were obtained from Stratagene (La Jolla, CA, USA). Ni-NTA spin columns and imidazole were purchased from Qiagen (Chatsworth, CA, USA). DNA oligonucleotide primers were synthesised using an Applied Biosystems DNA Synthesiser (Foster City, CA, USA). Restriction enzymes, DNA ligase, and Vent DNA Polymerase were purchased from New England Biolabs (Beverly, MA, USA). The 1kb DNA molecular mass standards were from Gibco BRL (Gaithersburg, MD, USA). Barley (1→3,1→4)-β-glucan was obtained from Deltagen (Wicklow, Ireland), and laminarin was from Sigma (St Louis, MO, USA). Thrombin from human plasma was obtained from Boehringer Mannheim (Sandhofer Strasse, Mannheim, Germany), and the Bresa-Clean kit was purchased from Geneworks (Adelaide, South Australia). Protein molecular mass markers were from Pharmacia LKB Biotechnology (Uppsala, Sweden), and the Coomassie Protein Assay Reagent was purchased from Pierce (Rockford, IL, USA). All general chemicals were purchased from Sigma unless otherwise stated.

2.2.2 *Construction of Expression Vectors*

(i) Preparation of the (1→3,1→4)-β-glucanase isoenzyme EII cDNA insert:

The DNA sequence encoding the mature barley (1→3,1→4)-β-glucanase isoenzyme EII was amplified by PCR using the corresponding full-length cDNA (Fincher *et al.*, 1986; Slakeski *et al.*, 1990) as a template. The following two primers were used for the PCR reaction:

P1: 5'-CCC AGC GTG CAT ATG ATC GGG GTG TGC TAC GGC-3' (Forward)

P2: 5'-CGA GCA CCG CGG AAC TAA GAA GTT GAT GGG G-3' (Reverse)

Primer P1 (forward) corresponds to the DNA sequence encoding the first six amino acids of barley (1→3,1→4)-β-glucanase isoenzyme EII, and has a 15 bp extension at its 5' end to incorporate an *Nde*I restriction site (underlined) into the PCR product (Figure 2.2). Primer P2 (reverse) is complementary to the last 13 bp of the cDNA coding region, and has a 5' extension of 19 bp which incorporates a *Sac*II restriction site (underlined) into the PCR product. In addition, the 5' extension of primer P2 encodes part of the thrombin cleavage recognition site (Figure 2.1). The positions of primers P1 and P2 with respect to the cDNA template are shown in Figure 2.2. The PCR reaction mix contained 50 ng template DNA, 100 pmol of each primer, 100 μM dNTPs, 5 mM $MgSO_4$, 10% (v/v) DMSO, 1 unit of Vent DNA polymerase, and 1X Vent polymerase buffer in a total volume of 50 μl. After a drop of mineral oil was added, the mixture was heated for 5 min at 94°C and subjected to 35 cycles of the following temperature regime: 94°C for 40 sec, 50°C for 40 sec, and 72°C for 1 min. A final 10 min extension was performed at 72°C, and the amplified PCR fragment was purified using Bresa-Clean according to the product literature. The purified fragment was digested with *Nde*I and *Sac*II for 2 h at 37°C. After digestion, the PCR fragment was purified with phenol:chloroform and the aqueous phase was precipitated with 0.1 vol 3 M sodium acetate buffer, pH 5.2, and 2.5 vol ethanol (Sambrook *et al.*, 1989). The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in sterile water. The purified PCR fragment was now ready for ligation into the pET3a-HT expression vector.

(ii) Preparation of the (1→3)-β-glucanase isoenzyme GII cDNA insert:

The DNA sequence encoding the mature barley (1→3)-β-glucanase isoenzyme GII was amplified by PCR using the corresponding full-length cDNA (Høj *et al.*, 1989; Xu *et al.*, 1992) as a template. The first six amino acids of (1→3)-β-glucanase isoenzyme GII and (1→3,1→4)-β-glucanase isoenzyme EII are identical (IGVCYG), and so primer P1 was again used as the forward primer. Differences at the 3' ends of the respective cDNAs meant that an additional primer, designated P3, was needed to introduce the *Sac*II site

(Figure 2.2; underlined) and thrombin cleavage recognition site into the 3' end of the PCR fragment:

Primer P3: 5'-GC TAG <u>CCG CGG</u> AAC TAA GAA CTG GAT GTT G-3'

The PCR amplification was performed essentially as described for barley (1→3,1→4)-β-glucanase isoenzyme EII. The positions of primers P1 and P3 with respect to the cDNA template are shown in Figure 2.2. Because the cDNA encoding (1→3)-β-glucanase isoenzyme GII contains an internal *Sac*II restriction site, it was necessary to perform a partial *Nde*I/*Sac*II digest to produce the full-length 943 bp fragment containing a *Nde*I site at the 5' end and a single *Sac*II site at the 3' end. The full-length PCR fragment was purified from the gel using the Bresa-Clean kit in preparation for ligation into the pET3a-HT expression vector.

(iii) Ligation of PCR products into the pET vector:

The pET3a-HT vector (Figure 2.1) was digested with *Nde*I and *Sac*II restriction enzymes at 37°C for 3 h. Following digestion, plasmid DNA was separated by 1% agarose gel electrophoresis, excised, and purified using Bresa-Clean. For ligation, the vector DNA and insert DNA (at a molar ratio of approximately 1:2) were incubated with T4 DNA ligase in 1X T4 DNA Ligase buffer, at 14°C for 16 h. The ligated DNA was now ready for transformation into *E. coli.*

(iv) Preparation of competent cells:

E. coli XL1-Blue cells were grown overnight at 37°C with shaking at 250 rpm in LB containing 15 μg/ml tetracycline. A 1 ml aliquot of the overnight culture was aseptically transferred to a 500 ml conical flask containing 100 ml LB and 15 μg/ml tetracycline, and the flask was incubated at 37°C with shaking at 250 rpm until the A_{600} reached approximately 0.6. Cells were transferred to 50 ml centrifuge tubes and centrifuged at 3,000 rpm for 15 min at 4°C. Cell pellets were resuspended in 30 ml buffer A (30 mM potassium acetate buffer, pH 5.8, containing 100 mM KCl, 60 mM $CaCl_2$ and 0.15% (v/v) glycerol) and incubated on ice for 1 h. Cells were harvested by centrifugation,

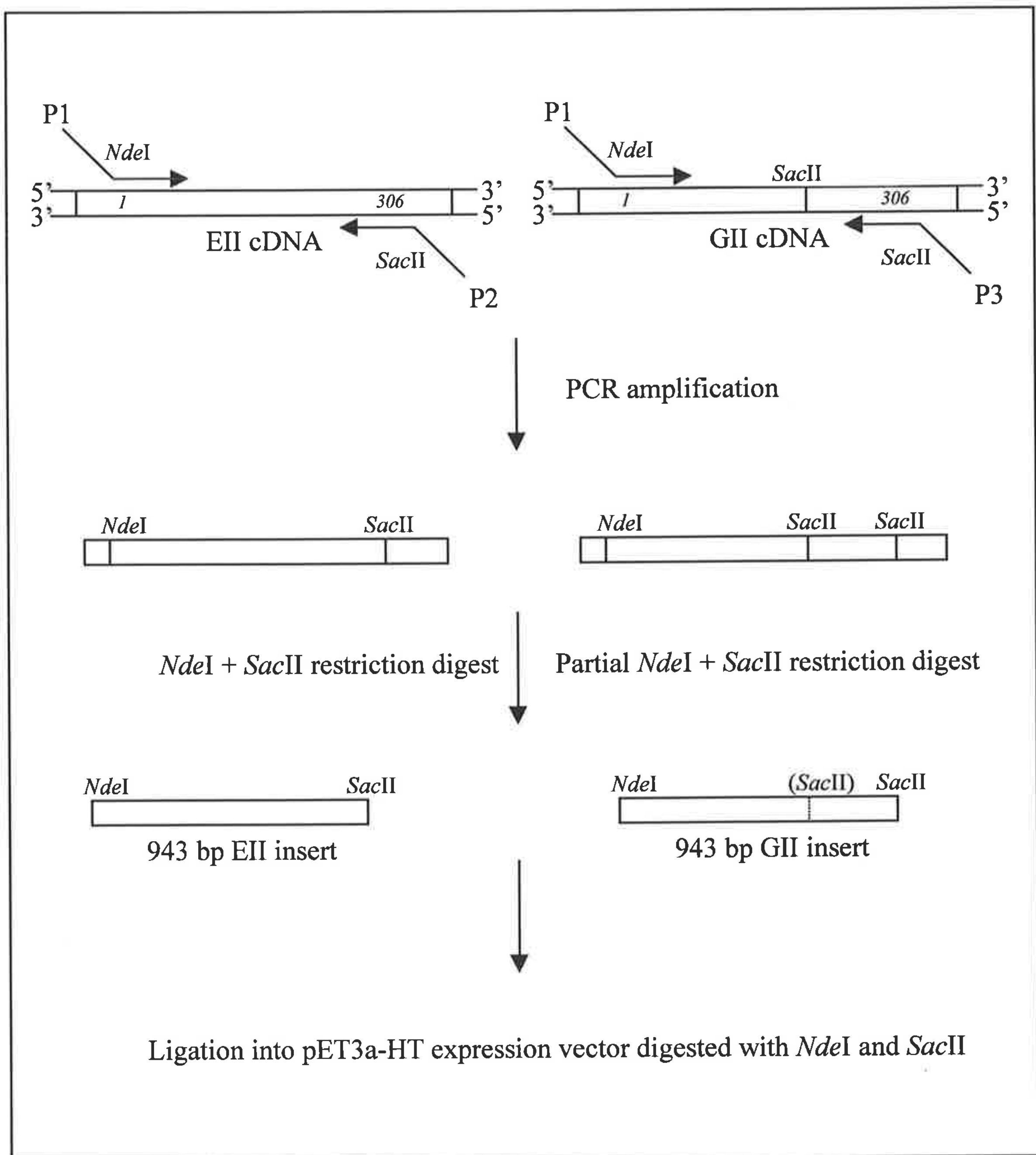


Figure 2.2 Preparation of the cDNAs encoding (1→3,1→4)-β-glucanase isoenzyme EII and (1 →3)-β-glucanase isoenzyme GII for ligation into the expression vector pET3a-HT. PCR was used to introduce *Nde*I and *Sac*II restriction enzyme sites at the 5' and 3' ends of each cDNA, respectively. Following digestion with *Nde*I and *Sac*II, the cDNAs were independently ligated into the pET3a-HT expression vector. P1, P2, and P3 refer to the DNA oligonucleotide primers used for PCR (see Section 2.2.2 for details).

resuspended in 4 ml buffer B (10 mM MOPS buffer, pH 6.8, containing 10mM KCl, 75 mM $CaCl_2$ and 0.15% (v/v) glycerol), and incubated on ice for 15 min. Competent cells were snap-frozen in liquid N_2 and stored in 100 µl aliquots at -80°C.

***(v) DNA transformation*:**

Frozen competent cells (100 µl) of *E. coli* XL1-Blue were thawed on ice and gently mixed. DNA ligation mix (10 µl) was added to the cell suspension and incubated on ice for a further 30 min. Cells were heat-shocked for 1 min at 42°C and immediately returned to ice for 2 min. One ml of LB was added to the tubes which were subsequently incubated for 1 h at 37°C with shaking at 160 rpm. Cells were harvested by centrifugation at 10,000 rpm for 1 min, resuspended in 100 µl LB, and spread onto LB agar plates containing 100 µg/ml ampicillin, 40 µg/ml X-gal, and 12.5 µg/ml IPTG. Plates were incubated overnight at 37°C, and white colonies were picked using sterile toothpicks and transferred to LB media containing 100 µg/ml ampicillin. Liquid cultures were incubated at 37°C overnight with shaking.

***(vi) Plasmid DNA preparation*:**

Cells (2ml) were harvested by centrifugation at 10,000 rpm for 15 min and resuspended in 200 µl GTE buffer (25 mM Tris-HCl buffer, pH 8.0, containing 50 mM glucose and 10 mM EDTA). Following incubation on ice for 5 min, 300 µl freshly prepared 0.2 M NaOH/1% SDS solution was added to the cells and gently mixed. After a further 10 min on ice, 3 M potassium acetate buffer, pH 4.8, was added to the tube with gentle mixing for 10 min. The soluble cell lysate was collected by centrifugation at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and 2 µl 10 mg/ml RNase A was added. Tubes were incubated at 37°C for 20 min and the digestion mixture was extracted twice with 400 µl chloroform. The resulting aqueous (upper) phase was transferred to a fresh tube and plasmid DNA was precipitated using 1 vol 100% isopropanol. The DNA was collected by centrifugation at 10,000 rpm for 10 min and the resulting pellet was washed with 70% (v/v) ethanol and air-dried. The dried pellet was resuspended in 32 µl sterile water and 8 µl 4 M NaCl, and plasmid DNA was precipitated by adding 40 µl of 13%

PEG_{8000}. Tubes were mixed thoroughly, incubated on ice for 20 min, and centrifuged at 10,000 rpm for 30 min at 4°C. The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in sterile water. To check that the plasmid contained an insert of the expected size, plasmid DNA (0.5 μg) was digested with *Nde*I and *Sac*II restriction enzymes for 1 h at 37°C. The digested DNA was mixed with 0.1 vol DNA loading buffer containing 25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, and 40% (w/v) sucrose. Samples were analysed by 1% agarose gel electrophoresis essentially as described by Sambrook *et al.* (1989). The 1kb ladder (Gibco BRL) was used as a DNA standard. Expression plasmids containing the (1→3)-β-glucanase isoenzyme GII cDNA and the (1→3,1→4)-β-glucanase isoenzyme EII cDNA, were designated pET3a-HT/GII and pET3a-HT/EII, respectively.

***(vii) Nucleotide sequence analysis*:**

Expression plasmids pET3a-HT/EII and pET3a-HT/GII were sequenced to confirm their identity. Sequencing was performed using a DNA sequencing kit (Sequenase Version 2.0, US Biochemical Corporation) according to the manufacturer's instructions. The sequencing kit is based on the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), and incorporates [α-^{35}S]-dATP as the radioactive label. Plasmid DNA templates were denatured in 2M NaOH/1mM EDTA for 30 min and recovered using Sepharose CL6B (Pharmacia LKB Biotechnology) spin columns. Denatured DNA was used as a template for the sequencing reaction and reaction products were separated by electrophoresis using 6% (w/v) polyacrylamide gels containing 46% (w/v) urea. Alternatively, automated DNA sequencing was carried out by the Nucleotide and Protein Chemistry Analysis Unit in the Department of Plant Science at the University of Adelaide, South Australia. Automated sequencing reactions were performed using the Applied Biosystems PRISM Dye Terminator Cycle Sequencing Reaction Kit. Computer analysis of automated sequencing data was performed using Version 1.0.3 of the SeqEd program (Applied Biosystems).

2.2.3 *Expression of Recombinant Fusion Proteins*

For expression, pET3a-HT/GII and pET3a-HT/EII were independently transformed into *E. coli* BL21 (DE3) pLysS using the standard transformation protocol (Section 2.2.2) with the following modifications: *E. coli* BL21 (DE3) pLysS cells were grown in the presence of 25 μg/ml chloramphenicol and, following transformation, cells were plated out on LB agar plates containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Plates were incubated overnight at 37°C.

An entire plate of transformed cells was resuspended in 3-4 ml LB. Expression medium was prepared as follows: 50 ml of a sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 was added to 450 ml of autoclaved Terrific Broth (containing 6 g bacto-tryptone, 12 g bacto-yeast extract, and 2 ml glycerol in 450 ml sterile water) in a 2 l Erlenmeyer conical flask. One ml of the cell suspension was added to the media, and cells were grown at 37°C in the presence of 100 μg/ml ampicillin and 25 μg/ml chloramphenicol, with shaking at 250 rpm. When the OD_{600} reached approximately 0.6, a 1 ml aliquot of the cells was centrifuged and the pellet was resuspended in 50 μl SDS-loading buffer (50 mM Tris-HCl buffer, pH 6.8, containing 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% (v/v) glycerol). The resuspended cells, representing the 'uninduced' sample, were frozen at -20°C. Expression was induced with the addition of IPTG to a final concentration of 1.0 mM or 0.25 mM, and incubation was continued at either 23°C or 37°C for up to 4 h. At the end of the incubation period, a 1 ml aliquot was centrifuged and the pellet was resuspended in 100 μl SDS-loading buffer and stored at -20°C as the 'induced' sample. The remaining cells were transferred to 50 ml centrifuge tubes and harvested by centrifugation at 3,000 rpm for 10 min at 4°C. Cell pellets were drained and stored at -20°C.

2.2.4 *Purification of Recombinant Fusion Proteins*

For clarity, the fusion proteins expressed from plasmids pET3a-HT/EII and pET3a-HT/GII will be referred to as recombinant (1→3,1→4)-β-glucanase and recombinant (1→3)-β-

glucanase, respectively. Frozen cell pellets containing recombinant (1→3,1→4)-β-glucanase or (1→3)-β-glucanase were thawed on ice for 15 min and resuspended in 1 ml 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl, 25 mM imidazole and 1 mM PMSF. The viscous cell suspension was incubated on ice for a further 30 min, and complete cell lysis was facilitated by sonication for 30 sec. The resulting cell lysate was centrifuged at 10,000 rpm for 20 min at 4°C to remove insoluble cell debris and inclusion bodies. To purify the expressed protein under non-denaturing conditions, 600 μl of the soluble cell lysate was loaded onto a Ni-NTA spin column which had been pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl and 25 mM imidazole (Buffer I). The column was centrifuged for 2 min at 2,000 rpm, and washed twice with 600 μl Buffer I at room temperature. The bound fusion protein was eluted from the column using 200 μl Buffer I adjusted to 200 mM imidazole. The eluted protein was collected in a 2 ml plastic tube and stored on ice. Protein yield was determined spectrophotometrically using the Coomassie Protein Assay Reagent (Pierce), and bovine serum albumin (BSA) as a standard.

2.2.5 Cleavage of Barley (1→3,1→4)-β-Glucanase Fusion Protein with Thrombin

The polyhistidine tag was removed from the COOH-terminus of the recombinant (1→3,1→4)-β-glucanase by proteolytic cleavage with thrombin. Purified recombinant (1→3,1→4)-β-glucanase (0.5-1 mg) was concentrated and transferred to thrombin cleavage buffer (20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, and 2.5 mM $CaCl_2$) using ultrafiltration (Amicon). The cleavage reaction was performed at 20°C in a 1 ml reaction volume of thrombin cleavage buffer, using a thrombin:recombinant (1→3,1→4)-β-glucanase molar ratio of approximately 1:100. Aliquots were removed periodically for up to 24 h and the extent of protein cleavage was measured by SDS-PAGE. To remove the histidine tag and any uncleaved recombinant (1→3,1→4)-β-glucanase, the cleavage reaction mixture was passed down a Ni-NTA column (Qiagen). Thrombin was removed from the unbound protein fraction using a benzamidine resin (Pharmacia LKB Biotechnology) which specifically binds trypsin-like proteases, including

thrombin. Binding of thrombin to the resin was performed in a 2 ml plastic tube for 30 min at 4°C with gentle rocking. The benzamidine resin was collected by centrifugation at 2,000 rpm for 1 min. The supernatant (unbound fraction) consisted of cleaved, purified recombinant (1→3,1→4)-β-glucanase.

2.2.6 SDS-PAGE

SDS-PAGE was performed on a 12.5% polyacrylamide gel essentially as described by Laemmli (1970). Samples were mixed with an equal volume of SDS-loading buffer (0.12 M Tris-Cl buffer, pH 6.8, containing 20% (v/v) glycerol, 4% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) Bromophenol Blue), boiled for 5 min, loaded onto a polyacrylamide gel, and separated by electrophoresis at a constant current of 30 mA. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in ethanol:acetic acid:water (40:7:53) and destained in a solution containing 20% (v/v) ethanol and 7% (v/v) acetic acid. Protein molecular weight markers (Pharmacia LKB Biotechnology) contained phosphorylase b (M_r 95,000), BSA (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,000), and α-lactalbumin (M_r 14,400).

2.2.7 (1→3,1→4)-β-Glucanase and (1→3)-β-Glucanase Activity

(1→3,1→4)-β-Glucanase activity was assayed reductometrically (Nelson, 1944; Somogyi, 1952) at 37°C using 0.2% (w/v) barley (1→3,1→4)-β-glucan (Deltagen) as a substrate. The substrate was dissolved by boiling in 50 mM sodium acetate buffer, pH 5.0, for 30 min. After boiling, the substrate was centrifuged briefly at 2,000 rpm to remove any undissolved material, and transferred to a fresh tube. After cooling the dissolved substrate to room temperature, BSA was added to a final concentration of 200 μg/ml. (1→3)-β-Glucanase activity was assayed reductometrically under the same conditions, using 0.2% (w/v) laminarin from *Laminaria digitata* (Sigma) as a substrate. Activity assays were performed at 37°C by adding 50 μl of appropriately diluted enzyme to 250 μl substrate in glass test tubes. Reactions were stopped with 250 μl alkaline copper reagent (Somogyi, 1952) and tubes were boiled for 10 min. After cooling in cold water, 250 μl arsenomolybdate reagent (Nelson, 1944) was added to develop the blue colour, which is

indicative of reducing sugar production. Absorbance was measured at 660 nm against substrate and enzyme blanks. Glucose was used as a standard. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol reducing equivalents per minute. Enzyme assays were performed using barley (1→3,1→4)-β-glucan as a substrate over the concentration range 0.2 to 2.0 mg/ml in 50 mM sodium acetate buffer, pH 5.0, at 37°C. The kinetic parameters K_m, V_{max} and k_{cat} were derived from Lineweaver-Burk plots.

2.3 Results and Discussion

2.3.1 Construction of Expression Plasmids

The cDNA coding regions of barley (1→3,1→4)-β-glucanase isoenzyme EII (Fincher *et al.*, 1986; Slakeski *et al.*, 1990) and (1→3)-β-glucanase isoenzyme GII (Høj *et al.*, 1989; Xu *et al.*, 1992) were amplified by PCR in order to introduce *Nde*I and *Sac*II restriction enzyme sites at their 5' and 3' ends, respectively (Figure 2.2). The PCR products were digested with *Nde*I and *Sac*II, and the digested fragments were ligated into the corresponding *Nde*I and *Sac*II sites of the pET3a-HT expression vector (Figure 2.1). The resulting expression vectors, pET3a-HT/EII and pET3a-HT/GII, were used to direct the expression of the recombinant (1→3,1→4)-β-glucanase isoenzyme EII, and recombinant (1→3)-β-glucanase isoenzyme GII, respectively. At the 3' end of the cDNA insertion site, both expression plasmids contained a sequence encoding a thrombin cleavage recognition site and a polyhistidine tag (Figure 2.1). Automated DNA sequencing of both strands showed that the ligation sites and cDNA inserts of pET3a-HT/GII and pET3a-HT/EII contained no errors (data not shown).

2.3.2 Expression and Purification of Recombinant Fusion Proteins

The pET3a-HT/EII and pET3a-HT/GII expression vectors were independently transformed into *E. coli* BL21 (DE3) cells for expression. Four hours after the induction of expression, cells were collected by centrifugation and lysed. Expressed recombinant proteins were purified from the soluble lysate by affinity chromatography using Ni-NTA resin. Uninduced, induced, soluble and pure protein fractions were analysed by SDS-PAGE (Figure 2.4).

The pET3a-HT/EII plasmid directed the synthesis of a protein with an approximate molecular mass of 33.5 kDa (Figures 2.3 and 2.4). This value corresponds with the calculated molecular mass for barley (1→3,1→4)-β-glucanase isoenzyme EII (approximately 32.5 kDa) fused to the polyhistidine tag (approximately 1.0 kDa). Four

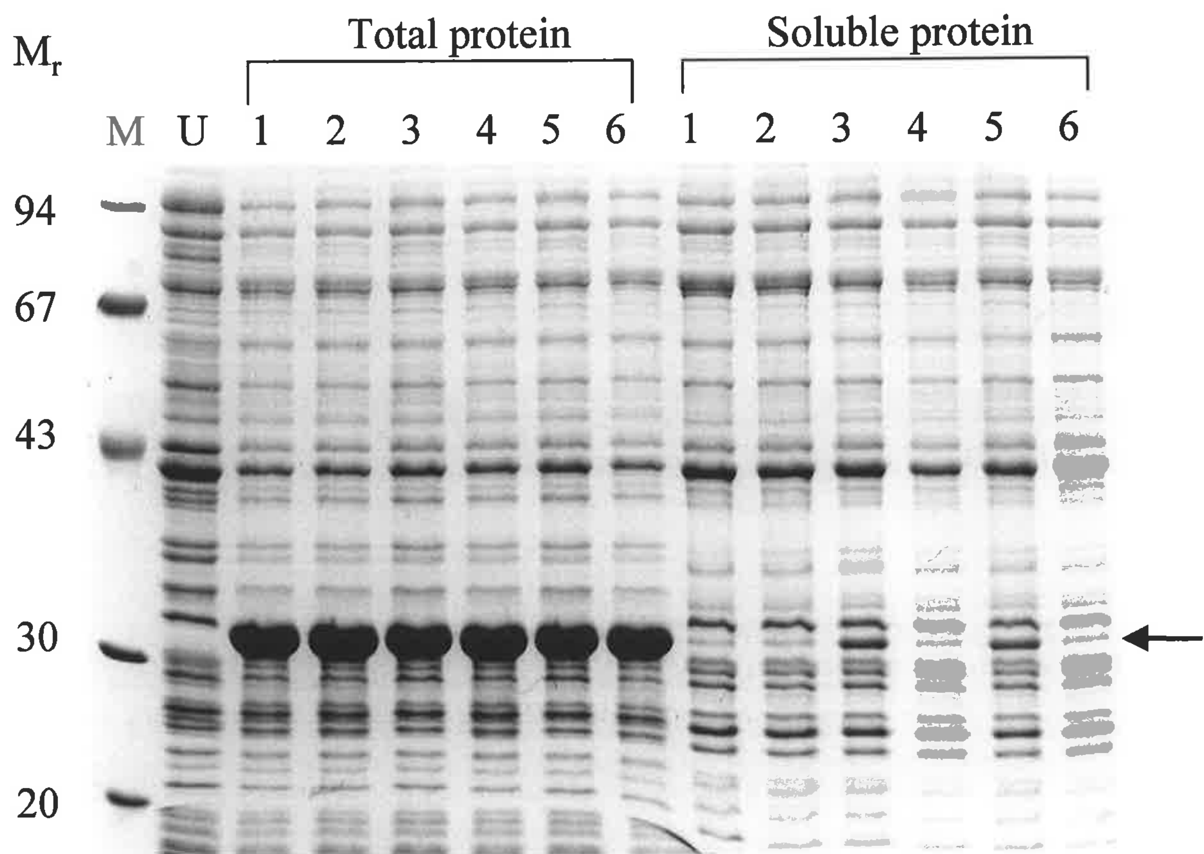
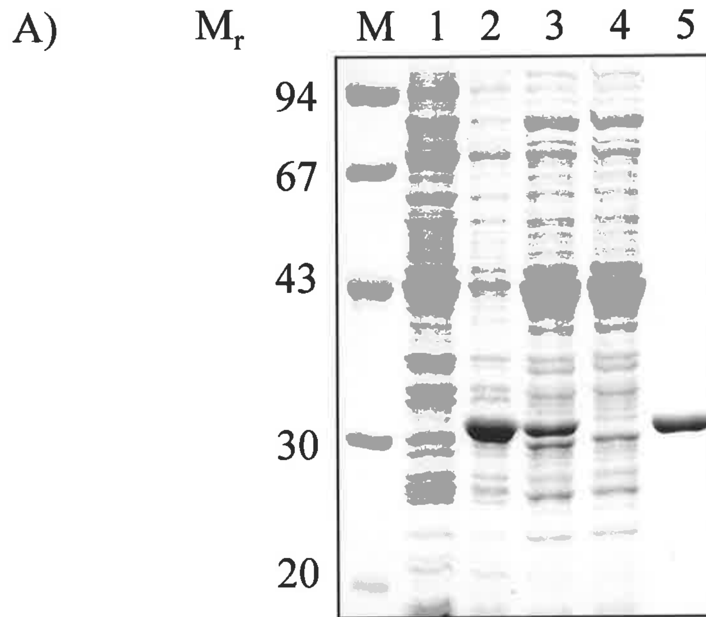


Figure 2.3 The heterologous expression of barley (1→3,1→4)-β-glucanase isoenzyme EII in *E. coli*. SDS-PAGE analysis of total and soluble proteins in the bacterial cell lysate after expression was induced with IPTG for 3 h under the following conditions: 1, 1 mM IPTG at 37°C with 660 mM glycine betaine; 2, 1 mM IPTG at 37°C in LB; 3, 1 mM IPTG at 22°C in Terrific Broth; 4, 1 mM IPTG at 37°C in Terrific Broth; 5, 0.25 mM IPTG at 22°C in Terrific Broth; 6, 0.25 mM IPTG at 37°C in Terrific Broth. U, bacterial cell lysate prior to induction. The arrow indicates the band corresponding to the expressed fusion protein. M represents Protein molecular weight markers.



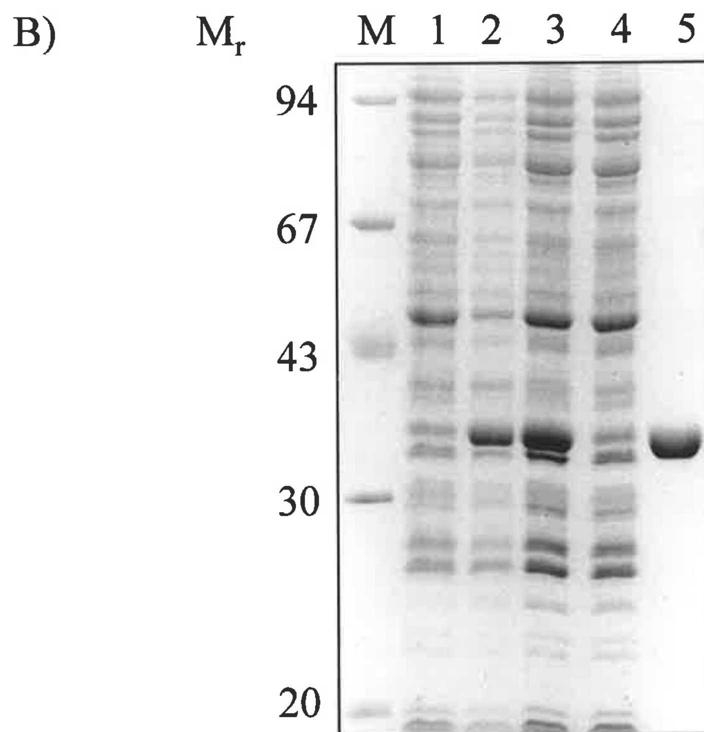


Figure 2.4 The heterologous expression and purification of barley (1→3,1→4)-β-glucanase isoenzyme EII (A) and barley (1→3)-β-glucanase isoenzyme GII (B). SDS-PAGE was used to analyse the following protein fractions: Lane 1, total cell lysate before induction; Lane 2, total cell lysate after induction; Lane 3, soluble fraction of the bacterial cell lysate; Lane 4, Ni-NTA column unbound fraction; Lane 5, eluted protein (bound fraction); M, molecular weight markers.

hours after the induction of expression at 37°C, more than 85% of the (1→3,1→4)-β-glucanase fusion protein was found in the insoluble fraction as inclusion bodies (data not shown). In an attempt to increase the proportion of active recombinant (1→3,1→4)-β-glucanase in the soluble fraction, adjustments were made to the expression conditions, including IPTG concentration, medium composition and temperature.

Studies have shown that the presence of glycine betaine can increase the solubility of expressed proteins in *E. coli* by placing the cells under osmotic stress (Blackwell and Horgan, 1991). However, the addition of glycine betaine (660 mM) to the expression medium had little effect on the solubility of the recombinant (1→3,1→4)-β-glucanase at 37°C (Figure 2.3). Lowering the IPTG concentration slows the rate of expression in *E. coli*, which can significantly increase the solubility of expressed foreign proteins (Qiaexpressionist product literature, Qiagen). However, inducing expression of the recombinant (1→3,1→4)-β-glucanase using 0.25 mM IPTG instead of 1 mM IPTG, had no effect on the solubility of the expressed protein (Figure 2.3). Similarly, replacing the normal growth media (Terrific Broth) with minimal media (LB) did not change protein solubility as anticipated (Figure 2.3; Moore *et al.*, 1993).

The solubility of expressed proteins in *E. coli* can be increased by growing the cells at temperatures below 37°C (Schein and Noteborn, 1988; Haase-Pettingell and King, 1988; Piatak *et al.*, 1988). For example, human interferon-α2 is completely insoluble when produced in *E. coli* at 37°C, but at growth temperatures below 30°C the majority of protein is soluble and active (Schein and Noteborn, 1988). The solubility of several plant proteins expressed in *E. coli* using the pET system has also been dramatically improved using low growth temperatures. For example, expression of a recombinant wheat serpin in *E. coli* at 37°C resulted in the predominant formation of inclusion bodies, but when the expression temperature was reduced to 15°C, more than 50% of the recombinant protein was soluble and active (Rasmussen *et al.*, 1996). In another study with the pET expression system, the specific activity of a de-branching enzyme from maize (*Zea mays* L.) was increased two-

fold when the expression temperature was reduced from 37°C to 25°C (Guan *et al.*, 1994). In contrast, lowering the cell growth temperature to as low as 16°C did not improve the solubility of the inherently unstable barley (1→4)-β-xylanase isoenzyme X-I when expressed in *E. coli* using the pET system (Banik, 1996).

In an attempt to increase the solubility of the recombinant (1→3,1→4)-β-glucanase, the expression temperature was decreased from 37°C to 23°C. Lowering the temperature significantly increased the solubility of the recombinant (1→3,1→4)-β-glucanase, with at least five times the amount of recombinant protein residing in the soluble fraction following expression at 23°C, compared with 37°C (Figures 2.3 and 2.4). For subsequent experiments involving recombinant (1→3,1→4)-β-glucanases, expression was routinely induced with 0.25 mM IPTG at 23°C for four hours.

The pET3a-HT/GII vector directed the synthesis of a protein with a molecular mass of approximately 34 kDa protein (Figure 2.4), which is consistent with the calculated molecular mass for barley (1→3)-β-glucanase isenzyme GII (approximately 33 kDa) fused to the thrombin cleavage recognition site and polyhistidine tag (approximately 1 kDa). Although a majority of the recombinant (1→3)-β-glucanase formed insoluble aggregates following expression at 37°C (data not shown), there was sufficient protein located in the soluble fraction to allow the purification of relatively large amounts of active enzyme (up to 8 mg/litre culture). Expression of recombinant (1→3)-β-glucanases was routinely induced with 0.25 mM IPTG for 3 hours at 37°C.

2.3.3 *(1→3,1→4)-β-Glucanase and (1→3)-β-Glucanase Activity and Kinetics*

The recombinant (1→3)-β-glucanase fusion protein, which was purified in yields of up to 12.0 mg of protein per litre of bacterial culture, had a specific activity of 128.0 units/mg (Table 2.1). In a previous study, barley (1→3)-β-glucanase isoenzyme GII was expressed in *E. coli* as a fusion protein with the Maltose Binding Protein (MBP) fused to its NH_2-terminus. That fusion protein had a specific activity of 125 units/mg (Chen *et al.*, 1993,

Table 2.1 Kinetic parameters and specific activities for recombinant and native barley (1→3,1→4)-β-glucanase isoenzyme EII and (1→3)-β-glucanase isoenzyme GII. Activity assays were performed reductometrically and 1 unit of activity is defined as the amount of enzyme required to release 1 μmol of reducing equivalents per minute at 37°C. 'EII' and 'GII' refer to (1→3,1→4)-β-glucanase isoenzyme EII and (1→3)-β-glucanase isoenzyme GII, respectively. 'Rec.' denotes recombinant enzymes which were produced in *E. coli.* Recombinant enzymes were fused to either a poly-histidine tag (+histidine) or a Maltose Binding Protein (+MBP). The native barley enzymes were extracted from germinated barley grain using conventional purification methods.

Enzyme:	Specific Activity (units/mg)	K_m (mg/ml)	k_{cat} (sec^{-1})
(1→3,1→4)-β-Glucanases			
Native barley isoenzyme EII[c]	16.0	3.4	193.0
Rec. EII + histidine tag	14.5	1.2	156.0
Rec. EII – histidine tag	13.8	n/d	n/d
(1→3)-β-Glucanases			
Native barley isoenzyme GII[b]	145.0	0.7	85.0
Rec. GII + MBP[a]	125.0	0.8	97.0
Rec. GII + histidine tag	128.0	n/d	n/d

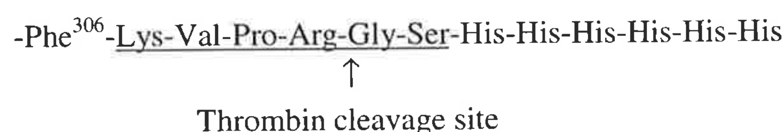
[a] From Chen *et al.*, 1993, 1995
[b] From Høj *et al.*, 1989
[c] From Woodward and Fincher, 1982a
n/d: not determined

1995) which is similar to the value obtained for the recombinant (1→3)-β-glucanase in the current study. The specific activities reported for the recombinant (1→3)-β-glucanase are slightly lower than the 145.0 units/mg reported for native barley (1→3)-β-glucanase isoenzyme GII (Table 2.1; Høj *et al.*, 1989). However, the conditions under which enzyme activity and protein yield are measured can vary between experiments, and the specific activity of the recombinant (1→3)-β-glucanases was therefore considered to be satisfactory.

The recombinant (1→3,1→4)-β-glucanase was purified from *E. coli* in yields of approximately 5.0 mg of protein per litre of bacterial culture, and had a specific activity of 14.5 units/mg, which is similar to the specific activity (16.0 u/mg) of the native barley (1→3,1→4)-β-glucanase isoenzyme EII (Table 2.1; Woodward and Fincher, 1982b). The kinetic parameters for the recombinant (1→3,1→4)-β-glucanase were determined at 37°C using 0.2-2.0 mg/ml barley (1→3,1→4)-β-glucan as a substrate. The recombinant (1→3,1→4)-β-glucanase had a K_m value of 1.3 mg/ml, which is slightly lower than that of native barley (1→3,1→4)-β-glucanase isoenzyme EII, which has a K_m of 3.4 mg/ml (Woodward and Fincher, 1982b). The k_{cat} values for the recombinant (1→3,1→4)-β-glucanase and the native barley (1→3,1→4)-β-glucanase isoenzyme EII, were 156.0 sec^{-1} and 193.0 sec^{-1}, respectively (Table 2.1). It should be noted that for different enzyme analyses, substrate preparation methods and assay procedures are often different and direct comparisons of kinetic parameters between experiments must therefore be made with a degree of caution.

2.3.4 Removal of the Polyhistidine Tag with Thrombin

The recombinant (1→3,1→4)-β-glucanase expressed in *E. coli* contained a thrombin cleavage recognition site (underlined) and polyhistidine tag (6 x His) fused to its COOH-terminal residue (Phe^{306}) as follows:



Analysis of the three-dimensional structure of barley (1→3,1→4)-β-glucanase isoenzyme EII (Figure 4.1) reveals that the terminal carboxyl group of Phe^{306} in the native enzyme is pointing outwards into the solvent. The COOH- terminal fusion of the recombinant (1→3,1→4)-β-glucanase, which remains essentially uncharged at pH 8.0, should therefore protrude into the solvent. In addition, the COOH-terminal residue Phe^{306} of the native enzyme is positioned on the opposite side of the enzyme to the substrate-binding groove, and so the presence of a polyhistidine extension should not interfere with substrate binding, and would therefore not adversely affect enzyme activity.

To confirm predictions that the presence of the polyhistidine tag would not interfere unduly with the behaviour of the recombinant (1→3,1→4)-β-glucanase, the tag was removed by proteolytic cleavage with thrombin. Because thrombin cleaves between the arginine (R) and glycine (G) residues of the thrombin recognition sequence LVPRGS (Stryer, 1988), the LVPR residues remain fused to the COOH-terminus of the (1→3,1→4)-β-glucanase following proteolytic cleavage. Thus, thrombin effectively removes only 8 of 12 residues that constitute the COOH-terminal fusion.

The extent of cleavage of the recombinant protein with thrombin was followed by SDS-PAGE over 24 hours (Figure 2.5). Partial cleavage of the polyhistidine tag is evident after six hours of digestion, but 24 hours of digestion is required to achieve complete cleavage (Figure 2.5). After 24 hours of digestion, the products were passed through a Ni-NTA spin column and the cleaved recombinant (1→3,1→4)-β-glucanase was collected in the non-bound fraction. Thrombin was removed using benzamidine resin, which specifically binds serine proteases including thrombin. There was no significant difference between the specific activities of the cleaved and uncleaved recombinant (1→3,1→4)-β-glucanase (Table 2.1). Thus, removal of the polyhistidine tag prior to performing (1→3,1→4)-β-glucanase activity assays was deemed unnecessary.

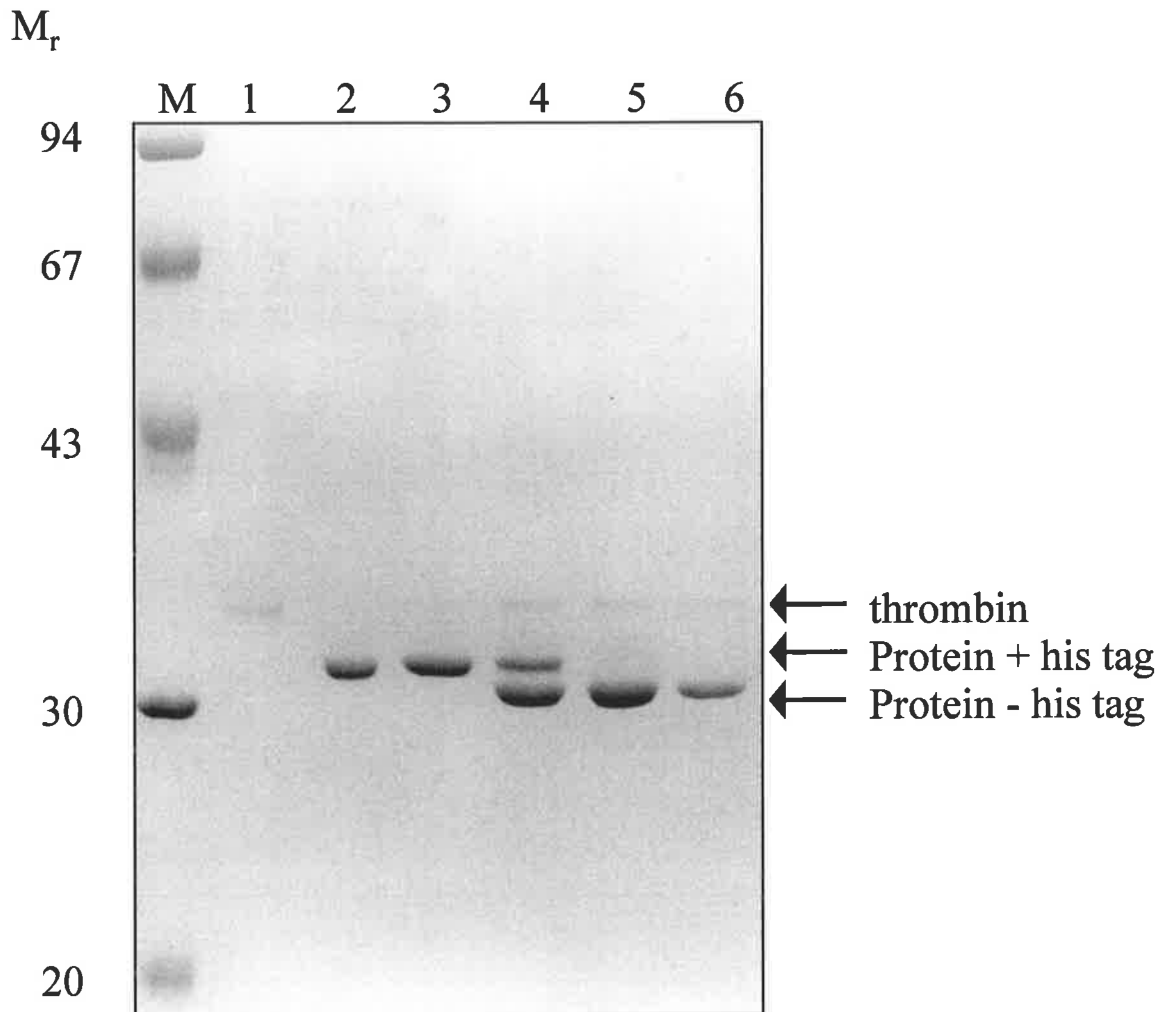


Figure 2.5 Removal of the histidine tag from a purified (1 →3,1 →4)-β-glucanase fusion protein. Barley (1 →3,1 →4)-β-glucanase isoenzyme EII was expressed in *E. coli* as a fusion protein containing a polyhistidine tag at its COOH-terminus to facilitate purification (see text for details). The histidine tag was removed by digestion with the protease thrombin, and the reaction was analysed by SDS-PAGE. 1, pure thrombin; 2, pure (1→3,1→4)-β-glucanase fusion protein; 3, digestion time of 0 h; 4, digestion time of 6 h; 5, digestion time of 24 h; 6, the protein fraction after passing the digested protein mix through a Ni-NTA column to remove the cleaved polyhistidine tag; M, molecular weight markers.

2.4 Summary and Conclusions

The pET system was successfully used to express cDNAs encoding barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII in *E. coli*, and to purify the enzymes in an active form. The recombinant (1→3)-β-glucanase had a similar specific activity to (1→3)-β-glucanase isoenzyme GII purified from barley. The recombinant (1→3,1→4)-β-glucanase also displays similar kinetic parameters to those of the native barley (1→3,1→4)-β-glucanase isoenzyme EII. Furthermore, the specific activity of the recombinant (1→3,1→4)-β-glucanase was not significantly affected by the presence of the polyhistidine tag with thrombin. It was concluded that the pET system represented an ideal method for the expression and purification of recombinant barley (1→3)- and (1→3,1→4)-β-glucanases in relatively high yields, and that thrombin cleavage prior to enzyme assays was not necessary.

CHAPTER THREE:

ALTERING THE SUBSTRATE SPECIFICITY OF BARLEY (1→3)-β-GLUCANASE ISOENZYME GII

3.1 Introduction

The three-dimensional structures of barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII have been solved to high resolution by x-ray crystallography (Varghese *et al.*, 1994). The C^{α} chains of the two enzymes are almost 100% superimposable, with a mean rms deviation of 0.65Å over 280 residues (Figure 3.1). Furthermore, the enzymes share 52% positional identity (Figure 3.2) and there is now compelling evidence to suggest that they result from the divergent evolution of a common ancestral enzyme (Varghese *et al.*, 1994; Høj and Fincher, 1995). Despite the similarities in their primary sequences and three-dimensional structures, the two enzymes have vastly different functions. (1→3,1→4)-β-Glucanases in germinated barley are primarily involved in endosperm cell wall degradation (Section 1.3.1.1), whereas the (1→3)-β-glucanases are probably involved in protecting the grain against pathogen attack (Section 1.4).

In describing the high degree of structural similarity between the (1→3)-β-glucanase and (1→3,1→4)-β-glucanase and their obvious evolutionary relatedness, Varghese *et al.* (1994) noted that the differences in substrate specificity and function were achieved without any major changes in the conformation of the polypeptide backbone (Figure 3.1). Instead, detailed analyses of the substrate-binding grooves of barley (1→3)-β-glucanase isoenzyme GII and barley (1→3,1→4)-β-glucanase isoenzyme EII (Figure 3.3) reveal that a relatively small number of amino acid differences are probably responsible for the distinct substrate specificities of the enzymes (Varghese *et al.*, 1994). The structural similarities between the two enzymes provide an opportunity to simulate evolution by changing the substrate specificity of a barley (1→3)-β-glucanase to that of a (1→3,1→4)-β-glucanase (Høj and Fincher, 1995). Furthermore, because barley (1→3)-β-glucanase isoenzyme GII is significantly more thermostable than barley (1→3,1→4)-β-glucanase isoenzyme EII (Figure 3.4; Chen *et al.*, 1995), altering the specificity of (1→3)-β-glucanase isoenzyme GII could result in the production of a 'hybrid' barley (1→3,1→4)-β-glucanase with increased thermostability.

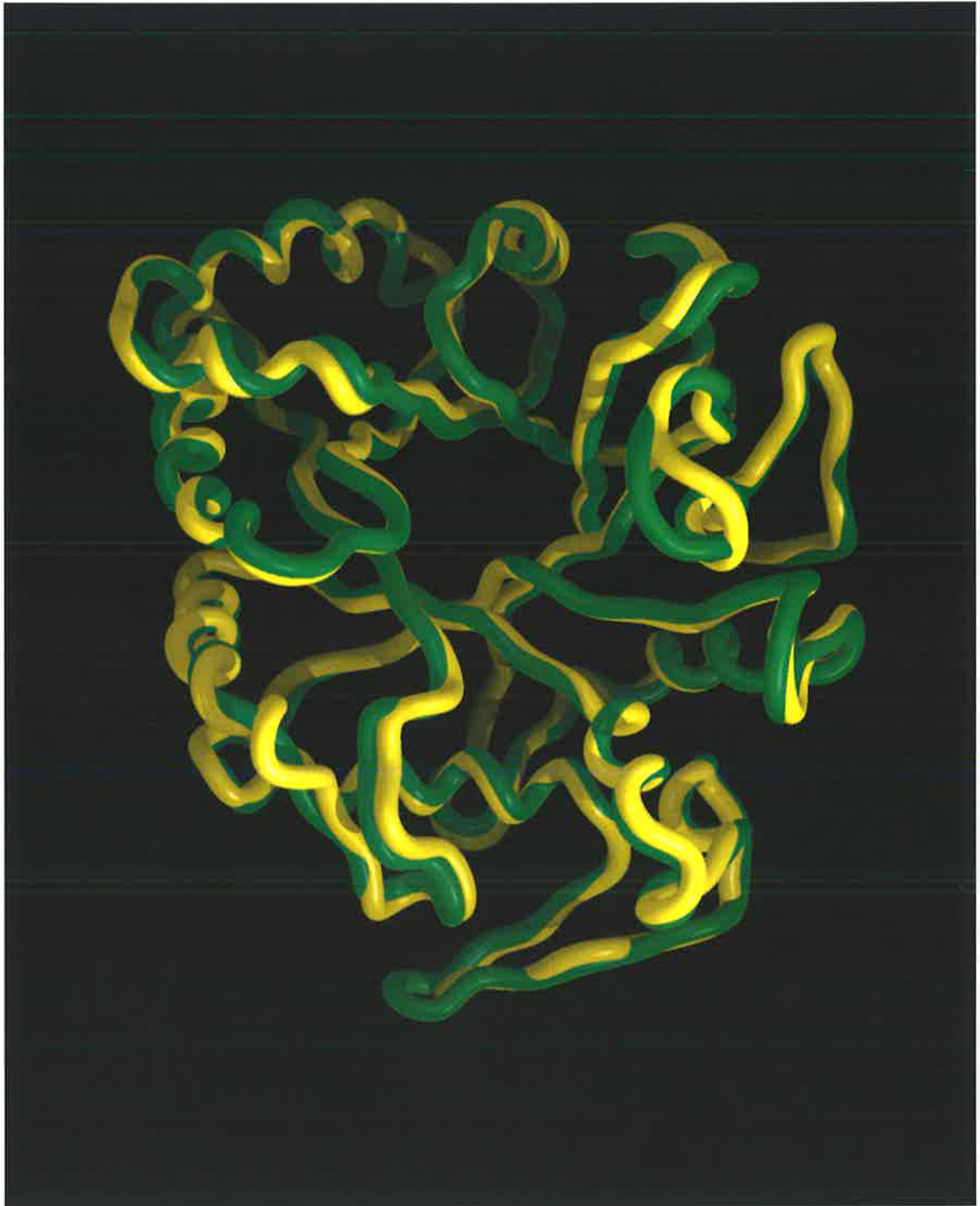


Figure 3.1 A superimposition of the polypeptide backbone of barley (1→3)-β-glucanase isoenzyme GII (green) and barley (1→3,1→4)-β-glucanase isoenzyme EII (yellow) showing the similarity of the two backbones. This figure was reproduced from Varghese *et al.* (1994).

Figure 3.2 An alignment of the amino acid sequences of barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII. The alignment was performed using PILEUP in the University of Wisconsin GCG suite of programs (Devereux *et al.*, 1984). A boxed cross (☒) marks positions where amino acid substitutions were introduced into barley (1→3)-β-glucanase isoenzyme GII. The conserved catalytic amino acids for both enzymes are marked with an asterix (*), and amino acid residues are numbered as follows: e2 residue / g2 residue. Identical amino acid residues are shown in bold.

```
    1                                                                                     60/60
e2  IGVCYGMSAN NLPAASTVVS MFKSNGIKSM RLYAPNQAAL QAVGGTGINV VVGAPNDVLS
g2  IGVCYGVIGN NLPSRSDVVQ LYRSKGINGM RIYFADGQAL SALRNSGIGL ILDIGNDQLA

    61/61                                                                            119/120
e2  NLAASPAAAA SWVKSNIQAY .PKVSFRYVC VGNEVAGGAT RNLVPAMKNV HGALVAAGLG
g2  NIAASTSNAA SWVRNNVRPY YPAVNIKYIA AGNEVQGGAT QSILPAMRNL NAALSAAGLG
                                       *

    120/121                                                                          179/177
e2  HIKVTTSVSQ AILGVFSPPS AGSFTGEAAA FMGPVVQFLA RTNAPLMANI YPYLAWAYNP
g2  AIKVSTSIRF DEVANSFPPS AGVFK..N.A YMTDVARLLA STGAPLLANV YPYFAYRDNP
             ⊠         ⊠                                     ⊠

    180/178                                                                          236/235
e2  SAMDMGYALF NASGTVVRD. ..GAYGYQNL FDTTVDAFYT AMGKHGGSSV KLVVSESGWP
g2  GSISLNYATF QPGTT.VRDQ NNG.LTYTSL FDAMVDAVYA ALEKAGAPAV KVVVSESGWP
                                       ⊠                        *

    237/236                                                                          295/295
e2  SGGGTAATPA NARFYNQHLI NHVGRGTPRH PGAIETYIFA MFNENQK.DS GVEQNWGLFY
g2  SAGGFAASAG NARTYNQGLI NHVGGGTPKK REALETYIFA MFNENQKTGD ATERSFGLFN
                                                           *

    296/296    306/306
e2  PNMQHVYPINF
g2  PDKSPAYNIQF
```

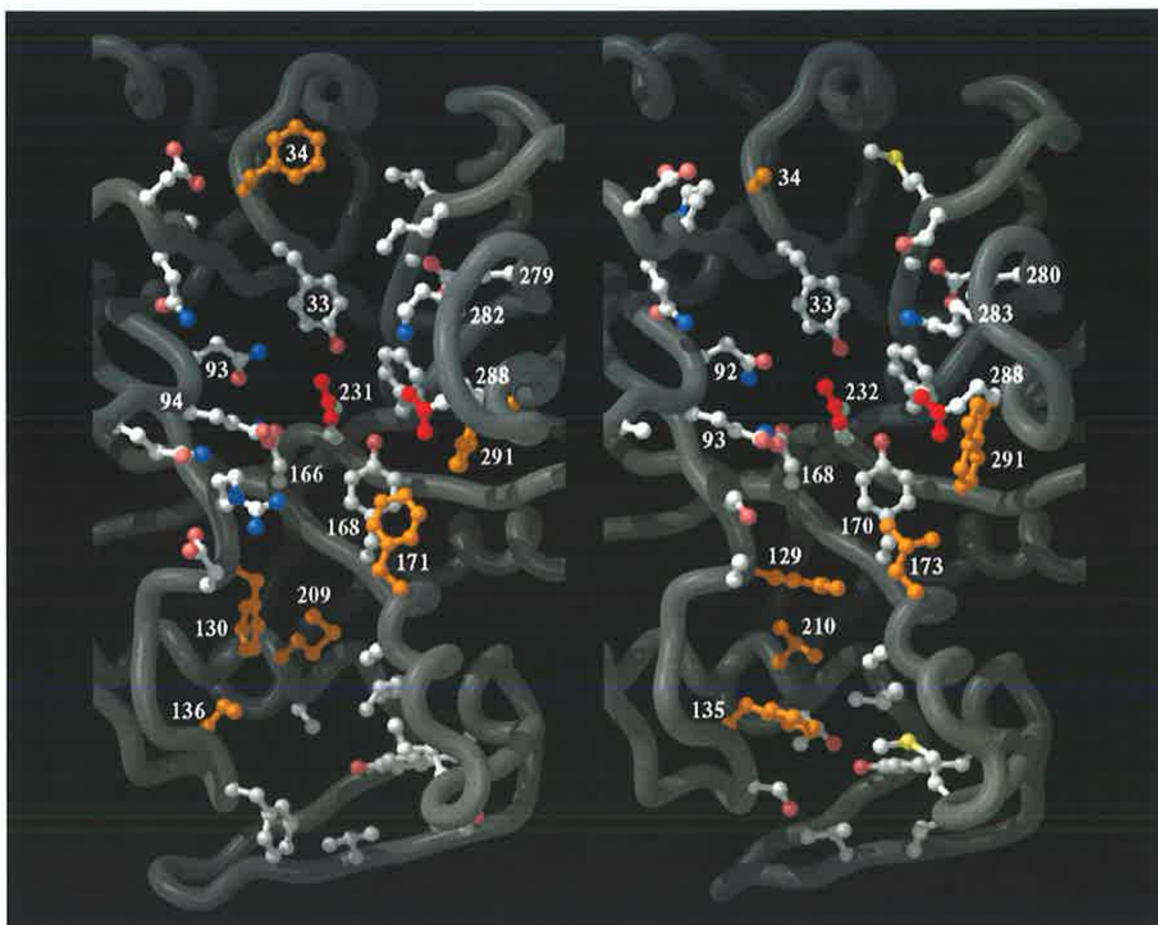


Figure 3.3 The catalytic grooves of barley (1→3)-β-glucanase isoenzyme GII (left) and barley (1→3, 1→4)-β-glucanase isoenzyme EII (right). The catalytic amino acids are shown in red, and the amino acids which potentially affect binding are shown in orange. Side chains of other amino acids which line the grooves are coloured by atom type: C, white; N, blue; O, pink, and S, yellow. This figure was reproduced from Varghese *et al*. (1994).

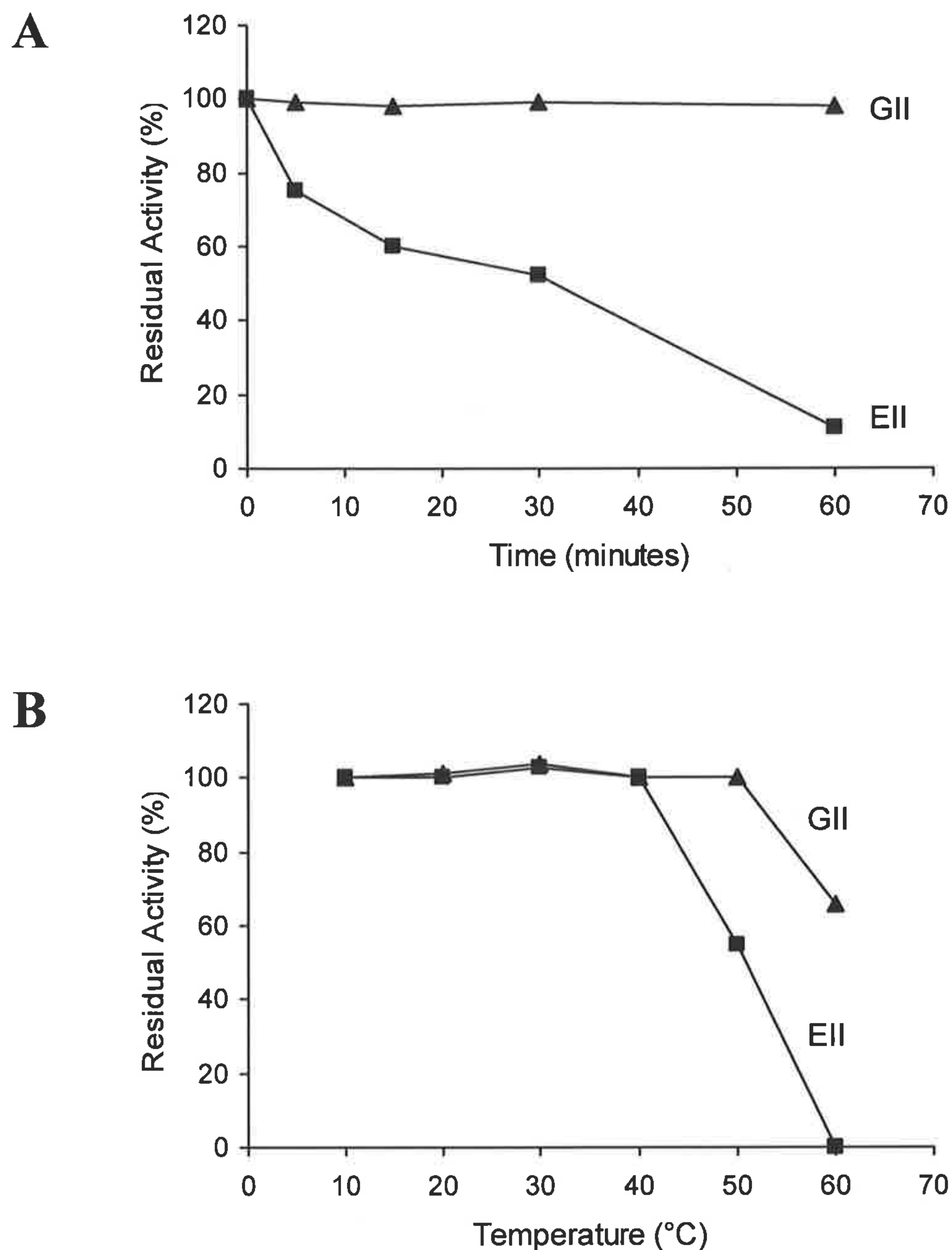


Figure 3.4 The thermostabilities of barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII. A, purified enzymes were incubated at 50°C in 50 mM sodium acetate buffer, pH 5.0, containing 0.5 mg/ml BSA. Samples were taken at regular time intervals and assayed for enzyme activity using 2% (w/v) barley (1→3,1→4)-β-glucan or 2% (w/v) laminarin as a substrate. Residual activity is expressed as a percentage of the initial activity. B, purified enzymes were incubated for 15 min at a range of different temperatures, and assayed as described above. This figure is based on results by Chen *et al.* (1995).

Changing the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII would involve introducing appropriate amino acid substitutions into the substrate-binding groove of (1→3)-β-glucanase isoenzyme GII, and analysing the resulting mutant enzymes for their ability to hydrolyse barley (1→3,1→4)-β-glucan. These amino acid substitutions can be introduced at the cDNA level using site-directed mutagenesis. There are many different site-directed mutagenesis techniques available, most of which are based on the use of PCR (Sarker and Sommer, 1990), or oligonucleotide primer selection (Olsen and Eckstein, 1990, Deng and Nickoloff, 1992).

The Unique Site Elimination (USE) method for site-directed mutagenesis (Deng and Nickoloff, 1992) was chosen for the introduction of mutations into the barley (1→3)-β-glucanase isoenzyme GII cDNA (Figure 3.5). This method utilises two oligonucleotide primers; the 'selection' primer is used to eliminate a unique restriction site from the template DNA plasmid, and the 'mutagenic' primer is used to introduce the desired mutation into the cDNA insert (Deng and Nickoloff, 1992). The template plasmid is first denatured, and the selection and mutagenic primers are annealed simultaneously to the same strand of the single-stranded denatured template DNA. A T4 DNA polymerase is used to direct the synthesis of a new DNA strand containing both mutations, and T4 DNA ligase circularises the strand. The circular DNA is replicated, and plasmids originating from the wildtype strand are linearised by digestion at the unique restriction enzyme site, using the appropriate enzyme. Plasmids originating from the mutant strand do not contain the original unique restriction enzyme site, and therefore survive the digestion. Finally, the mixture of circular mutant DNA and linearised wildtype DNA is transformed into *E. coli*, and because the transformation efficiency of circular DNA is more than 2000 times higher than that of linear DNA (Deng and Nickoloff, 1992), the *E. coli* cells are preferentially transformed with the mutated DNA. Finally, mutant plasmids can be further selected by restriction digest analysis.

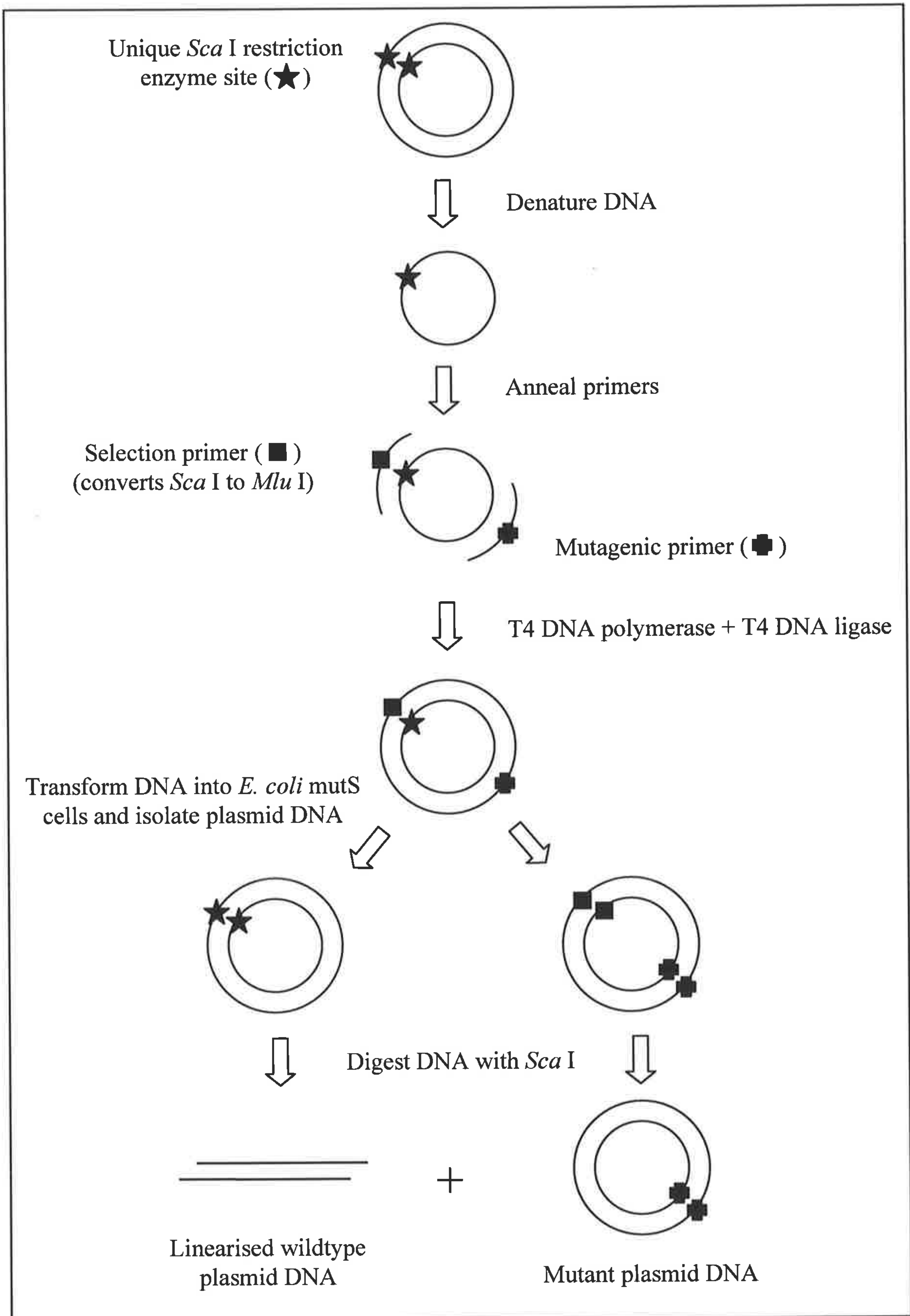


Figure 3.5 The Unique Site Elimination (USE) method for site-directed mutagenesis. This figure was adapted from the USE Mutagenesis Kit product literature (Pharmacia LKB Biotechnology).

The USE method is relatively quick and has a reported mutation rate of greater than 90% (USE Mutagenesis Kit product literature, Pharmacia LKB Biotechnology). The USE method has been used to introduce mutations into the cDNA encoding barley (1→3)-β-glucanase isoenzymc GII in a previous study in our laboratory (Chen *et al.*, 1995).

Attempts to change the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII to generate a relatively heat-stable (1→3,1→4)-β-glucanase, using the USE site-directed mutagenesis method, are described in this Chapter.

3.2 Materials and Methods

3.2.1 Materials

Restriction enzymes and polynucleotide kinase were purchased from New England Biolabs. *E. coli* strains XL1-Blue and BL21 (DE3) were obtained from Stratagene, and Qiagen, respectively. The USE Mutagensis Kit and *E. coli* strain BMH71-18 mutS were from Pharmacia LKB Biotechnology. Coomassie Protein Reagent was from Pierce and protein molecular weight markers were from BioRad. Ni-NTA spin columns were from Qiagen. Barley (1→3,1→4)-β-glucan and laminarin were purchased from Deltagen and Sigma, respectively. Unless otherwise stated, all general chemicals were purchased from Sigma.

3.2.2 The Substrate Binding Grooves of Barley (1→3)- and (1→3,1→4)-β-Glucanases

The substrate binding grooves of barley (1→3)-β-glucanase isoenzyme GII and barley (1→3,1→4)-β-glucanase isoenzyme EII were examined in detail using the 'O' program (Jones *et al.*, 1991), to identify amino acids that are most likely to be involved in substrate specificity. Protein modelling was performed using a Silicon Graphics Indigo Elan 4000 workstation, and major contributions by Drs J.N. Varghese and T.P.J. Garrett (Biomolecular Research Institute, Parkville, Victoria, Australia) are gratefully acknowledged. An amino acid sequence alignment of barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII was performed using PILEUP in the University of Wisconsin GCG suite of programs (Devereux *et al.*, 1984), in order to determine the positional identity of the two enzymes.

3.2.3 Site-Directed Mutagenesis

The USE method for site-directed mutagenesis (Figure 3.5; Deng and Nickoloff, 1992) was used to introduce amino acid substitutions into barley (1→3)-β-glucanase isoenzyme GII at the cDNA level, using plasmid pET3a-HT/GII (Section 2.2.2) as a template. Four mutagenic primers were designed to introduce the amino acid substitutions F130Q, S136F,

F17L and M209T, and a selection primer was designed to convert the unique *Sca*I restriction enzyme site to a *Mlu*I site on the pET3a-HT/GII plasmid (Figure 3.5). Standard nomenclature for the mutants and standard one-letter amino acid codes are used throughout. For example, F130Q indicates a mutant in which a phenylalanine (F) residue at position 130 in the native enzyme is altered to a glutamine (Q) in the mutant enzyme.

All primers were synthesised on an Applied Biosystems DNA Synthesiser and phosphorylated at their 5' end with polynucleotide kinase. Primer sequences are shown below, with the *Mlu*I restriction site in the selection primer and altered codons in the mutagenic primers underlined.

Selection primer (*Sca*I → *Mlu*I):

	5'-CTG TGA CTG GTG ACG CGT CAA CCA AGT C-3'	**AGTACT → ACGCGT**

Mutagenic primers:

F130Q	5'-GGC CAC CTC GTC CTG CCG GAT GGA GG-3'	**TTC → CAG**
F171L	5'-GTC ACG GTA CGC GAG GTA GGG GTA GAC G-3'	**TTC → CTC**
S136F	5'-C GGA GGG CGG GAA GAA GTT GGC CAC CTC G-3'	**TCC → TTC**
M209T	5'-C GGC GTC CAC CGT CGC GTC GAA CAG GG-3'	**ATG → ACG**

For the site-directed mutagenesis reaction, template DNA (0.025 pmol) was mixed with 25 pmol of both the selection and mutagenic primers in 1 X OPA$^+$ buffer (10 mM Tris acetate buffer, pH 7.5, containing 10 mM magnesium acetate and 50 mM potassium acetate) in a total volume of 25 μl. The reaction was boiled for 5 min and immediately cooled on ice for 5 min. Primers were annealed to the denatured plasmid DNA at room temperature for 30 min, before nucleotide mix (7 μl) and reaction mix (3 μl) were added. Nucleotide mix contained 4.34 mM ATP, 1.5 X OPA$^+$ buffer, and 2.86 mM dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology). Reaction mix contained 3 kU/ml T4 DNA ligase, 3 kU/ml T4 DNA polymerase, and 0.6 mg/ml T4 Gene 32 protein (Pharmacia LKB Biotechnology). After thorough mixing, the reaction mixture was incubated at 37°C for 1 h, terminated by heating to 85°C for 15 min, and stored on ice.

The reaction sample was digested with 20 U *Sca*I in a total volume of 50 µl for 4 h. The digested mix (25 µl) was used to transform *E. coli* strain BMH71-18 mutS (Pharmacia LKB Biotechnology) using the standard procedure described in Section 2.2.2 (iv). Transformed cells were added to 4 ml LB containing 100 µg/ml ampicillin and cultured overnight at 37°C with shaking at 200 rpm. Plasmid DNA was isolated from the cell cultures as described in Section 2.2.2 (vi), and 0.5 µg was digested with *Sca*I in a total volume of 50 µl water containing 1X *Sca*I buffer. The digested DNA (25 µl) was used to transform *E. coli* strain XL1-Blue as previously described (see Section 2.2.2, iv-v). Colonies were grown overnight at 37°C on LB agar (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl and 15 g bacto-agar per litre) containing 100 µg/ml ampicillin. Individual colonies were transferred to liquid LB, and cultures were grown overnight at 37°C with shaking at 200 rpm. Plasmid DNA was isolated from cell cultures and digested with 0.5 U *Mlu*I in a total volume of 30 µl water containing 1X *Mlu*I buffer. Plasmids in which the unique restriction enzyme site *Sca*I had been converted to an *Mlu*I site, potentially contained the desired mutations and were therefore selected for automated DNA sequence analysis (see Section 2.2.2, vii).

3.2.4 Expression and Purification of Mutant (1→3)-β-Glucanase Fusion Proteins

Plasmids containing the desired base changes were transformed into *E. coli* strain BL21 (DE3) pLysS using standard procedures (Section 2.2.2, iv-v). Cells were grown in FTB (500 ml) to an OD_{600} of 0.6, and expression was induced using 0.25 mM IPTG as previously described for barley (1→3)-β-glucanase isoenzyme GII (Section 2.2.3). Expression was allowed to proceed for 3 h at 37°C, before cells were collected by centrifugation and stored at –20°C. The mutant and wildtype recombinant (1→3)-β-glucanases were purified using Ni-NTA spin columns as described in Section 2.2.4. Purified proteins were analysed by SDS-PAGE, and diluted with 50 mM sodium acetate buffer, pH 5.0, in preparation for activity assays.

3.2.5 *(1→3,1→4)-β-Glucanase and (1→3)-β-Glucanase Activity*

Purified recombinant (1→3)-β-glucanases were analysed for both (1→3)-β-glucanase and (1→3,1→4)-β-glucanase activity, using as substrates 0.2% (w/v) laminarin (from *L. digitata*) and 0.2% (w/v) barley (1→3,1→4)-β-glucan, respectively. Both substrates were dissolved in 50 mM sodium acetate buffer, PH 5.0, containing 200 μg/ml BSA. Activity assays were performed reductometrically using the Somoygi-Nelson procedure as described in Section 2.2.7. Protein concentration was estimated by the Bradford method (Bradford, 1976) using the Coomassie Protein Reagent (Pierce) and BSA as a standard. Purified enzymes were stored on ice in 50 mM sodium acetate buffer, pH 5.0, for several weeks with no significant decrease in specific activity.

3.3 Results and Discussion

3.3.1 Proposed Amino Acid Substitutions for Changing Substrate Specificity

Barley (1→3)-β-glucanase isoenzyme GII and barley (1→3,1→4)-β-glucanase isoenzyme EII share almost 100% similarity at the structural level (Figure 3.1), yet have different substrate specificities, which are determined by a relatively small number of amino acid differences in the substrate-binding grooves (Varghese *et al.*, 1994). A diagrammatic representation of the substrate binding grooves of the two enzymes, highlighting the amino acids which are potentially involved in substrate binding and catalysis, is shown in Figure 3.3.

Four key amino acid differences that may be responsible for the distinct substrate specificities of barley (1→3)-β-glucanase isoenzyme GII and barley (1→3,1→4)-β-glucanase isoenzyme EII were identified. Near the southern end of the (1→3)-β-glucanase isoenzyme GII groove (as oriented in Figure 3.3), the carbon ring of residue Phe^{130} is almost parallel with the left-hand side of the groove, where it would probably not interfere with substrate binding. In contrast, the corresponding residue in (1→3,1→4)-β-glucanase isoenzyme EII, Gln^{129}, is perpendicular to, and therefore physically blocks, the substrate binding groove (Figure 3.3). This suggests that the introduction of amino acid substitution F130Q into (1→3)-β-glucanase isoenzyme GII may facilitate the binding of a (1→3,1→4)-β-glucan molecule. The amino acid substitution S136F, which is also located at the left-hand side of the southern portion of the groove (as oriented in Figure 3.3), may have a similar affect on substrate binding. Residue Met^{209} of (1→3)-β-glucanase isoenzyme GII, which lies at the bottom of the groove with its side chain pointing upwards (Figure 3.3), whereas the corresponding residue in (1→3,1→4)-β-glucanase isoenzyme EII, Thr^{210}, has a relatively short side chain group which is tucked away at the bottom of the groove (Figure 3.3). Residue Phe^{171} lies in a prominent position at the top of the southern end of the (1→3)-β-glucanase isoenzyme GII binding cleft, but in (1→3,1→4)-β-glucanase isoenzyme EII, this position is occupied by the smaller residue Leu^{173} (Figure 3.3). Based

on these observations, the amino acid substitutions F130Q, S136F, F171L, and M209T were selected for introduction into barley (1→3)-β-glucanase isoenzyme GII in preliminary attempts to change its substrate specificity to that of a (1→3,1→4)-β-glucanase.

3.3.2 Site-Directed Mutagenesis

The USE method for site-directed mutagenesis was successfully used to introduce the amino acid substitutions F130Q, S136F, F171L, and M209T into (1→3)-β-glucanase isoenzyme GII at the cDNA level. Automated DNA sequencing confirmed that the appropriate nucleotide changes were present, and that no additional changes had been introduced (data not shown). The mutation frequency was defined as the percentage of plasmids that contained both the restriction site modification *Sca*I to *Mlu*I (as evident by restriction digest analysis), and the desired codon changes (as evident from DNA sequence analysis). Mutation frequencies for substitutions F130Q, S136F, F171L, and M209T were approximately 80%, 25%, 50%, and 100%, respectively. Thus, the USE mutagenesis kit (Pharmacia LKB Biotechnology) enabled the production of all four desired mutations, and no inadvertent base changes were introduced. However, mutation frequencies were not always as high as those claimed by the product manufacturer.

3.3.3 Production of Recombinant Wildtype and Mutant (1→3)-β-Glucanases

Plasmids containing the four individual amino acid substitutions, in addition to wildtype plasmid pET3a-HT/GII, were transformed into *E. coli* BL21 (DE3) pLysS and expressed for 3 h at 37°C. The five expressed enzymes were purified in yields of between 6.0 and 12.0 mg protein per litre of bacterial culture, and analysed by SDS-PAGE (Figure 3.6). All five expressed proteins were of relatively high purity, estimated to be greater than 95%. With the exception of F130Q, the expressed enzymes all had an approximate apparent molecular mass of 34 kDa, which is the expected mass for recombinant barley (1→3)-β-glucanase isoenzyme GII with a polyhistidine tag (Figure 3.6). However, mutant enzyme F130Q migrated through the polyacrylamide gel appreciably faster than the other four enzymes and had an apparent molecular mass of approximately 31 kDa (Figure 3.6). This

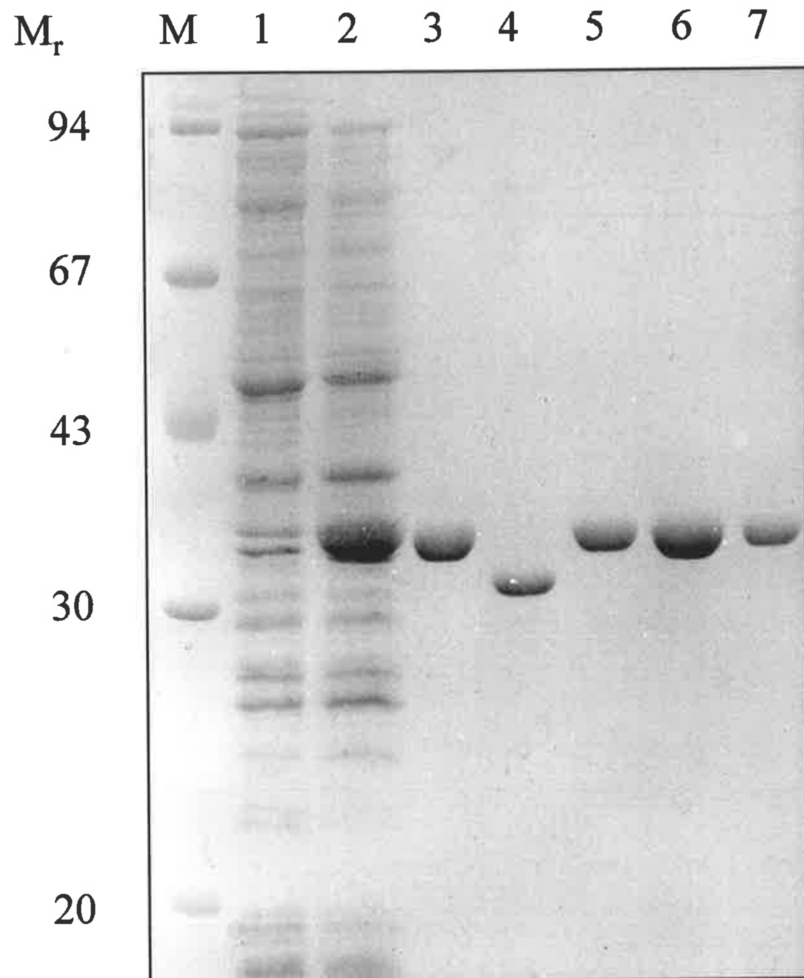


Figure 3.6 SDS-PAGE analysis of (1→3)-β-glucanase isoenzyme GII mutants. Four mutant (1→3)-β-glucanase fusion proteins were expressed in *E. coli* and purified using a Ni-NTA column. Lane 1, bacterial cell lysate prior to induction; Lane 2, bacterial cell lysate following induction of expression; Lane 3, pure wildtype barley (1→3)-β-glucanase isoenzyme GII fusion protein; Lane 4, pure mutant F130Q; Lane 5, pure mutant S136F; Lane 6, pure mutant F171L; Lane7, pure mutant M209T; M, molecular weight markers.

result suggested that the plasmid encoding recombinant (1→3)-β-glucanase F130Q may contain an error, causing the premature termination of protein translation. However, careful examination of the sequencing data for the expression plasmid encoding mutant F130Q revealed that no errors were present at the DNA level. Electrospray mass spectrometry analysis of the mutant (1→3)-β-glucanase F130Q and the corresponding wildtype (1→3)-β-glucanase confirmed that both enzymes were of the expected molecular mass (Rutten, 1997). The reason for the difference in electrophoretic mobility between the mutant (1→3)-β-glucanase F130Q and the other mutant enzymes is therefore unclear.

3.3.4 (1→3)-β-Glucanase and (1→3,1→4)-β-Glucanase Activity of Mutant Enzymes

The wildtype recombinant (1→3)-β-glucanase and the four mutants F130Q, S136F, F171L and M209T, were assayed for both (1→3)-β-glucanase and (1→3,1→4)-β-glucanase activity. The relative specific activities of the four mutant (1→3)-β-glucanases, compared with the corresponding wildtype enzyme, are shown in Table 3.1. Mutant enzymes containing the F130Q, S136F, and F171L substitutions all displayed a significant reduction in (1→3)-β-glucanase activity, compared with the wildtype recombinant (1→3)-β-glucanase. The amino acid substitution M209T caused a 95% reduction in (1→3)-β-glucanase activity, which suggests that Met^{209} is important for determining the shape of the substrate binding cleft and hence for the binding of a (1→3)-β-glucan. Despite the fact that all four amino acid substitutions decreased activity of the wildtype recombinant (1→3)-β-glucanase against laminarin, the mutant enzymes did not hydrolyse barley (1→3,1→4)-β-glucan. Thus, no change in the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII was observed.

Table 3.1 Specific activities of wildtype and mutant forms of barley (1→3)-β-glucanase isoenzyme GII expressed in *E. coli* as fusion proteins.

Enzyme	Relative Specific Activity [b, c] (%)	
	(1→3)-β-Glucanase [a]	(1→3,1→4)-β-Glucanase [a]
Wildtype	100	n/d
F130Q	57	n/d
S136F	64	n/d
F171L	23	n/d
M209T	5	n/d

[a] Activity was assayed at 37°C using 0.2% (w/v) barley (1→3,1→4)-β-glucan or 0.2% (w/v) laminarin from *L. digitata* as a substrate.

[b] A unit of activity is defined as the amount of enzyme required to release 1 μmol of glucose equivalents per min at 37°C from the appropriate substrate (see above).

[c] Specific activity is expressed as units per mg of protein.

n/d: Not detectable.

3.4 Summary and Conclusions

In this study, attempts were made to convert the substrate specificity of a relatively heat-stable (1→3)-β-glucanase to a (1→3,1→4)-β-glucanase, in the expectation that the newly formed (1→3,1→4)-β-glucanase would retain the relatively high heat stability of the 'parent' enzyme. The introduction of the single amino acid substitutions F130Q, F171L, S136F, and M209T decreased the specific activity of barley (1→3)-β-glucanase isoenzyme GII, but did not change its substrate specificity to that of a (1→3,1→4)-β-glucanase. The amino acid substitution F130Q involved changing all three nucleotides of the corresponding codon (TTC→CAG) which, from an evolutionary viewpoint, indicates that this substitution may be significant in determining substrate specificity. Gln^{130} is conserved in barley (1→3,1→4)-β-glucanase isoenzymes EI and EII, which further indicated that the residue may be required to accommodate a (1→3,1→4)-β-glucan molecule. It can be concluded from the results presented in this Chapter that altering the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII is likely to require multiple amino acid changes, or may involve replacing entire regions of the barley (1→3)-β-glucanase isoenzyme GII primary sequence with the corresponding regions from barley (1→3,1→4)-β-glucanase isoenzyme EII.

Designing the correct strategy for changing the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII to enable it to bind a (1→3,1→4)-β-glucan would be facilitated by a more comprehensive understanding of the binding and catalytic mechanisms of barley (1→3)-β-glucanase isoenzyme GII and barley (1→3,1→4)-β-glucanase isoenzyme EII. Subsite mapping experiments have shown that the substrate binding groove of barley (1→3)-β-glucanase isoenzyme GII contains eight β-glucosyl binding subsites, where each site binds a single glucosyl residue of an extended (1→3)-β-glucan (Hrmova *et al.*, 1995). The catalytic amino acids lie between the third and fourth binding subsites, numbering from the non-reducing end of the polysaccharide. The binding energies associated with the subsites adjacent to the catalytic site are negative, which suggests that some distortion of the substrate is effected in these regions to facilitate hydrolytic cleavage of the substrate

(Hrmova *et al.*, 1995). However, subsite mapping experiments have not been undertaken for the barley (1→3,1→4)-β-glucanase, nor is there any information on substrate-enzyme interactions in the substrate binding cleft of this enzyme.

Changing the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII to that of a (1→3,1→4)-β-glucanase will therefore require a more detailed examination of the binding interactions which occur between the enzymes and their substrates. Such binding interactions between enzymes and their polysaccharide substrates can be predicted using sophisticated molecular modelling programs such as AutoDock 2.1 (Goodsell and Olsen, 1990; Coutinho *et al.*, 1997). Automated docking programs can be used to simulate the docking of a carbohydrate substrate into the substrate-binding groove of an enzyme and to calculate the strength of potential molecular interactions between the substrate and amino acids on the enzyme surface. More direct evidence as to which amino acid residues are involved in determining substrate binding and catalysis, and how they interact with the glucosyl residues of the polysaccharide substrate, can be obtained by co-crystallisation of enzyme-substrate complexes and subsequent crystallographic analyses. Several (1→3)- and (1→3,1→4)-β-glucan substrates and substrate analogues have been chemically synthesised for this purpose (Hrmova *et al.*, 1998b). Because crystallisation procedures are complex and long-term in nature, the strategy for increasing (1→3,1→4)-β-glucanase thermostability was shifted towards the alteration of specific amino acid residues in barley (1→3,1→4)-β-glucanase isoenzyme EII. These alterations did indeed enhance the thermostability of the enzyme, and the corresponding experiments are described in Chapter 4.

CHAPTER FOUR:

USING PROTEIN ENGINEERING TO INCREASE THE THERMOSTABILITY OF BARLEY (1→3,1→4)-β-GLUCANASE ISOENZYME EII

4.1 Introduction

In Chapter 3 of this thesis, attempts to produce a thermostable barley (1→3,1→4)-β-glucanase by changing the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII to that of a (1→3,1→4)-β-glucanase, using site-directed mutagenesis, were described. Although the resulting mutant enzymes displayed lower specific activities than the wildtype barley (1→3)-β-glucanase isoenzyme GII, they did not hydrolyse barley (1→3,1→4)-β-glucan. An alternative approach for producing a thermostable barley (1→3,1→4)-β-glucanase would involve strengthening the existing intramolecular forces within the (1→3,1→4)-β-glucanase, so that the enzyme would retain its active three-dimensional conformation at elevated temperatures. This approach could be implemented through the strategic introduction of amino acid substitutions into the enzyme, provided its three-dimensional structure was known. The three-dimensional structure of barley (1→3,1→4)-β-glucanase isoenzyme EII has indeed been solved to high resolution (Varghese *et al.*, 1994), and this enzyme therefore represents a logical target for attempts to engineer increased heat stability into barley (1→3,1→4)-β-glucanases through rational protein design. Furthermore, barley (1→3,1→4)-β-glucanase isoenzyme EII can be expressed in *E. coli* and purified using a single affinity chromatography step (Chapter 2). This facilitates the rapid production and screening of mutant enzymes. In this Chapter, attempts to increase the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII using protein engineering are described.

4.1.1 Factors Affecting Protein Stability

Predicting which amino acid substitutions are needed to increase the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII requires a comprehensive understanding of the factors which affect protein folding and stability in general. Protein engineering studies, together with structural comparisons of proteins from thermophilic and mesophilic organisms, have provided valuable information about the stability of proteins (Grütter *et al.*, 1979; Nosoh and Sekiguchi, 1990; Fersht and Serrano, 1993; Vriend and Eijsink, 1993; Fágáin, 1995; Matthews, 1996; Querol *et al.*, 1996; Vieille and Zeikus, 1996). Although

the principles governing protein stability are not completely understood, some general rules have emerged. These rules are discussed below.

(i) Surface lysine to arginine substitutions

Thermophilic and mesophilic organisms are those which have optimum growth temperatures of greater than 55°C, and less than 55°C, respectively. Proteins isolated from thermophilic organisms have a substantially higher ratio of arginine to lysine residues than similar proteins isolated from mesophilic organisms (Menéndez-Arias and Argos, 1989; Vieille and Zeikus, 1996), which suggests that lysine to arginine substitutions could play an important role in protein stability. Indeed, arginine is more hydrophilic than lysine, and therefore is more likely to form favourable interactions with the polar solvent at the protein surface. In addition, arginine has two more nitrogen atoms than lysine and can therefore form extra hydrogen bonds with neighboring atoms. The introduction of lysine to arginine substitutions by site-directed mutagenesis can significantly increase protein thermostability (Mrabet *et al.*, 1992). For example, the strategic introduction of a single lysine to arginine substitution into a D-xylose isomerase from *Actinoplanes missouriensis* increased the half-life of the enzyme at 84°C from 3 hours to 4.5 hours (Mrabet *et al.*, 1992).

(ii) Changes in conformational entropy

Glycine has a higher degree of conformational freedom than the other amino acids because it does not contain a β-carbon side-chain or substituent (Branden and Tooze, 1991). Thus, the replacement of glycine can reduce the conformational entropy of the unfolded state of the protein, leading to a decreased 'unfolding' energy gradient, and hence to an increase in stability. For example, a glycine to alanine substitution introduced into bacterial T4 lysozyme by site-directed mutagenesis (Matthews *et al.*, 1987) increased the half-life of the enzyme at 65°C from 10 minutes to 18 minutes. The largest increases in stability generally occur when the glycines are replaced within the structure of an α-helix (Serrano *et al.*, 1992).

In contrast to glycine, proline has very little conformational freedom, because its side chain group is covalently linked to the nitrogen atom of the polypeptide backbone (Branden and Tooze, 1991). Thus, proline residues can reduce the flexibility of the polypeptide backbone and concomitantly lower the conformational entropy of the unfolded protein (Matthews *et al.*, 1987). This again means that the difference in entropy between the folded and unfolded states is lowered, and the energy gradient for unfolding is therefore reduced. As a result, the folded form of the enzyme is more stable at elevated temperatures. For these reasons, the strategic introduction of proline residues can have a significant effect on protein thermostability (Matthews *et al.*, 1987; Hardy *et al.*, 1993; Watanabe *et al.*, 1994; Eijsink *et al.*, 1995; Watanabe, 1996; Watanabe and Suzuki, 1998). For example, Matthews *et al.* (1987) increased the half-life of bacterial T4 lysozyme at 65°C from 10 minutes to 55 minutes through the introduction of a single alanine to proline substitution. Similarly, the thermostability of a thermolysin-like neutral protease from *Bacillus stearothermophilus* was increased through the introduction of an alanine to proline substitution (Hardy *et al.*, 1993; Eijsink *et al.*, 1995). The T_{50} of the mutant protease was 5.6°C higher than that of the wildtype enzyme, where T_{50} was defined as the temperature at which 50% of the initial enzyme activity remained after 30 minutes incubation.

Similarly, the introduction of disulphide bonds can have a stabilising effect which are attributed to a decrease in the conformational entropy of the denatured protein (Pantoliano *et al.*, 1987; Van den Berg *et al.*, 1993; Wakarchuk *et al.*, 1994; Zhang *et al.*, 1994; Pace *et al.*, 1996; Mansfeld *et al.*, 1997). Mansfeld *et al.* (1997) engineered a disulphide bond into a thermolysin-like protease from *B. stearothermophilus* and consequently increased the T_{50} of the enzyme by 16°C. The heat stability of the bacterial protease subtilisin BPN was significantly enhanced through the introduction of a disulphide bond; the thermal inactivation rate for the engineered enzyme containing the disulphide bond was only half that of the corresponding wildtype enzyme (Pantoliano *et al.*, 1987).

(iii) Increasing packing density and hydrophobicity

During folding, most hydrophobic residues become buried in the interior of the protein, while hydrophilic residues remain exposed to the solvent (Branden and Tooze, 1991). It is believed that this preferred arrangement represents one of the fundamental driving forces behind protein folding. However, there are two additional considerations with respect to the sequestering of hydrophobic residues away from the solvent. Firstly, amino acids at the interior of the protein are relatively tightly packed (Dill, 1990), but there are inevitably cavities present in the hydrophobic core (Rashin *et al.*, 1986; Karpusas *et al.*, 1989). These cavities can be large enough to accommodate solvent molecules that will interrupt the hydrophobicity of the core, and are therefore considered energetically unfavourable (Pakula and Sauer, 1989). Mutations designed to fill cavities in the core of a thermolysin-like neutral protease from *B. stearothermophilus* resulted in T_{50} increases of up to 4°C, compared with the wildtype enzyme (Eijsink *et al.*, 1992). In contrast, the replacement of hydrophobic residues located at the protein core with smaller residues can reduce the overall stability of the protein (Sandberg and Terwilliger, 1989; Shortle *et al.*, 1990).

Secondly, although non-polar residues are normally buried in the protein core, they are sometimes organised as hydrophobic clusters on the protein surface (Burley and Petsko, 1985). These hydrophobic regions are involved in protein interactions with carbohydrates, membranes, substrate molecules, and other proteins. The thermostability of lactate dehydrogenase from *B. stearothermophilus* was significantly improved by replacing a surface isoleucine with an arginine, in a substitution that was designed to reduce the area of solvent-accessible hydrophobic surface residues (Wigley *et al.*, 1987).

(iv) Salt bridges

Salt bridges between positively and negatively charged amino acid residues, also referred to as 'ion pairs', can exert a significant influence on protein stability (Perutz, 1978; Anderson *et al.*, 1990; Davies *et al.*, 1993; Ishikawa *et al.*, 1993; Kelly *et al.*, 1993). Salt bridges which link individual elements of secondary structure can increase protein stability

(Ishikawa *et al.*, 1993), while the creation of salt bridges between two consecutive turns of a single α-helix can help stabilize the helix and hence the overall protein structure (Marqusee and Baldwin, 1987). Further evidence for the stabilising nature of salt bridges is provided by a comparison of 3-phosphoglycerate kinases from the thermophilic organism *B. stearothermophilus* and the mesophilic organism *S. cerevisiae*. There are 15 additional salt bridges present in the relatively thermostable enzyme from *B. stearothermophilus*, all of which are involved in cross-linking secondary structural elements (Davies *et al.*, 1993).

(v) Glycosylation

Glycosylated proteins are generally more thermostable than their unglycosylated counterparts, and the introduction of extra glycosylation sites can therefore be used to increase protein thermostability (Olsen and Thompsen, 1991; Doan and Fincher, 1992; Meldgaard and Svendsen, 1994). For example, Doan and Fincher (1992) used site-directed mutagenesis to introduce an *N*-glycosylation site into barley (1→3,1→4)-β-glucanase isoenzyme EI and this resulted in a small but significant increase in thermostability. Furthermore, the removal of a glycosylation site from the naturally more thermostable barley (1→3,1→4)-β-glucanase isoenzyme EII decreased the heat stability of the enzyme (Doan and Fincher, 1992). These results provide evidence that glycosylation is an important determinant of protein thermostability.

4.1.2 The Intrinsic Thermostability of Plant (1→3)-β-Glucanases

Another approach which may give insight into which amino acid substitutions will be required to increase the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII involves comparisons with (1→3)-β-glucanases from a number of higher plant species. Plant (1→3)-β-glucanases have been classified among the pathogenesis-related (PR) proteins because they are generally secreted as part of the plant defence strategy against invading pathogens (Boller, 1987; Mauch *et al.*, 1988; Leah *et al.*, 1991; Stintzi *et al.*, 1993). In the extracellular environment, plant (1→3)-β-glucanases encounter relatively harsh conditions of pH and temperature and are subject to attack by degradative enzymes

of microbial origin. It has therefore been suggested that in many cases these enzymes have evolved to be inherently more stable at extremes of pH and temperature (Stintzi *et al.*, 1993). Indeed, barley (1→3)-β-glucanase isoenzyme GII is significantly more thermostable than barley (1→3,1→4)-β-glucanase isoenzyme EII (Figure 3.4; Chen *et al.*, 1995).

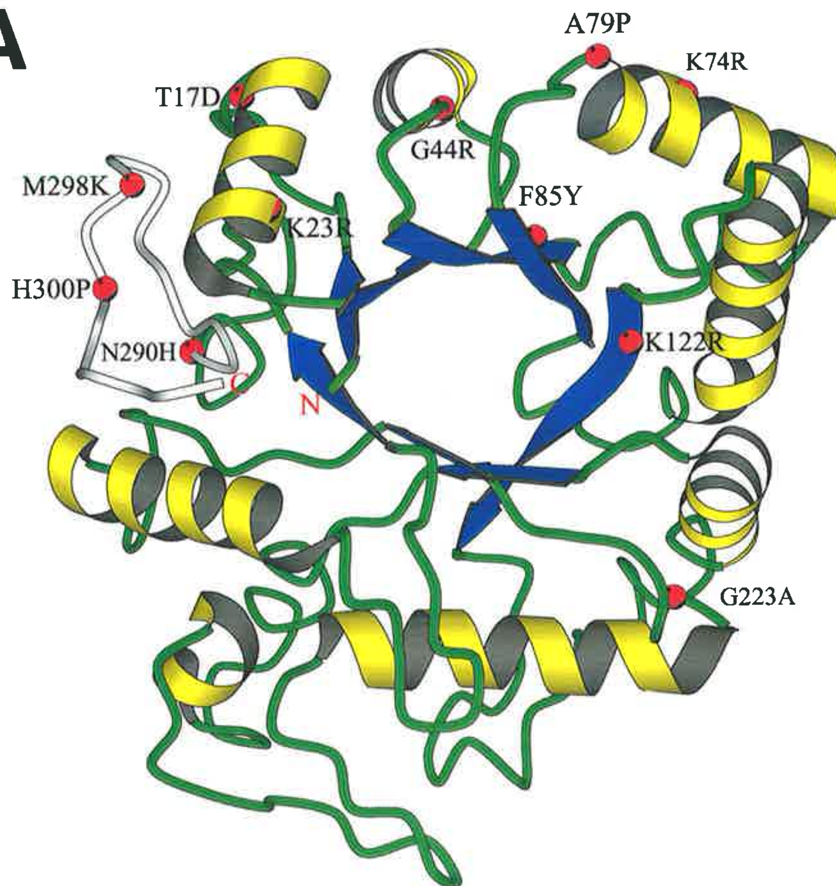
Barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII have almost identical secondary structures (Varghese *et al.*, 1994), and the higher thermostability of the (1→3)-β-glucanase must therefore result from specific amino acid differences between the two enzymes (Figure 4.2). Those residues which are highly conserved in the relatively heat-stable plant (1→3)-β-glucanases but are not present in the relatively heat-labile barley (1→3,1→4)-β-glucanase isoenzymes EI and EII, may play an important role in protein stability. To identify these residues, the amino acid sequences of (1→3)-β-glucanases from several different plant species were aligned (see Section 4.2.2 for details). It should be noted that although three-dimensional structures have not been solved for the majority of the plant (1→3)-β-glucanase sequences that were aligned, we can assume that their tertiary structures are similar because of their high degree of amino acid sequence identity (Varghese *et al.*, 1994). Although little thermostability data is available for these enzymes, we can assume that they have evolved to be relatively heat-stable (Stintzi *et al.*, 1993), as is clearly the case for barley (1→3)-β-glucanase isoenzyme GII (Chen *et al.*, 1995).

4.1.3 Enhancing the Thermostability of Barley (1→3,1→4)-β-Glucanase Isoenzyme EII

The strategy described in this Chapter for enhancing the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII was based primarily on our knowledge of the factors which affect protein stability in general. The amino acid substitutions designed for enhancing the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII were further rationalised by comparing the amino acid sequence of barley (1→3,1→4)-β-glucanase isoenzyme EII with those of several different plant (1→3)-β-glucanases,

Figure 4.1 A schematic view of barley (1→3,1→4)-β-glucanase isoenzyme EII showing secondary structure and the positions of various amino acid substitutions. The three-dimensional structure of barley (1→3,1→4)-β-glucanase isoenzyme EII is shown with α-helices as yellow tapes, β-strands as blue arrows, and loop regions as green coils. The NH_2- and COOH-termini are labelled 'N' and 'C', respectively (red), and the COOH-terminal loop region is shown in white. The various amino acid substitutions described in this Chapter are indicated in black. A) a 'bottom' view of the enzyme looking from the underside of the substrate binding cleft, B) a 'side' view of the enzyme showing the substrate binding cleft, and represents a 90° rotation of the enzyme compared with the orientation in frame A. This figure was constructed using MOLSCRIPT.

A



B

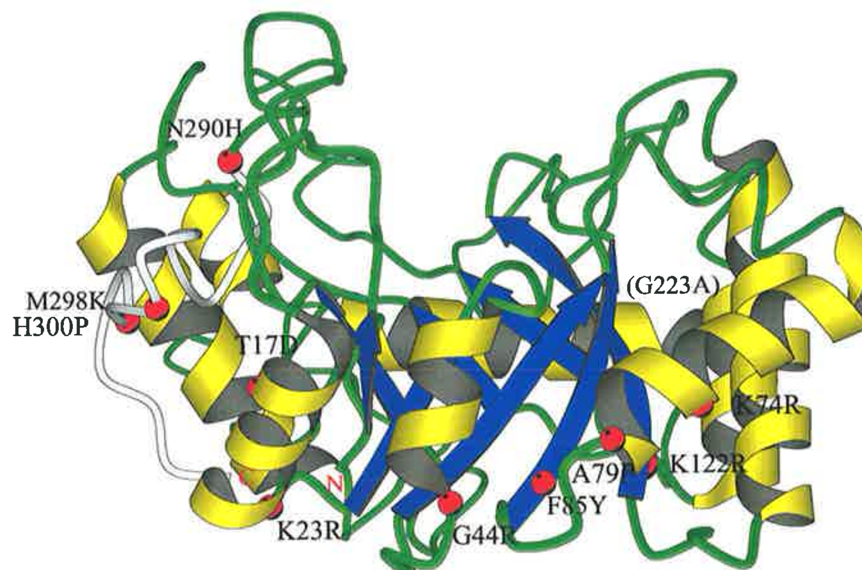


Figure 4.2 An alignment of the amino acid sequences of barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII. The alignment was performed using PILEUP in the University of Wisconsin GCG suite of programs (Devereux *et al.*, 1984). A black dot (●) marks positions where amino acid substitutions were introduced into barley (1→3,1→4)-β-glucanase isoenzyme EII. The conserved catalytic amino acids for both enzymes are marked with an asterix (*), and amino acid residues are numbered as follows: e2 residue / g2 residue. Identical residues are shown in bold.

```
   1                                                              60/60
e2 IGVCYGMSAN NLPAASTVVS MFKSNGIKSM RLYAPNQAAL QAVGGTGINV VVGAPNDVLS
g2 IGVCYGVIGN NLPSRSDVVQ LYRSKGINGM RIYFADGQAL SALRNSGIGL ILDIGNDQLA
                      ●          ●                    ●

   61/61                                                        119/120
e2 NLAASPAAAA SWVKSNIQAY .PKVSFRYVC VGNEVAGGAT RNLVPAMKNV HGALVAAGLG
g2 NIAASTSNAA SWVRNNVRPY YPAVNIKYIA AGNEVQGGAT QSILPAMRNL NAALSAAGLG
                 ●       ●      ●          *

   120/121                                                      179/177
e2 HIKVTTSVSQ AILGVFSPPS AGSFTGEAAA FMGPVVQFLA RTNAPLMANI YPYLAWAYNP
g2 AIKVSTSIRF DEVANSFPPS AGVFK..N.A YMTDVARLLA STGAPLLANV YPYFAYRDNP

   180/178                                                      236/235
e2 SAMDMGYALF NASGTVVRD. ..GAYGYQNL FDTTVDAFYT AMGKHGGSSV KLVVSESGWP
g2 GSISLNYATF QPGTT.VRDQ NNG.LTYTSL FDAMVDAVYA ALEKAGAPAV KVVVSESGWP
                                                 ●          *

   237/236                                                      295/295
e2 SGGGTAATPA NARFYNQHLI NHVGRGTPRH PGAIETYIFA MFNENQK.DS GVEQNWGLFY
g2 SAGGFAASAG NARTYNQGLI NHVGGGTPKK REALETYIFA MFNENQKTGD ATERSFGLFN
                                                         *  ●

   296/296   306/306
e2 PNMQHVYPINF
g2 PDKSPAYNIQF
     ●  ●
```

including barley (1→3)-β-glucanase isoenzyme GII (Figure 4.2). Thus, if there were several possible substitutions in a particular region of the enzyme and one of these was conserved in the relatively stable (1→3)-β-glucanases, that substitution was chosen in preference to other possibilities. Molecular modelling was used to confirm that the substitutions did not destabilize the enzyme's three-dimensional conformation. Finally, the proposed amino acid substitutions were introduced into barley (1→3,1→4)-β-glucanase isoenzyme EII at the cDNA level using site-directed mutagenesis, and the resulting mutant enzymes were expressed in *E. coli*, purified, and analysed for thermostability.

4.2 Materials and Methods

4.2.1 Materials

The Sequenase Version 2.0 DNA Sequencing Kit was from the US Biochemical Company, and DNA restriction enzymes and polynucleotide kinase were from New England Biolab. DNA oligonucleotides were synthesised using an Applied Biosystems 392 DNA Synthesiser. Barley (1→3,1→4)-β-glucan was from Deltagen. Ni-NTA columns and *E. coli* strain BL21 pLysS were from Qiagen, and the USE Mutagenesis Kit was purchased from Pharmacia LKB Biotechnology. Unless otherwise stated, all general chemicals were purchased from Sigma.

4.2.2 Choosing Amino Acid Substitutions for Enhancing Protein Stability

To identify those amino acids which might be responsible for the apparent stability of plant (1→3)-β-glucanases, an amino acid sequence alignment of 41 related plant β-glucanases was performed using PILEUP in the University of Wisconsin GCG suite of programs (Table 4.1; Devereux *et al.*, 1984). The aligned sequences encoded (1→3)-β-glucanases from barley (7 isoforms), potato (3 isoforms), tomato (4 isoforms), tobacco (10 isoforms), *Nicotiana* species (3 isoforms), *Arabidopsis* species (4 isoforms), and *Brassica* species (2 isoforms), and one enzyme each from soybean, pea, and bean. Also included in the alignment were sequences encoding barley (1→3,1→4)-β-glucanase isoenzymes EI and EII, and one related enzyme each from wheat, maize, and oats which have not been unequivocally assigned a substrate specificity. The aligned sequences were obtained from GenBank/EMBL DNA databases using the following codes in the 'BrowseCode' search program in the University of Wisconsin GCG suite of programs (Devereux *et al.*, 1984); blyglcnhi, blygeh, hv13geiii, blyglu2x, blyglcnhiv, blyglcnhv, mze13bglcn, blygcnhvi, hvglb1, blyglb2, tabetglua, asbglucan, tobpr2a, tobglucb, ntsp41b, ntsp41a, tobgl153a, tobb13g, tomb13glua, peabetaglu, pv13bdgl, tobgcbreg, npglucb, npb13gg, tobglb13b, tobgluca, u01900, u01901, u01902, tomb13glub, ntec32139, leqa, leqb, soyb13endg, athbg1a, athbg2a, athbg3a, bcbgl, ata6glua, bna6bglu, and ntbgluc.

Table 4.1 Summary of an amino acid alignment of 41 plant β-glucanases. The deduced amino acid sequences of 41 different plant β-glucanases were aligned using PILEUP in the University of Wisconsin GCG suite of programs (Devereux *et al.*, 1984), and the number of sequences containing a particular residue at each position is shown (see text for details). Residue positions are numbered according to the barley (1→3,1→4)-β-glucanase isoenzyme EII primary structure, and only those positions at which amino acid substitutions were made in this study are shown. The amino acids residues for barley (1→3,1→4)-β-glucanase iosenzyme EII and barley (1→3)-β-glucanase isoenzyme GII at the selected positions are shown in bold. Standard one-letter codes are used to describe amino acids.

Residue Number	17	23	44	74	79	85	122	223	290	298	300
Residue Type and Frequency	E 16	K 26	R 22	Q 26	N 12	F 24	K 40	G 30	H 28	K 19	P 25
	D 16	R 5	G 4	R 8	A 7	I 15	P 1	A 3	N 7	M 8	A 6
	T 4	N 7	S 5	K 5	D 7	C 1		T 3	F 4	Q 5	H 4
	Q 4	Q 2	K 5	H 2	P 7	V 1		I 2	S 1	R 5	K 3
	K 1	D 1	N 3		S 6			Y 2	Y 1	G 3	L 1
			A 1		Y 1			F 1		L 1	Q 1
			H 1		K 1						S 1
EII	**T**	**K**	**G**	**K**	**A**	**F**	**K**	**G**	**N**	**M**	**H**
GII	**D**	**R**	**R**	**Q**	**P**	**I**	**K**	**A**	**S**	**K**	**P**

Files of crystal structure coordinates describing the three-dimensional structures of barley (1→3,1→4)-β-glucanase isoenzyme EII and (1→3)-β-glucanase isoenzyme GII were obtained from the Brookhaven Protein Database, and the two protein structures were analysed using the 'O' software (Jones *et al.*, 1991) on a Silicon Graphics Iris Indigo Elan 4000 workstation. Potential substitutions were introduced 'hypothetically' into the enzyme and rationalised using the 'O' program (Jones *et al.*, 1991). Those which caused steric hindrance with atoms of neighbouring residues or disrupted hydrogen bonding were discarded from the proposed list. Substitutions involving residues in close proximity to substrate binding or catalytic regions were also avoided. The prediction and rationalisation of amino acid substitutions were carried out in consultation with Drs J.N. Varghese and T.P.J. Garrett (Biomolecular Research Institute, Parkville, Victoria, Australia).

4.2.3 Rationalizing the Proposed Amino Acid Substitutions

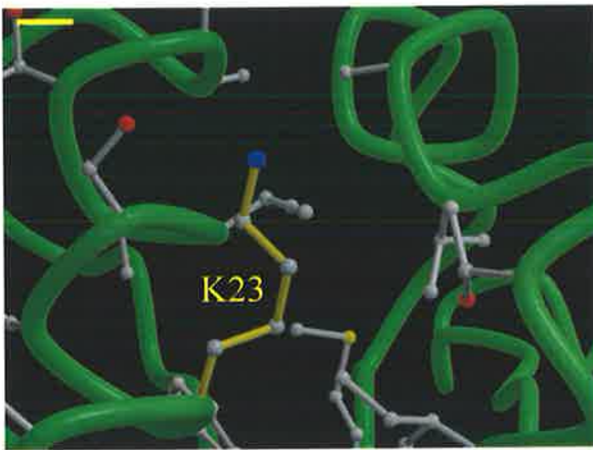
Ten different amino acid substitutions were designed for increasing the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII, and specific descriptions of how each substitution was rationalized are presented below.

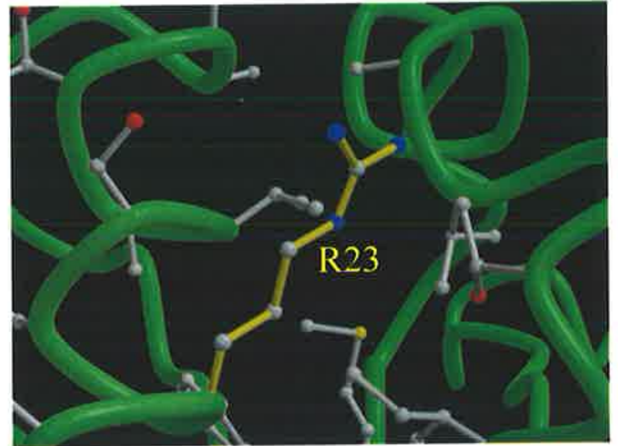
(i) Surface lysine to arginine substitutions: K23R, K74R, and K122R

Amino acid residues K23, K74, and K122 are all located on the surface of (1→3,1→4)-β-glucanase isoenzyme EII where their side chain groups are exposed to the solvent (Figure 4.1). Barley (1→3)-β-glucanase isoenzymes GI-GIV all have an arginine residue at amino acid position 23, which indicates that the presence of this residue may be important for protein stability. Although arginine is not highly conserved at position 122 in the plant β-glucanases (Table 4.1), it potentially creates a hydrogen bond with the carbonyl oxygen of amino acid residue161 (Figure 4.3). Substitutions K23R and K122R were successfully introduced into barley (1→3,1→4)-β-glucanase isoenzyme EII using the USE site-directed mutagenesis procedure, but several attempts to introduce the substitutions K74R were not successful. This was probably due to annealing problems between the K74R oligonucleotide and the template DNA (see Section 4.3.2 for details).

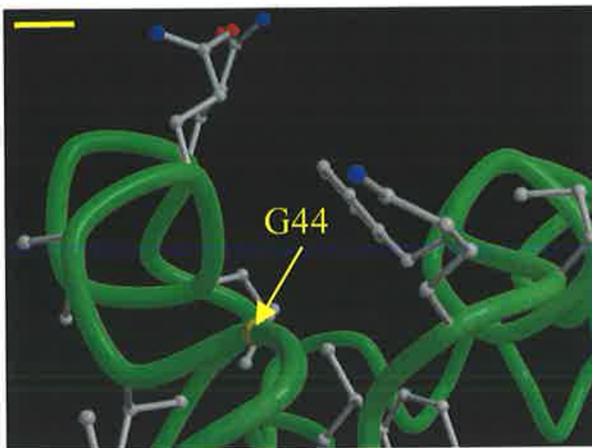
Figure 4.3 A schematic representation of the amino acid substitutions which were introduced into barley (1→3,1→4)-β-glucanase isoenzyme EII. For each substitution, the left panel shows the wildtype enzyme and the right panel shows the mutant enzyme which contains the amino acid substitution(s). Only those amino acids which are within 5 Å of the substituted residue are shown, because molecular interactions do not normally occur beyond this distance. The amino acids which are involved in substitutions are coloured yellow for clarity. Nitrogen, carbon, sulphur, and oxygen atoms are coloured blue, grey, yellow, and red, respectively. Carbonyl oxygens (O^c) are shown only where they are involved in hydrogen bonding, and the main chain nitrogen is shown for substitutions involving proline. For the G44R substitution, the C^{α} for glycine 44 is highlighted in yellow. Otherwise, only side chain atoms are displayed. Potential hydrogen bonds are represented by a dotted yellow line and labelled 'H', and for the ion pair substitution, '+' and '–' indicate charge. The scale bar represents a distance of 1.5 Å. This figure was constructed using MOLSCRIPT.

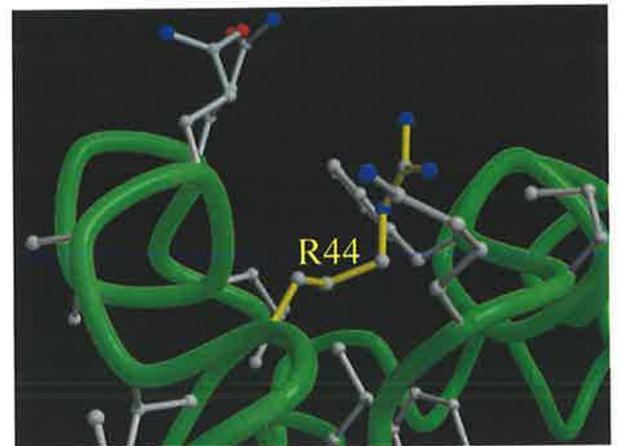
K23R



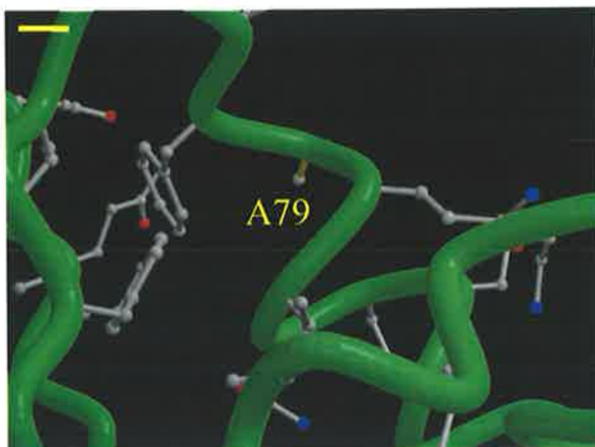


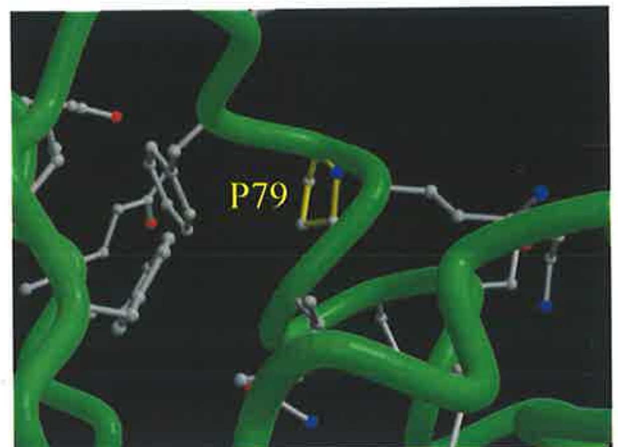
G44R



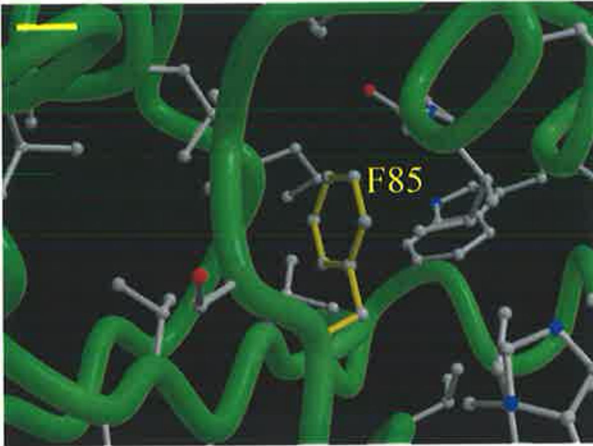


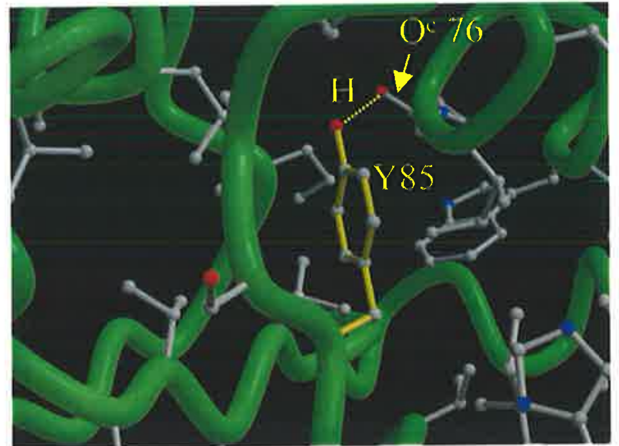
A79P



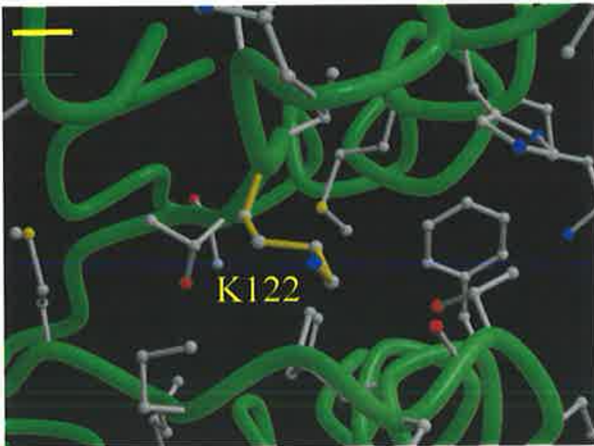


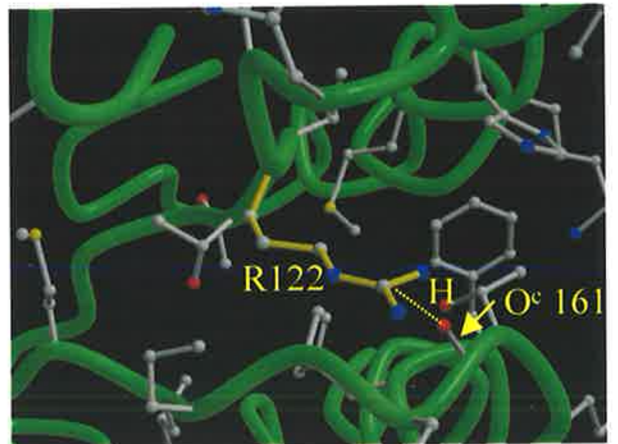
F85Y



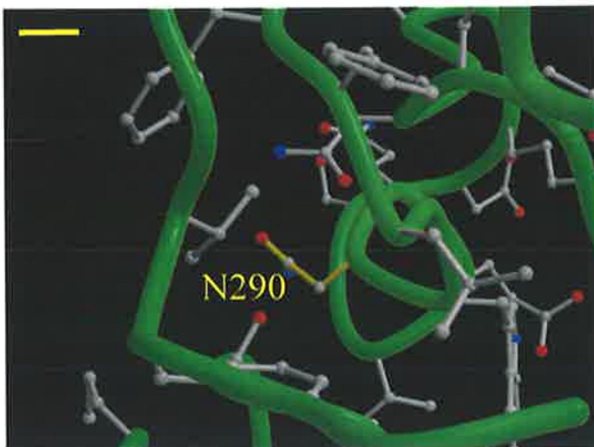


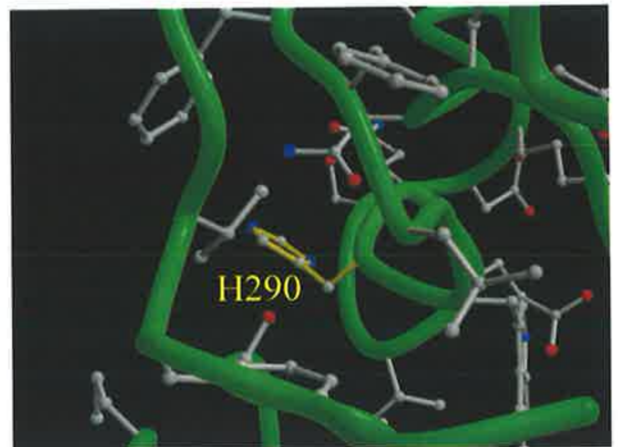
K122R



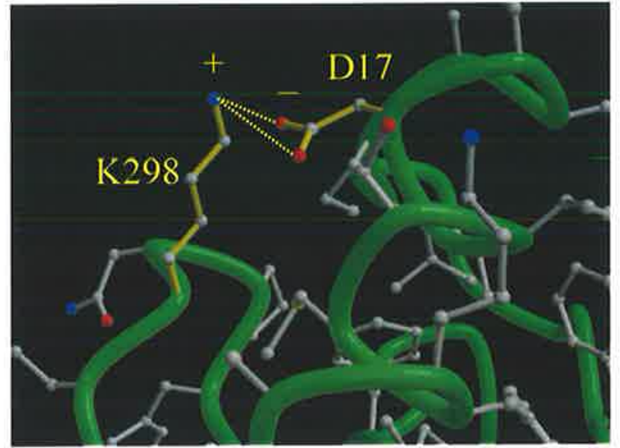


N290H

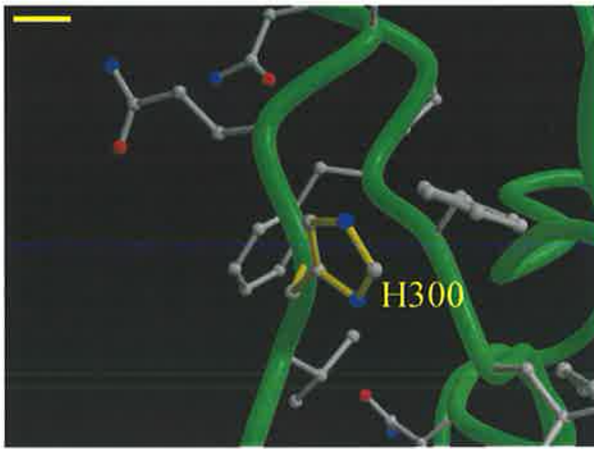


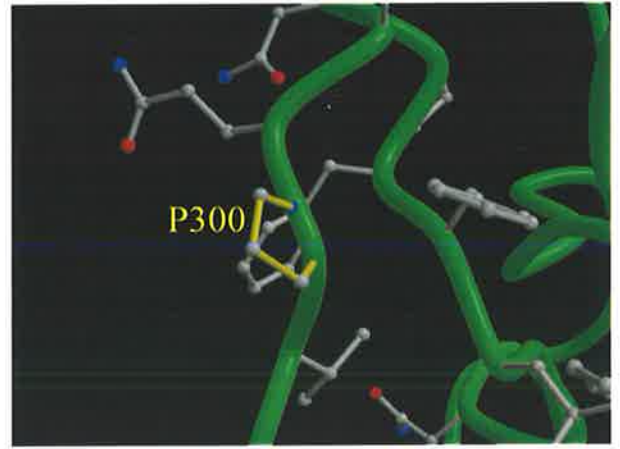


M298K;T17D



H300P





(ii) Changes in conformational entropy: G44R, A79P, G223A, and H300P

The amino acid substitutions G44R, A79P, and H300P were independently introduced into barley (1→3,1→4)-β-glucanase isoenzyme EII in an attempt to decrease the entropy of the unfolded state and hence to increase protein stability (Matthews *et al.*, 1987). Barley (1→3)-β-glucanase isoenzymes GI, GII, GV, and GVI all have an arginine residue at amino acid position 44 (Figures 3.3 and 4.2), and arginine is also highly conserved at this position in other plant β-glucanases (Table 4.1). Although 74% of the aligned plant (1→3)-β-glucanases have a glycine at position 223, 43% of the barley (1→3)-β-glucanases, including isoenzyme GII, have an alanine at this position. The G223A substitution was chosen on this basis, but attempts to create the substitution at the cDNA level were unsuccessful, again because of DNA annealing problems (see Section 4.3.2 for details). The A79P substitution was chosen because a proline is well conserved in this region in the barley (1→3)-β-glucanases (Table 4.1), including the intrinsically thermostable isoenzyme GII (Figure 4.2). Also, the A79P substitution is located at the end of a major α-helix which is a common position for stabilizing substitutions of this type (Serrano *et al.*, 1992). The H300P substitution was chosen on the basis that proline is conserved at this position in 5 of the 7 barley (1→3)-β-glucanases, and in 60% of the aligned plant (1→3)-β-glucanases examined, including barley (1→3)-β-glucanase isoenzyme GII (Table 4.1).

(iii) Increasing packing efficiency and hydrophobicity: F85Y, N290H

In barley (1→3,1→4)-β-glucanase isoenzyme EII, the amino acid substitution F85Y potentially creates a hydrogen bond between the hydroxyl group of the introduced tyrosine and the carbonyl oxygen of amino acid 76 or 80 (Figure 4.3), and may thereby help in the packing efficiency of the hydrophobic core (Rose and Wolfenden, 1993). Furthermore, phenylalanine represents the most common residue type at this position in the plant β-glucanases (Table 4.1), which suggests that residues with relatively large side chains may be important here for packing efficiency. Another substitution located in the protein core,

N290H, was selected on the basis that there is a strong bias for histidines (67%) at the corresponding position in other plant β-glucanases (Table 4.1).

(iv) Salt bridges: M298K;T17D

Residues K298 and D17 potentially constitute an ion pair which links the COOH-terminal loop in the relatively thermostable barley (1→3)-β-glucanases isoenzyme GII (Figures 4.1 and 4.3). These residues are not observed at the corresponding positions of barley (1→3,1→4)-β-glucanase isoenzyme EII. In addition, 52% of the plant β-glucanases examined have an aspartic or glutamic acid at position 17 *and* a lysine or arginine at position 298. Furthermore, 5 out of the 7 barley (1→3)-β-glucanases contain a potential ion pair at positions 298 and 17. The amino acid substitutions M298K and T17D were therefore introduced into (1→3,1→4)-β-glucanase isoenzyme EII to form a salt bridge and hence to stabilize the COOH-terminal loop region.

(v) The triple mutant: H300P, N290H, and M298K;T17D

The effects of single amino acid substitutions which give increases in protein stability are often additive (Matsumura and Aiba, 1985; Matsumura *et al.*, 1988; Watanabe *et al.*, 1994; Eijsink *et al.*, 1995). The three substitutions which increased the thermostability of barley (1 →3,1→4)-β-glucanase isoenzyme EII (N290H, H300P and M298K;T17D; Figure 4.6), were combined in an attempt to take advantage of any additive effects on thermostability.

4.2.4 Site-Directed Mutagenesis

The USE method for site-directed mutagenesis was employed to introduce the proposed amino acid substitutions into barley (1→3,1→4)-β-glucanase isoenzyme EII at the cDNA level, essentially as described in Chapter 3. The pET3a-HT/EII plasmid was used as a template for mutagenesis, and the *Sca*I→*Mlu*I primer was used for the selection of mutated plasmids. The following primers were designed to introduce the desired mutations:

K23R	5'- CGA TTT GAT CCC GTT GGA CCT GAA CAT GCT CAC G -3'	**AAG→AGG**
G44R	5'- GAT GCC CGT GCC GCG GAC GGC CTG CAG CGC -3'	**GGC→CGC**
A79P	5'- GGA AAC CTT GGG GTA GGG CTG GAT GTT GC -3'	**GCC→CCC**

F85Y 5'- C GCA GAC GTA CCG GTA GGA AAC CTT GGG G -3' **TTC→TAC**

K122R 5'- C CGA CGT GGT CAC CCT GAT GTG GCC CAG CCC -3' **AAG→AGG**

N290H 5'- GAA GAG TCC CCA GTG CTG CTC CAC GCC -3' **AAC→CAC**

M298K 5'- GGG GTA GAC GTG CTG CTT GTT GGG GTA GAA GAG -3' **ATG→AAG**

T17D 5'- GCT CAC GAC GTC GCT CGC CGC-3' **ACC→GAC**

H300P 5'- GTT GAT GGG GTA GAC GGG CTG CAT GTT GGG -3' **CAC→CCC**

G223A 5'- G CTT CAC GCT GGA CGC GCC GTG CTT GCC C -3' **GGG→GCG**

K74R 5'- CGC CTG GAT GTT GCT CCT GAC CCA CGA GGC -3' **AAG→AGG**

where the standard single letter codes for amino acids are used, and their relative positions are numbered according to the primary structure of barley (1→3,1→4)-β-glucanase isoenzyme EII. DNA oligonucleotide primers are complementary to the coding strand of the template plasmid, and altered codons are underlined. Codon changes relative to the coding strand are shown in bold. All primers were phosphorylated at their 5' end with polynucleotide kinase before use. Following site-directed mutagenesis, individual mutations were confirmed using DNA sequencing as described in Section 2.2.2. Mutant plasmids are described here using the following nomenclature: pET3a-HT/*S*, where '*S*' refers to the introduced amino acid substitution.

The following mutagenic primer, which initiated mutations corresponding to both the M298K and H300P substitutions, was used to create a triple mutant (see Section 4.3 for details):

5'- GAA GTT GAT GGG GTA GAC GGG CTG CTT GTT GGG GTA GAA GAG TC -3'

For the triple mutant, site-directed mutagenesis was carried out using the pET3a-HT/N290H mutant cDNA as a template. This resulted in the production of a single cDNA containing the M298K, H300P, and N290H substitutions. A *Pst*I/*Mlu*I fragment containing the three amino acid substitutions was ligated into the corresponding sites of pET3a-HT/T17D, resulting in a mutant containing the N290H, M298K, T17D, and H300P substitutions. Because the M298K and T17D substitutions constitute a single electrostatic interaction, this mutant plasmid was designated pET3a-HT/triple, or the 'triple mutant'.

4.2.5 Expression and Purification of Mutant (1→3,1→4)-β-Glucanases

Mutant plasmids were transformed into *E. coli* strain BL21 (DE3) pLysS and expression was induced as described in Section 2.2.3. Expressed fusion proteins were purified using Ni-NTA resin and analysed by SDS-PAGE, as described in Section 2.2.4.

4.2.6 Thermostability, pH Stability, and Kinetic Parameters

The thermostabilities of mutant and wildtype (1→3,1→4)-β-glucanases were determined by incubating the enzymes in 50 mM sodium acetate buffer, pH. 5.0, containing 0.2 mg/ml BSA, for 15 min over a range of temperatures, and measuring the residual activity (Section 2.2.7). Thermostability was described by T_{50}, which represents the temperature at which 50% of the initial enzyme activity remains after incubation for 15 min. For mutant H300P, the progressive decline in enzyme activity at a constant temperature of 48°C was also measured. Kinetic parameters of mutant H300P were determined as described in Section 2.2.7. The pH optima of mutant H300P and the corresponding wildtype enzyme were measured in 0.1 M sodium citrate-sodium phosphate buffers (McIlvaine buffers) containing 200 mg/ml BSA, using 0.2% (w/v) barley (1→3,1→4)-β-glucan (Deltagen) as a substrate. Enzymes were incubated with the substrate for up to 20 min at 37°C and total activity was expressed as a percentage of the highest activity value, which occurred at pH 4.5. All incubations for the thermostability assays and pH optima assays, were performed in a Julabo VC F10 water-bath (Seelbach, West Germany) using thin-walled 2.5 ml plastic tubes (Eppendorf, Germany). The accuracy of the water-bath thermostat was checked using a standard mercury thermometer.

4.2.7 Simulated Mashing at 65°C

In preparation for simulated mashing studies, barley (cv. Schooner) was malted and kilned by Joe White Malting Company Ltd, Adelaide, South Australia, using standard Institute of Brewing (IOB) procedures, and subsequently milled to 0.7 mm using a Bühler Miag milling apparatus. The amount of endogenous (1→3,1→4)-β-glucanase activity present in the malt was determined by extracting the malt in water at room temperature and

measuring (1→3,1→4)-β-glucanase activity using the Barley Malt β-D-Glucanase Kit (Deltagen) as described in the product literature, using Azo Barley Glucan as a substrate (McCleary and Shameer, 1987). The decline in endogenous (1→3,1→4)-β-glucanase activity during mashing was measured as follows; Dry malt (25 g) was equilibrated to 65°C in a 25-head mash bath (Industrial Equipment and Control Ltd, Melbourne, Australia) with constant mixing, and mashing was initiated by adding distilled water (65°C) at a malt to water ratio of 3:1 (w/w). Regular samples were removed from the mash using a 5 ml pipette, cooled on ice, and centrifuged at 3,000 rpm for 10 min to remove insoluble material. The resulting supernatants were assayed for (1→3,1→4)-β-glucanase activity as described above. Residual activity was expressed as a percentage of the initial activity.

A separate experiment was carried out to determine the thermostability of (1→3,1→4)-β-glucanase mutant H300P during mashing, at 65°C. Kiln-dried malt was pre-incubated in water at 65°C for 20 min to inactivate endogenous (1→3,1→4)-β-glucanase activity, and recombinant wildtype barley (1→3,1→4)-β-glucanase isoenzyme EII, or the corresponding recombinant mutant enzyme H300P, was subsequently added to the heat-inactivated mash. Samples were removed at regular intervals and assayed for (1→3,1→4)-β-glucanase activity as described above. The amount of each recombinant enzyme added to the mash was equivalent to the amount of endogenous (1→3,1→4)-β-glucanase present in 25 g of dried malt, based on activity.

4.3 Results and Discussion

4.3.1 Introduction of Mutations using Site-Directed Mutagenesis

Mutations causing amino acid substitutions K23R, G44R, A79P, F85Y, K122R, N290H, M298K;T17D, and H300P were confirmed by DNA sequencing. The nucleotide sequence of the appropriate region of the triple mutant containing the amino acid substitutions H300P, N290H, M298K, and T17D, is shown in Figure 4.4. The DNA sequence that encodes the polyhistidine tag and thrombin cleavage site is also shown in Figure 4.4.

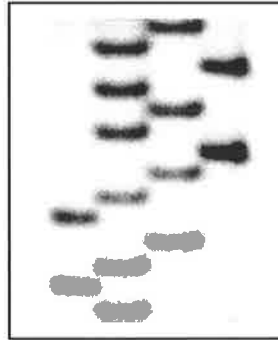
Although the product literature for the USE Mutagenesis Kit (Pharmacia LKB Biotechnology) claims mutation frequencies of greater than 90%, the success rate achieved in this study was generally less than 75%. Furthermore, numerous attempts to introduce the amino acid substitutions K74R and G223A using the USE Mutagenesis Kit were unsuccessful. Because the success of the mutagenesis was primer-specific, low mutation rates probably indicate that the primers formed intermolecular dimers or other secondary structures, preventing them from annealing to the template plasmid.

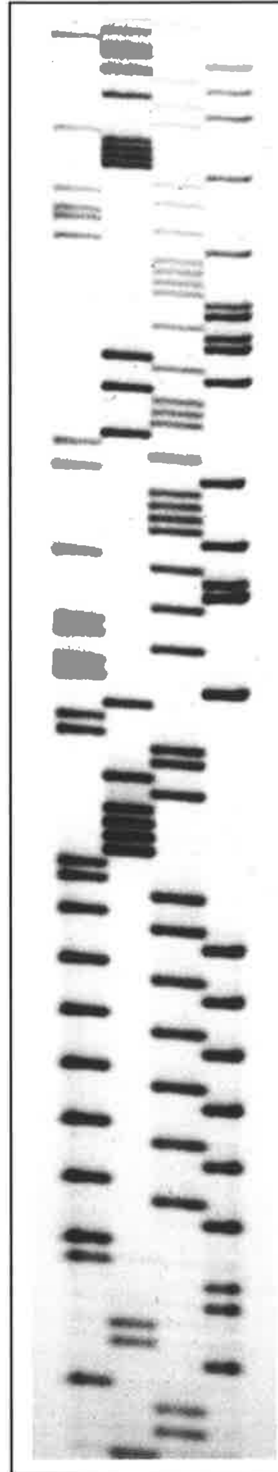
4.3.2 Expression and Purification of Mutant (1→3,1→4)-β-Glucanases

The nine mutant (1→3,1→4)-β-glucanases, including the 'triple' mutant, were expressed in *E. coli* BL21 pLysS as fusion proteins and purified using Ni-NTA resin, as described in Chapter 2. It was estimated from SDS-PAGE analysis of the four thermostable mutant enzymes (see Section 4.3.4) and the recombinant wildtype (1→3,1→4)-β-glucanase, that the five enzymes were all greater than 95% pure (Figure 4.5). Yields of the recombinant enzymes were generally between 2.0 and 5.0 mg protein per litre of bacterial culture, but varied for each of the mutants and between independent experiments. Mutants with low relative thermostabilities were generally less soluble, and were therefore purified in lower yields from the soluble bacterial cell lysate. The lower solubility of these mutant enzymes was probably a direct reflection of their compromised stability. All purified recombinant enzymes were stored on ice at a concentration of 0.25-1.0 mg/ml in 50 mM sodium

Figure 4.4 DNA nucleotide sequence analysis of a recombinant barley (1→3,1→4)-β-glucanase containing four amino acid substitutions. DNA sequencing was performed using the dideoxynucleotide method (Sanger *et al.*, 1977). The sequencing primer was complementary to the coding strand of the plasmid, and lanes are loaded in the order 'ACGT'. Because the sequencing primers were complementary to the coding strand of the plasmid DNA, the sequence is read upwards from the bottom of the gel in the in the 3' to 5' direction. A, sequence encoding the T17D substitution; B, sequence encoding the substitutions N290H, H300P, and M298K. The position of each altered codon is labelled with a vertical line, and the corresponding amino acid substitutions are also shown. DNA sequences encoding the thrombin recognition site (LVPRGS), polyhistidine tag, and stop codon are also labelled.

A **B**





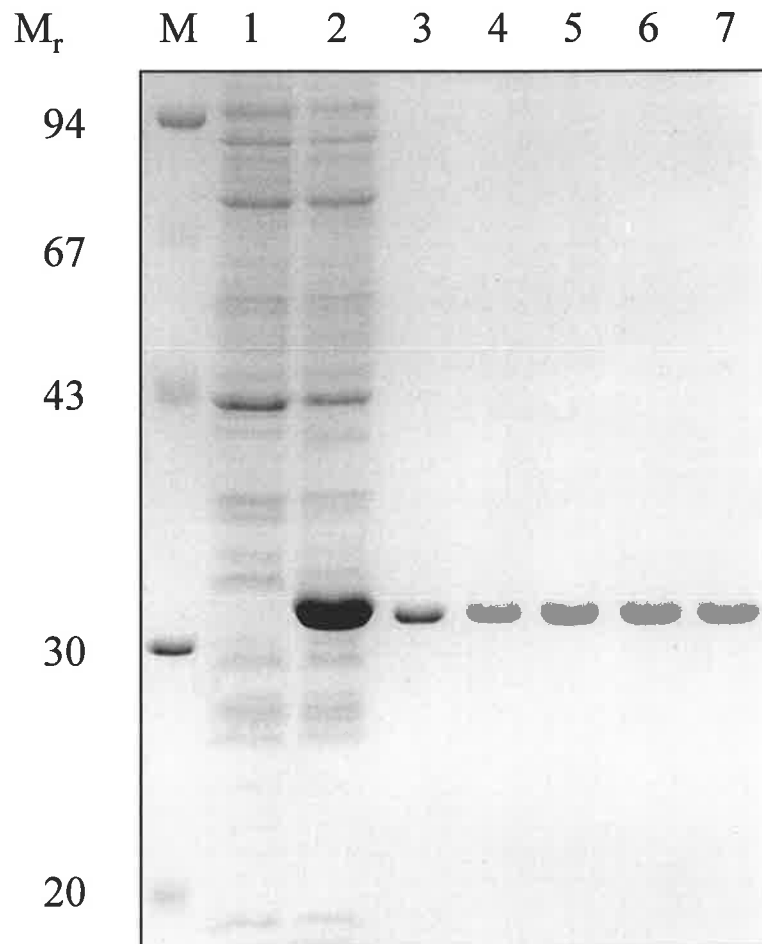


Figure 4.5 SDS-PAGE analysis of wildtype and mutant recombinant barley (1→3,1→4)-β-glucanases. Recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII and four thermostable mutants were expressed in *E. coli* as fusion proteins and purified using affinity chromatography (see text for details). 1, bacterial cell lysate prior to induction of expression; 2, bacterial cell lysate following expression of recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII; 3, purified recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII; 4, purified mutant N290H; 5, purified mutant M298K;T17D; 6, pure mutant H300P; 7, purified 'triple' mutant containing N290H, H300P, and M298K/T17D; M, molecular weight markers.

phosphate buffer, pH 7.8, containing 300 mM sodium chloride. Under these conditions, the enzymes showed no appreciable decrease in specific activity for several weeks (data not shown).

4.3.3 Thermostability of Mutant (1→3,1→4)-β-Glucanases

Thermostabilities of the mutant enzymes were defined using the T_{50} value which is the temperature (°C) at which 50% of the initial activity remains after incubation at that temperature for 15 minutes. As shown in Table 4.1, the wildtype recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII had a T_{50} of 47.5°C. This is consistent with results from a previous study, where barley (1→3,1→4)-β-glucanase isoenzyme EII was expressed in *E. coli* with a maltose-binding protein (MBP) fused to its NH_2-terminus (Chen *et al.*, 1995); this MBP fusion protein had a T_{50} value of 47.5°C. The native (1→3,1→4)-β-glucanase isoenzyme EII purified from germinated barley grain (Woodward and Fincher, 1982b) has a T_{50} of 52°C, which is 4.5°C higher than the recombinant wildtype enzyme purified from *E. coli*. This difference in thermostability is probably due to the glycosylation states of the respective enzymes. Barley (1→3,1→4)-β-glucanase isoenzyme EII is naturally glycosylated, whereas the recombinant enzyme expressed in *E. coli* remains unglycosylated. Doan and Fincher (1992) have demonstrated that glycosylation contributes significantly to the stability of barley (1→3,1→4)-β-glucanase isoenzyme EII. Results from related studies have also shown that glycosylation can increase protein stability (Olsen and Thomsen, 1991; Meldgaard and Svendsen, 1994).

(i) Amino acid substitutions which increased thermostability

Three of the eight mutant barley (1→3,1→4)-β-glucanases displayed enhanced thermostability. The substitutions N290H, M298K;T17D, and H300P, increased the T_{50} of the corresponding wildtype enzyme by 0.7°C, 0.4°C, and 3.7°C, respectively (Figure 4.6). The observed increases in stability were considered to be significant because at least four independent thermostability assays were carried out for each mutant enzyme. Furthermore, the T_{50} value for each mutant enzyme was always higher than that of the corresponding wildtype enzyme. To avoid potential errors during protein quantification, all enzymes were

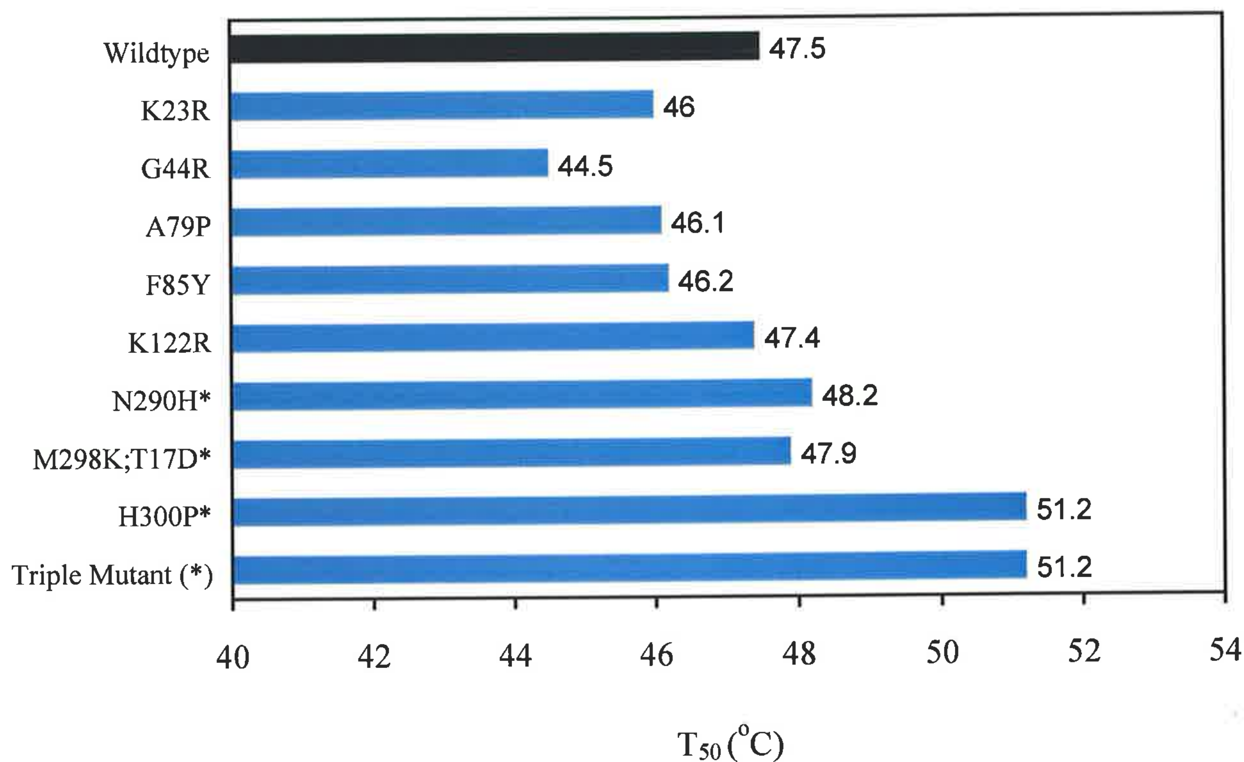


Figure 4.6 Thermostability of wildtype and mutant recombinant barley (1→3,1→4)-β-glucanases. Enzymes were expressed in *E. coli* as fusion proteins and purified using Ni-NTA resin (see text for details). Thermostability assays were performed in 50 mM sodium acetate buffer, pH 5.0, containing 200 μg/mL BSA. Enzymes were incubated for 15 min at a range of temperatures, and residual activity was measured. T_{50} represents the temperature (°C) at which 50% activity remains after heating for 15 min. Standard deviations for T_{50} values were invariably less than 0.2°C.

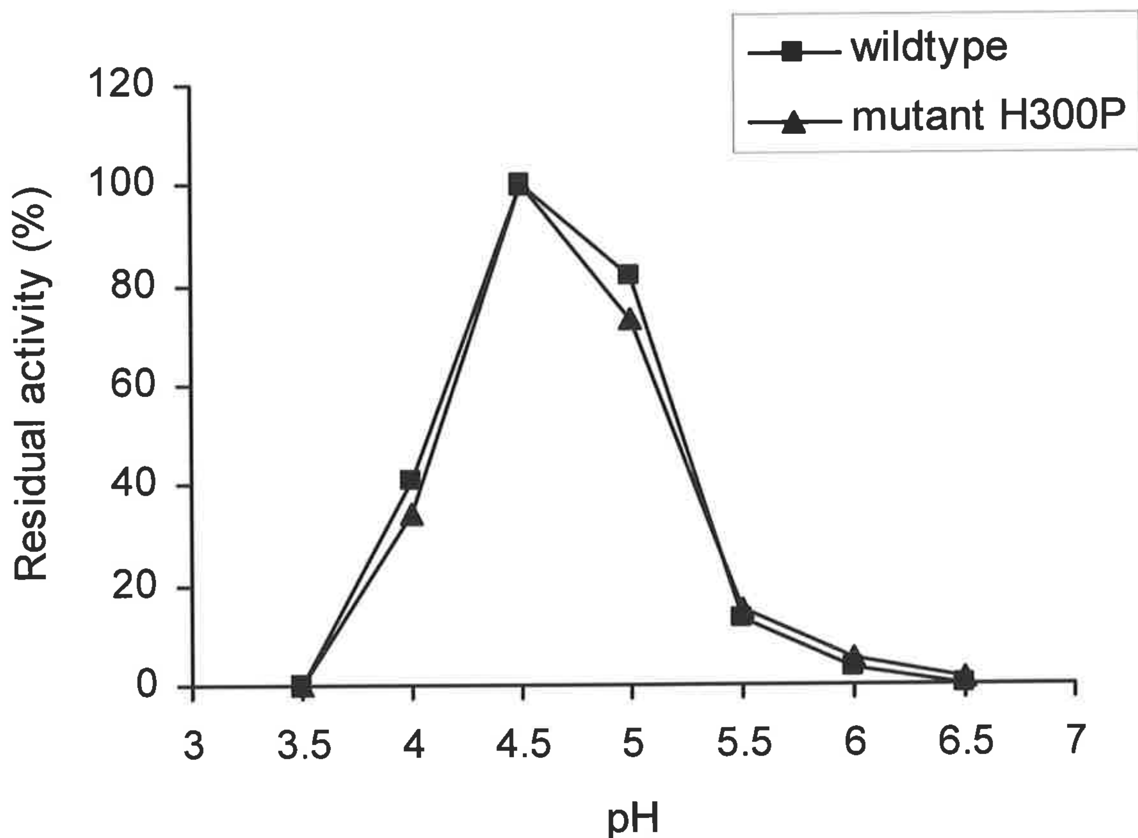


Figure 4.7 Effect of pH on the activity of recombinant barley (1→3,1→4)-β-glucanases isoenzyme EII and the thermostable mutant H300P. Enzyme activity was measured in 0.1 M sodium citrate-sodium phosphate buffers (McIlvane buffers) containing 200 μg/mL BSA, using 2% (w/v) barley (1→3,1→4)-β-glucan as a substrate. The total activity was measured after 20 min incubation at 37°C and expressed as a percentage of the highest activity value, which occurred at pH 4.5.

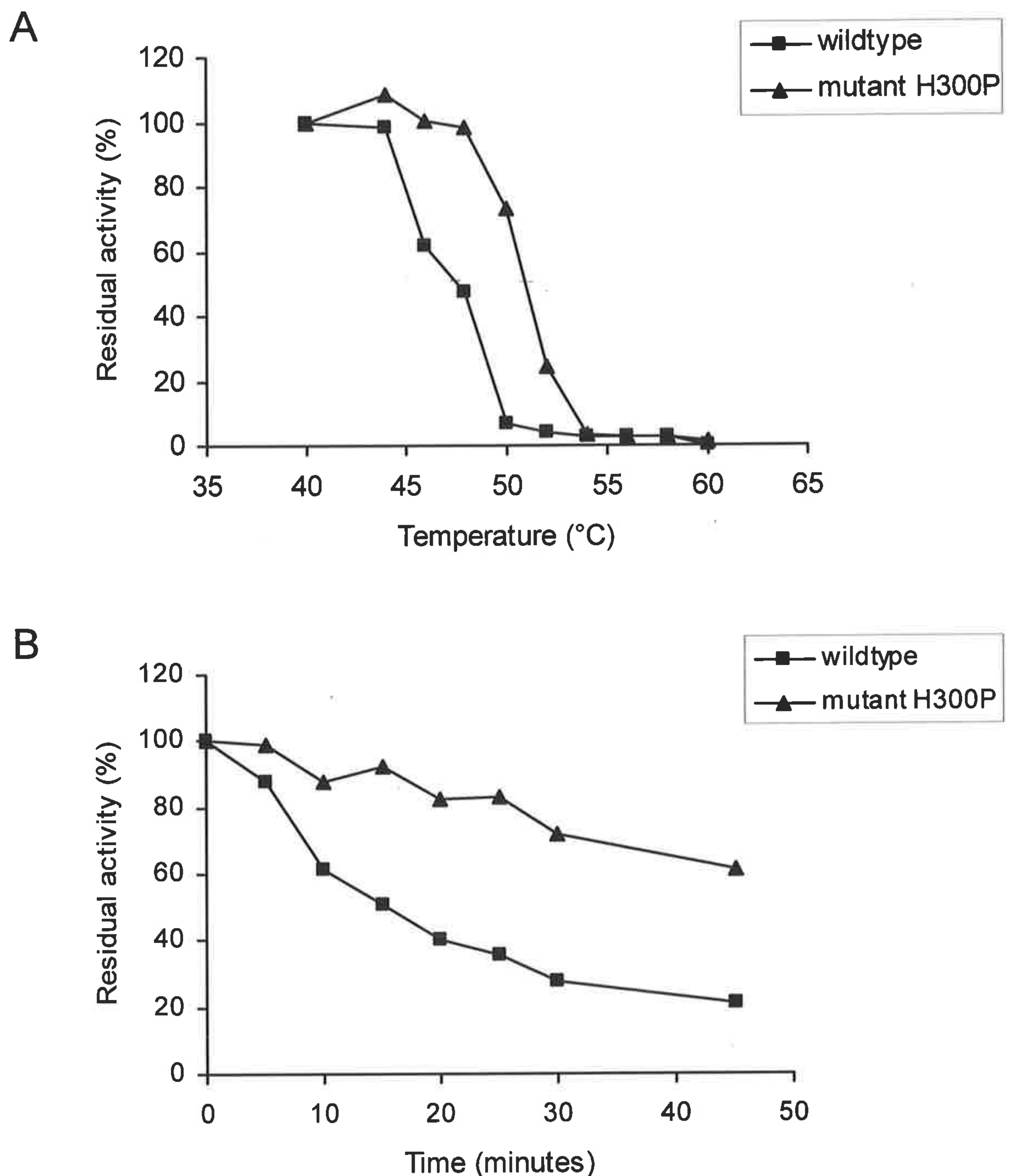


Figure 4.8 The thermostability of barley (1→3,1→4)-β-glucanase mutant H300P. Barley (1→3,1→4)-β-glucanase isoenzyme EII and a thermostable mutant form of the enzyme containing the amino acid substitution H300P were expressed in *E. coli* and purified using affinity chromatography. The thermostabilities of the two enzymes were determined by either measuring the residual activity after 15 minutes incubation at a range of different temperatures (A) or by measuring the progressive loss of activity over time at 48°C (B).

freshly purified prior to thermostability assays. Standard deviations were invariably less than 0.2°C.

The small increase in stability (0.7°C) that was associated with the N290H substitution may result from an increase in the packing efficiency of the protein due to the larger size of the histidine residue compared with the asparagine. Alternatively, the nitrogen atoms of the histidine may be form extra hydrogen bonds with neighbouring atoms. There are several examples where increased packing efficiency of the protein core has lead to increased protein stability (Davies *et al.*, 1993; Rose and Wolfenden, 1993).

The M298K;T17D substitution was designed to stabilize the COOH-terminal loop (Figure 4.3) by forming a salt bridge between the top of the loop and the body of the enzyme (Figure 4.3). Although salt bridges can make a major contribution to the stability of proteins (Anderson *et al.*, 1990; Dao-Pin *et al.*, 1991; Davies *et al.*, 1993; Ishikawa *et al.*, 1993; Kelly *et al.*, 1993), the increase in thermostability due to the M298K;T17D substitution was only 0.2°C (Figure 4.6).

The amino acid substitution H300P showed the largest increase in thermostability (Figures 4.6 and 4.8). This effect can be attributed to a reduction in the entropy of the unfolded state of the enzyme (Matthews *et al.*, 1987; Hardy *et al.*, 1993; Watanabe *et al.*, 1994; Eijsink *et al.*, 1995; Watanabe, 1996; Watanabe and Suzuki, 1998). Because mutant enzyme H300P displayed the largest increase in thermostability compared to the wildtype enzyme, it was chosen as the target enzyme for simulated mashing studies and barley transformation experiments. To this end, it was further analysed in terms of its activity decay at 48°C and its kinetic properties. The thermostable mutant enzyme H300P retained activity for more than three times longer than the corresponding wildtype enzyme at 48°C (Figure 4.8). The K_m and k_{cat} values for mutant H300P were 1.0 mg/ml and 166.0 sec^{-1}, respectively, which were similar to the values of 1.2 mg/ml and 156.0 sec^{-1}, obtained for the recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII (Table 2.1). The pH

optimum for both the wildtype and mutant H300P enzymes was approximately 4.5 (Figure 4.7).

(ii) Amino acid substitutions which decreased thermostability

The four amino acid substitutions G44R, A79P, F85Y, and K23R had negative effects on the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII (Figure 4.6). For substitutions A79P and G44R, the new residues are significantly different from the replaced residues in terms of size and polarity. Alanine and glycine have relatively small uncharged side-chains, whereas arginine and proline are relatively large amino acids. Thus, the G44R and A79P substitutions might have destabilized the enzyme by causing steric hindrance with the atoms of neighbouring residues or disrupting other non-covalent interactions, although this was not predicted by molecular modelling.

The F85Y substitution was designed to form an additional hydrogen bond with the carbonyl oxygen of residues 76 or 80 (Figure 4.3). The decreased thermostability resulting from the F85Y substitution may be due to the hydroxyl group of the tyrosine disrupting the hydrophobicity of the protein core. Indeed, it has been demonstrated that interruption of the spatial arrangement of hydrophobic residues in the core can decrease protein stability (Karpusas *et al.*, 1989; Sandberg and Terwilliger, 1989; Shortle *et al.*, 1990)

The amino acid substitution K23R also had a negative effect on the stability of barley (1→3,1→4)-β-glucanase isoenzyme EII. This substitution was proposed based on the conservation of arginine at this position in barley (1→3)-β-glucanases, including the relatively thermostable (1→3)-β-glucanase isoenzyme GII. Also, a computer-based structural analysis of barley (1→3,1→4)-β-glucanase isoenzyme EII suggested that an arginine at amino acid position 23 should be tolerated by the enzyme structure. Although it is unlikely that the relatively conservative lysine to arginine substitution caused conformational strain in the polypeptide backbone, it is possible that hydrogen bonds in the vicinity of the substitution were disrupted.

The K122R substitution had no apparent effect on stability (Figure 4.6) which suggests that while the arginine can be tolerated by the enzyme's structure, it may not form the anticipated hydrogen bond with the carbonyl oxygen of residue 161 (Figure 4.3).

(iii) Cumulative increases in thermostability

Because amino acid substitutions which increase thermostability are often additive (Watanabe *et al.*, 1994; Eijsink *et al.*, 1995; Matsumura *et al.*, 1995), a triple mutant containing the substitutions H300P, M298K;T17D, and N290H was engineered. However, the thermostability of the resulting triple mutant enzyme was the same as that of the enzyme containing the single substitution H300P (Figure 4.6). This non-cumulative effect is also evident in the neutral proteases from *Bacillus stearothermophilus*, where the mutational effects of stabilising substitutions are not always additive (Vriend and Eijsink, 1993). The relatively high ΔT_{50} value for mutant H300P (3.7°C), compared to that of M298K;T17D (0.4°C) and N290H (0.7°C), suggest that the H300P substitution had an overriding effect on the stability of the COOH-terminal loop, where each of these mutations were effected. These results suggest that the COOH-terminal loop, which includes residues 286 to 306 (Varghese *et al.*, 1994), is potentially unstable and could 'unfold' early in the thermal inactivation process.

4.3.4 Clustering of the Amino Acid Substitutions that Increased Thermostability

The three amino acid substitutions which increased the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII (H300P, N290H, and M298K;T17D), were all located in the COOH-terminal loop of the enzyme (Figure 4.1). This solvent-exposed loop region is clearly lacking in defined secondary structure (Varghese *et al.*, 1994), which suggests that it may represent a 'weak link' in the enzyme. Eijsink *et al.* (1995) found that the crucial interactions responsible for the relatively high thermostability of a bacterial thermolysin were all localised to a solvent-exposed loop region in the NH_2-terminal domain of the protein, suggesting that peripheral loops may be involved in the early steps of the unfolding process (Eijsink *et al.*, 1995). Similarly, Welfle *et al.* (1996) found that several amino acid substitutions which contributed to the thermostability of a hybrid

bacterial (1→3,1→4)-β-glucanase (Olsen *et al.*, 1991), were all located in the NH_2-terminal loop region. Finally, proline residues responsible for the extremely high thermostability of a (1→6)-glucosidase from *Bacillus thermoglucosidasius*, also occur with high frequency in the loop regions (Watanabe *et al.*, 1991).

4.3.5 Performance of Mutant H300P during Simulated Mashing at 65°C

The thermostability of the recombinant (1→3,1→4)-β-glucanase mutant H300P was tested under simulated mashing conditions at 65°C. Kiln-dried barley malt was pre-incubated in water at 65°C for 20 min to inactivate endogenous barley (1→3,1→4)-β-glucanase activity. Following the heat-inactivation step, the mash was spiked with recombinant wildtype barley (1→3,1→4)-β-glucanase isoenzyme EII or the recombinant (1→3,1→4)-β-glucanase mutant H300P, and (1→3,1→4)-β-glucanase activity was measured during subsequent mashing at 65°C. In a separate experiment, endogenous (1→3,1→4)-β-glucanase activity in the malt was measured during mashing at 65°C. Results from the two experiments were plotted on a single graph, as shown in Figure 4.9.

After 30 sec mashing, the level of endogenous (1→3,1→4)-β-glucanase activity was twice that of the recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII. This may be attributed to the fact that, unlike the recombinant wildtype (1→3,1→4)-β-glucanase isoenzyme EII, the endogenous (1→3,1→4)-β-glucanase isoenzyme EII is glycosylated (Doan and Fincher, 1992). For the (1→3,1→4)-β-glucanase mutant H300P, approximately 30% activity remained after 1 min mashing at 65°C, indicating that the mutant enzyme is significantly more thermostable than both the endogenous barley (1→3,1→4)-β-glucanases and the recombinant wildtype (1→3,1→4)-β-glucanase isoenzyme EII. The mutant enzyme H300P survived for up to 3 min in the mash at 65°C, whereas the corresponding recombinant wildtype (1→3,1→4)-β-glucanase isoenzyme EII was almost entirely inactivated after approximately 20 sec of mashing (Figure 4.9).

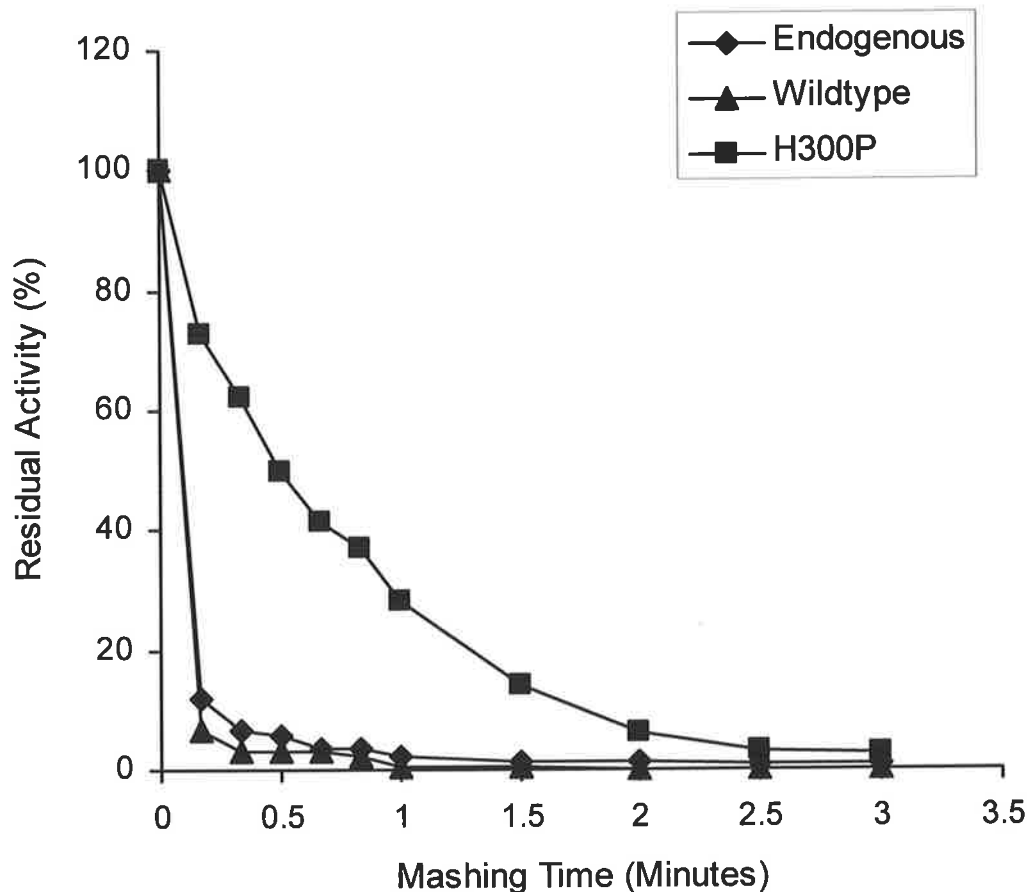


Figure 4.9 The thermostability of (1→3,1→4)-β-glucanase mutant H300P during simulated mashing at 65°C. Kiln-dried barley malt was subjected to simulated mashing at a constant temperature of 65°C and the decline in endogenous (1→3,1→4)-β-glucanase activity was measured over time. In a separate experiment, barley malt was incubated in water at 65°C for 20 min to inactivate endogenous (1→3,1→4)-β-glucanase activity, and the recombinant (1→3,1→4)-β-glucanase mutant enzyme H300P, or the corresponding recombinant wildtype enzyme, were added to the heat-inactivated mash, and (1→3,1→4)-β-glucanase activity was measured over 5 minutes. The amount of each recombinant enzyme which was added to the mash was equivalent to the amount of endogenous (1→3,1→4)-β-glucanase present in the malt, based on activity. Results from the two independent experiments were plotted on a single graph.

Although (1→3,1→4)-β-glucanase mutant H300P is more stable than the endogenous barley (1→3,1→4)-β-glucanases during mashing at 65°C, it is still significantly less stable than fungal and bacterial enzymes which are currently used in the brewing industry to hydrolyse residual (1→3,1→4)-β-glucan. Indeed, preliminary experiments show that the ability of (1→3,1→4)-β-glucanase mutant H300P to reduce the viscosity of the mash, and subsequently the wort, is very low when compared to that of a commercial fungal (1→4)-β-glucanase from Quest International (Bussum, Netherlands) and a commercial *Bacillus subtilis* lichenase from Deltagen (R.J. Stewart, D.C. Stewart and G.B. Fincher, unpublished data).

4.4 Summary and Conclusions

Eight amino acid substitutions were introduced into barley (1→3,1→4)-β-glucanase isoenzyme EII in an attempt to increase thermostability. Three of the resulting mutant enzymes displayed a higher thermostability than the corresponding wildtype enzyme. The replacement of the histidine at amino acid position 300 with a proline (H300P) gave the largest increase in thermostability. During simulated mashing at 65°C, the mutant enzyme H300P lasted at least five times longer than the corresponding wildtype enzyme. The three amino acid substitutions which caused an increase in heat stability were clustered at the COOH-terminal loop of barley (1→3,1→4)-β-glucanase isoenzyme EII, suggesting that this loop may represent an unstable region of the enzyme. The three substitutions were combined in an effort to further enhance stability, but the resulting enzyme displayed the same thermostability as the most stable individual mutant, H300P. Finally, it was decided that joining the COOH- and NH_2-termini of barley (1→3,1→4)-β-glucanase isoenzyme EII may stabilize the COOH-terminal loop and hence stabilize the enzyme. The potential existed that this coupling of the relatively unstable COOH-terminus could provide even larger increases in thermostability. Attempts to fuse the NH_2- and COOH-termini of barley (1→3,1→4)-β-glucanase isoenzyme EII are described in the next Chapter.

CHAPTER FIVE:

CIRCULAR PERMUTATION OF BARLEY $(1\rightarrow3,1\rightarrow4)$-$\beta$-GLUCANASE ISOENZYME EII

5.1 Introduction

In Chapter 4 of this thesis, it was shown that the amino acid substitutions N290H, M298K;T17D and H300P enhanced the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII. The three amino acid substitutions are all located in the COOH-terminal loop of the enzyme, and it is therefore possible that the COOH-terminal loop represents an unstable region in the enzyme's structure and may play a role in the initial unfolding events of the enzyme at elevated temperatures. Because the NH_2- and COOH- termini of barley (1→3,1→4)-β-glucanase isoenzyme EII are in close proximity (Varghese *et al.*, 1994), it was decided to fuse the termini in an attempt to stabilise the COOH-terminal region and hence increase the enzyme's thermostability. The rearrangement of a protein's primary structure in this way, such that the NH_2- and COOH- termini are fused and new termini are created at other positions in the polypeptide chain, is referred to as 'circular permutation' (Ponting and Russell, 1995).

Natural circular permutations exist in the bacterial (1→3,1→4)-β-glucanase protein family (Schimming *et al.*, 1992; Keitel *et al.*, 1993; Hahn *et al.*, 1995; Ponting and Russell, 1995; Ay *et al.*, 1998). A sequence alignment of (1→3,1→4)-β-glucanases from different species of bacteria reveal an amino acid identity of greater than 30%, with the notable exception of the enzyme from *Fibrobacter succinogenes*. The COOH-terminal portion of the (1→3,1→4)-β-glucanase from *F. succinogenes* aligns with the NH_2-terminal portion of the other bacterial (1→3,1→4)-β-glucanases (Schimming *et al.*, 1992). A hybrid (1→3,1→4)-β-glucanase from *Bacillus macerans*, which has a compact jelly-roll fold with its COOH- and NH_2- termini in close proximity, was used to create a circularly permutated *B. macerans* enzyme based on the sequence rearrangement present in the *F. succinogenes* (1→3,1→4)-β-glucanase (Hahn *et al.*, 1995). The resulting recombinant protein was enzymically active, and its three-dimensional structure was almost identical to that of the wildtype *B. macerans* enzyme (Keitel *et al.*, 1993; Hahn *et al.*, 1995). Results from a related study show that circular permutations of the (1→3,1→4)-β-glucanase from *B.*

macerans, other than those based on the *F. succinogenes* sequence, are also tolerated and lead to active protein (Ay *et al.*, 1998). It was concluded that the jelly-roll fold of the bacterial (1→3,1→4)-β-glucanases is sufficiently stable to accommodate several different circular permutations, and that the NH_2-terminal region in the native enzymes is not required for correct folding (Ay *et al.*, 1998).

Proteins with the traditional $(\alpha/\beta)_8$ barrel structure are ideal candidates for creating circular permutations because their NH_2- and COOH- termini are normally in close proximity, and there are a number of surface loop regions to which the termini can be relocated without disrupting the secondary structure of the protein (Goldenberg, 1989). A phosphoribosyl anthranilate (PRA) isomerase from *E. coli* has a typical $(\alpha/\beta)_8$ barrel structure (Priestle *et al.*, 1987) and shows 30% amino acid identity to a yeast PRA isomerase. It was assumed that the yeast PRA isomerase has an $(\alpha/\beta)_8$ barrel structure that is similar to the bacterial PRA isomerase, and a peptide sequence of the appropriate length and polarity was used to fuse the NH_2- and COOH-termini of the yeast enzyme (Crawford *et al.*, 1987). The new NH_2- and COOH- termini were located to a surface loop region distal to the active site to ensure that the enzyme's activity was unaffected (Crawford *et al.*, 1987). The wildtype and circularly permutated form of the yeast PRA displayed similar folding and activity characteristics (Crawford *et al.*, 1987).

Attempts to produce a circularly permutated form of barley (1→3,1→4)-β-glucanase isoenzyme EII are described in this Chapter.

5.2 Materials and Methods

5.2.1 Materials

Plasmid pSP73 was provided by Dr Helen Healy, Department of Plant Science, University of Adelaide. Monoclonal antibodies specific for barley (1→3,1→4)-β-glucanase isoenzyme EII were as described by Høj *et al.* (1990). Strains of *E. coli* XL1-Blue and BL21 (DE3) pLysS were from Stratagene and Novagene, respectively. Ni-NTA resin, imidazole and polyhistidine-specific monoclonal antibodies were purchased from Qiagen. The antibody conjugate IgG-HRP was from Bio-Rad and Horse Radish Peroxidase substrate was from Promega (Madison, WI, USA). *Taq* DNA polymerase was purchased from Gibco BRL (Gaithersburg, MD, USA). Restriction enzymes and DNA ligase were purchased from New England Biolabs. The BresaClean kit was from Geneworks, and DNA oligonucleotides were synthesised using an Applied Biosystems DNA Synthesiser. Trifluoroacetic acid and acetonitrile were from BDH Chemicals (Poole, Dorset, England).

5.2.2 Designing a Circular Permutation

The three-dimensional structure of barley (1→3,1→4)-β-glucanase isoenzyme EII was analysed using the 'O' program (Jones *et al.*, 1991) to select a linker peptide sequence with the appropriate length, polarity and dihedral angles to join the NH_2- and COOH-termini of the enzyme. The tri-peptide sequence Ala-Ala-Gly met these criteria, and was therefore used to join NH_2- and COOH-termini. Manipulations were performed using the *move_zone* and *merge_atom* commands, as described in the 'O' software manual. New NH_2- and COOH-termini were created at positions 222 and 226 of barley (1→3,1→4)-β-glucanase isoenzyme EII, respectively. This involved removing residues 223, 224 and 225 using the *delete* command, to allow room for the polyhistidine tag at the COOH- terminus. The new termini were located in a surface loop region which joined the major α-helix α7 with β-sheet β6 (Varghese *et al.*, 1994). The circularly permutated recombinant (1→3,1→4)-β-glucanase was designated NC-F ($\underline{N}H_2$-$\underline{C}OOH$ $\underline{f}$usion), and is shown in Figure 5.1.

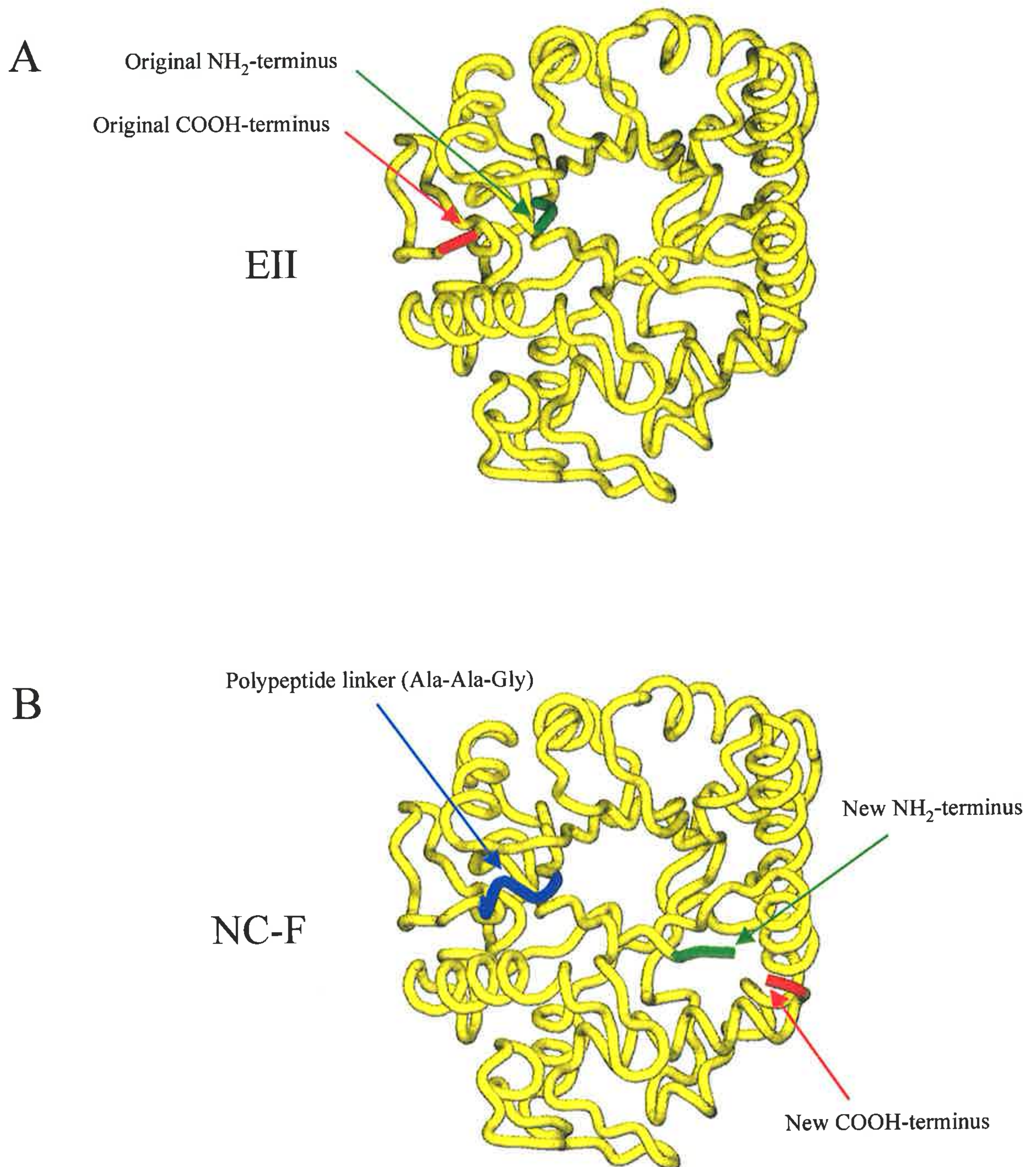


Figure 5.1 Diagram showing the positions of the NH_2- and COOH-termini of barley (1→3,1→4)-β-glucanase isoenzmye EII (A) and its corresponding circular permutation NC-F (B). The polypeptide chains of the two enzymes are shown in yellow and the NH_2- and COOH-terminal residues are coloured green and red, respectively. The polypeptide sequence Ala-Ala-Gly, which was used to fuse the termini of barley (1→3,1→4)-β-glucanase isoenzmye EII is shown in blue. This figure was created using the 'O' program (Jones *et al.*, 1991).

5.2.3 Creating a Circular Permutation at the cDNA Level

The cDNA encoding barley (1→3,1→4)-β-glucanase isoenzyme EII was rearranged using PCR to create the circular permutation NC-F (Figure 5.2). Plasmid pET3a-HT/EII was used as a template for the PCR and the following primers were used to amplify two separate DNA fragments:

*Pvu*II
NC1: 5'- CTT CGC AGC TGG GAT CGG GGT GTG CTA CGG C -3'

*Sac*II
NC2: 5'- CAC CAG CTT CCG CGG AAC TAA GCC GTG CTT GCC CAT GGC -3'

*Nde*I
NC3: 5'- CAC GGC GGC CAT ATG GTG AAG CTG GTG GGG -3'

*Pvu*II
NC4: 5'- CGA GCA CGA CCC AGC TGC GAA GTT GAT GGG GGA G -3'

Primers NC1 (forward) and NC2 (reverse) were used to amplify a 672 bp fragment designated NC-A (Figure 5.3), which encodes the Ala-Ala-Gly linker followed by amino acid residues 1 to 222 of the mature barley (1→3,1→4)-β-glucanase isoenzyme EII and a polyhistidine tag (Figure 5.2). Primer NC1 is complementary to the first 10 nucleotides of the (1→3,1→4)-β-glucanase isoenzyme EII cDNA coding region and has a 5' extension which encodes the Ala-Ala-Gly linker sequence and incorporates a *Pvu* II restriction site (underlined). Primer NC2 (reverse primer) is complementary to the last 10 nucleotides of the (1→3,1→4)-β-glucanase isoenzyme EII cDNA coding region and has a 5' overhang which encodes a thrombin cleavage recognition site and polyhistidine tag. The DNA sequence encoding the thrombin cleavage recognition site also incorporates a unique *Sac*II restriction enzyme site (underlined).

Primers NC3 (forward) and NC4 (reverse) were used to amplify a 235 bp fragment designated NC-B (Figures 5.2). Fragment NC-B encodes a methionine start codon which incorporates a unique *Nde*I site (underlined) followed by amino acid residues 226 to 306 of the mature barley (1→3,1→4)-β-glucanase isoenzyme EII. The 5' overhang of primer

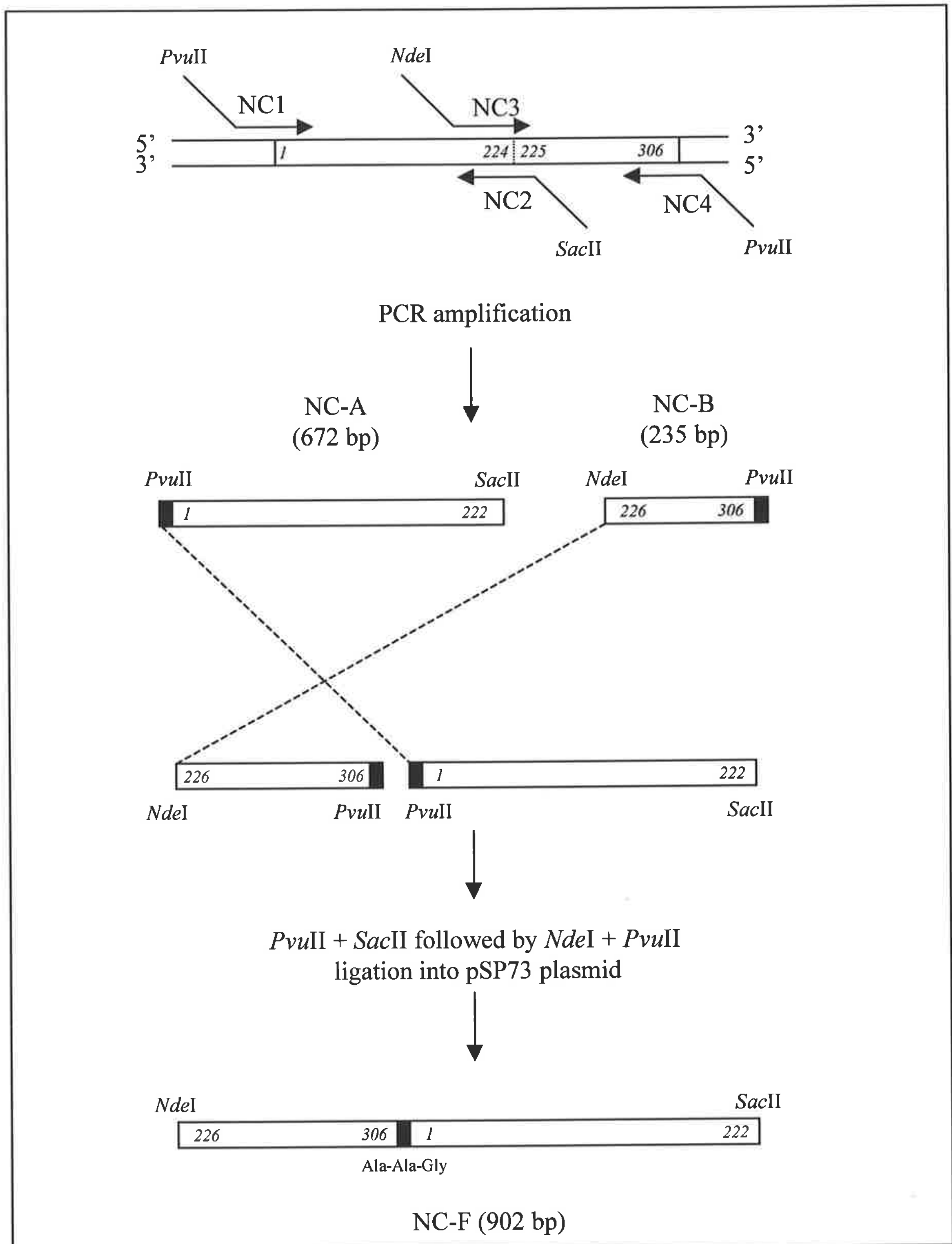


Figure 5.2 Strategy used to construct a circular permutation of barley (1→3,1→4)-β-glucanase isoenzyme EII. Primer pairs NC1/NC2 and NC3/NC4 were used to amplify fragments NC-A and NC-B, respectively. The black shaded box represents the DNA sequence encoding the Ala-Ala-Gly linker which also incorporates a unique *Pvu*II site (see text for details). Italicized numbers refer to amino acid positions in the original wildtype recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII.

NC4 encodes the tripeptide sequence Ala-Ala-Gly and also incorporates a *Pvu*II restriction enzyme site (underlined).

The PCR reaction mix contained 50 ng template DNA, 100 pmol of each primer, 100 μM dNTP, 5 mM $MgCl_2$, 10 % (v/v) DMSO, 1 unit *Taq* DNA polymerase, and 1X *Taq* polymerase buffer. A drop of mineral oil was added to each tube and the PCR was performed in a Perkin Elmer DNA Thermal Cycler. Tubes were heated for 94°C for 5 min and subjected to 35 cycles of the following temperature regime: 94°C for 40 sec, 50°C for 40 sec, and 72°C for 1 min. A final 10 min extension was performed at 72°C and the amplified PCR fragments were purified using BresaClean (Geneworks) according to the product literature.

In preparation for ligation, fragment NC-A was digested with *Pvu*II and *Sac*II, and fragment NC-B was digested with *Nde*I and *Pvu*II (Figure 5.2). The plasmid pSP73 was digested with *Pvu*II and *Sac*II, and NC-A was ligated into pSP73 using these sites. The ligation and transformation procedures were performed as described in Section 2.2.2 (iii). The ligated vector was digested with *Nde*I and *Pvu*II and fragment NC-B was ligated to produce a vector containing DNA fragment NC-B followed by fragment NC-A, which together encode the recombinant (1→3,1→4)-β-glucanase NC-F. Finally, the 912 bp fragment, designated NC-F (Figure 5.3), was excised from pSP73 with *Nde*I and *Sac*II and ligated into the pET3a-HT expression plasmid to produce pET3a-HT/NC-F. Automated DNA sequencing was performed as described in Section 2.2.2 (vi) to ensure the sequence of plasmid pET3a-HT/NC-F was correct.

5.2.4 Expression and Purification of (1→3,1→4)-β-Glucanase NC-F

Plasmid pET3a-HT/NC-F was used to direct the expression of (1→3,1→4)-β-glucanase NC-F in *E. coli*, essentially as described for barley (1→3,1→4)-β-glucanase isoenzyme EII in Section 2.2.3. Following cell lysis, inclusion bodies present in the insoluble fraction were dissolved in 5 ml of 0.01 M Tris-HCl buffer, pH 8.0, containing 8 M urea (Buffer A).

The recombinant (1→3,1→4)-β-glucanase NC-F was purified under denaturing conditions as follows: A Ni-NTA spin column was equilibrated with buffer A containing 20 mM imidazole, and centrifuged for 2 min at 2,000 rpm. Denatured protein (600 μl) was loaded onto a pre-equilibrated column and centrifuged for 2 min at 2,000 rpm. The column was washed twice with buffer A, and twice more with the same buffer adjusted to pH 6.7. Bound protein was eluted with 200 μl buffer A, adjusted to pH 6.7 and 200 mM imidazole. Eluted proteins were analysed by SDS-PAGE as described in Section 2.2.6.

5.2.5 Western Blot Analysis of (1→3,1→4)-β-Glucanase NC-F

For Western blot analysis, the denatured (1→3,1→4)-β-glucanase NC-F was resolved by 12.5 % SDS-PAGE (300 mm X 450 mm gel) and transferred to a nitrocellulose filter using a Bio-Rad Transblot Apparatus. The transfer was performed over 1 hour at 100 V using transblot buffer (25 mM Tris, 0.19 M glycine, 20% (v/v) methanol, 0.17 mM SDS). The filter was blocked with 5% (w/v) milk powder in 1 X PBS (5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.5 mM KCl) for 10 min with gentle shaking, and washed twice with 1 X PBS. Monoclonal antibodies specific for barley (1→3,1→4)-β-glucanase isoenzyme EII (Høj *et al.*, 1990) and for the polyhistidine tag sequence (Qiagen) were diluted 1:1000 in 1 X PBS containing 1% (w/v) BSA. Filters were incubated with antibody solution in a sealed plastic bag overnight at room temperature, washed three times in 1 X TPBS (1 X PBS containing 0.05% (v/v) Tween-20), and twice with 1 X PBS. Washed filters were incubated with goat anti-mouse IgG-horse radish peroxidase (HRP) conjugate (1:5000 dilution) in 1 X PBS and 1% (w/v) BSA for 2 h at room temperature. Finally, filters were washed three times with 1 X TPBS, twice with 1 X PBS, and developed at room temperature using TMB-stabilised HRP substrate (Promega).

5.2.6 HPLC Purification and NH_2-Terminal Protein Sequencing

Recombinant (1→3,1→4)-β-glucanase NC-F was further purified by reverse-phase High Performance Liquid Chromatography (HPLC) on a Hewlett Packard Series II 1090 Liquid Chromatograph. Approximately 6 μg denatured protein was separated using a Brownlee Aquapore C18 column (40.0 x 4.6 mm) at a flow rate of 0.6 ml/min, using 0.04% (v/v)

trifluoroacetic acid and 70% (v/v) acetonitrile as a solvent. Purified recombinant barley (1→3,1→4)-β-glucanase isoenzyme EII (see Chapter 2) was run independently as a positive control. Protein fractions corresponding to the major peak, which overlapped with the control peak, were pooled for protein sequencing. Protein sequencing was performed by the Nucleic Acid and Protein Chemistry Unit in the Department of Plant Science, University of Adelaide, South Australia. Approximately 4 μg HPLC-purified protein was sequenced using a Hewlett Packard G1000A Protein Sequencer, according to the manufacturer's instructions.

5.3 Results and Discussion

5.3.1 Creating a Circular Permutation of (1→3,1→4)-β-Glucanase Isoenzyme EII

The cDNA encoding barley (1→3,1→4)-β-glucanase isoenzyme EII was rearranged using PCR to create the circularly permutated cDNA, NC-F. The cDNA was effectively divided into fragments NC-A and NC-B (Figures 5.2 and 5.3) which encoded amino acids 1 to 222, and 226 to 306 of mature barley (1→3,1→4)-β-glucanase isoenzyme EII, respectively. A DNA linker encoding the tri-peptide Ala-Ala-Gly was used to join the fragments *via* a *Pvu*II restriction site, which was introduced at the 5' and 3' ends of fragments NC-A and NC-B, respectively. The resulting fragment, designated fragment NC-F (Figures 5.2 and 5.3), contained a *Nde*I at its 5' end and a *Sac*II site at its 3' end. These sites were used to clone fragment NC-F into the expression plasmid pET3a-HT, creating pET3a-HT/NC-F. Complete DNA sequencing confirmed that pET3a-HT/NC-F contained no errors.

5.3.2 Expression and Purification of Recombinant (1→3,1→4)-β-Glucanase NC-F

The pET3a-HT/NC-F plasmid was used to direct the expression of recombinant (1→3,1→4)-β-glucanase NC-F in *E. coli.* Cells containing the expressed recombinant enzyme were lysed, and the insoluble fraction, which contained cell debris and inclusion bodies, was separated from the soluble fraction by centrifugation. Attempts to purify the expressed recombinant NC-F from the soluble fraction using a Ni-NTA column were unsuccessful; no (1→3,1→4)-β-glucanase protein bands could be detected on SDS-PAGE gels. Furthermore, no (1→3,1→4)-β-glucanase activity could be detected in the soluble protein fraction (data not shown), indicating that the majority of the enzyme was insoluble.

In order to determine whether the expressed recombinant NC-F was forming inclusion bodies, the insoluble fraction pellet was solubilised in 8 M urea and purified using an Ni-NTA column. SDS-PAGE analysis of the denatured, purified fraction revealed a single major band with an approximate molecular mass of 33.0 kDa (Figure 5.4), which corresponds to the expected size for the recombinant (1→3,1→4)-β-glucanase NC-F. The

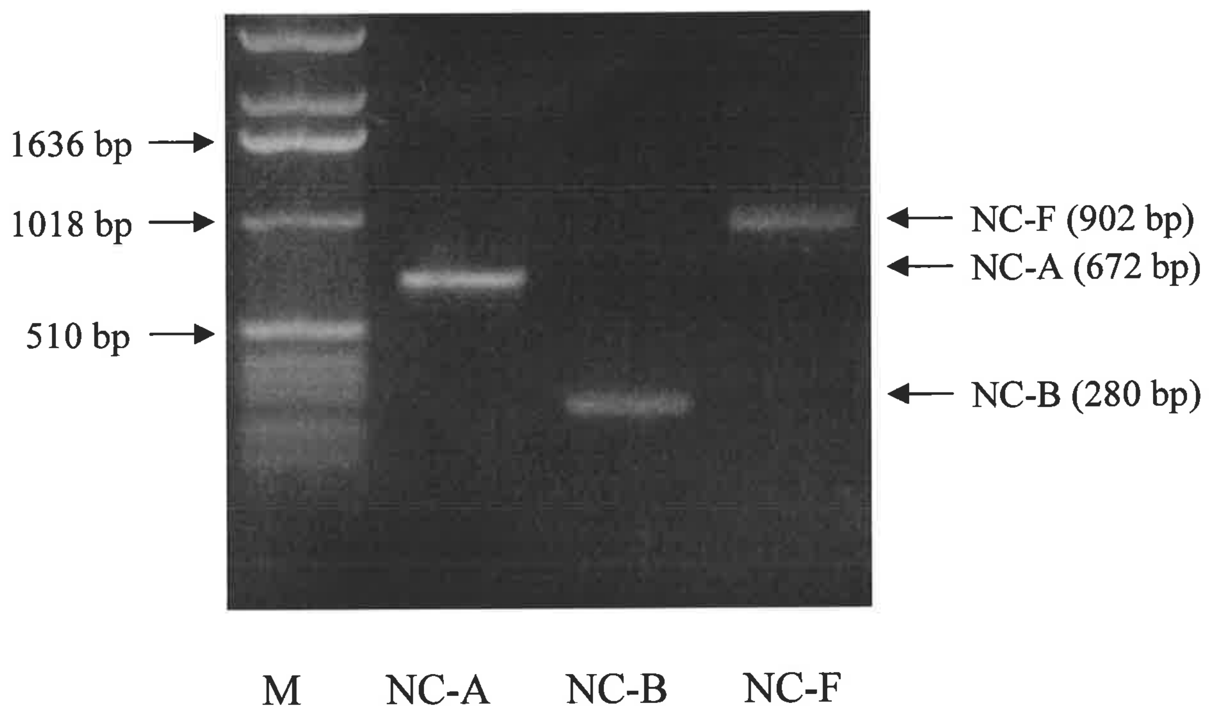


Figure 5.3 Analysis of the PCR products used to create a circular permutation of barley (1→3,1→4)-β-glucanase isoenzyme EII. Amplified PCR products (~0.5 ug) NC-A, NC-B, and NC-F were analysed by 1% agarose gel electropohoresis to confirm their size and purity (see text for details). M = standard 1 kb DNA ladder.

relatively high yield (approximately 1-2 mg per litre culture) and purity of the 33.0 kDa protein (Figure 5.4) indicates that it had a high affinity for the Ni-NTA resin, and therefore contained a polyhistidine tag. These results strongly suggest that the 33.0 kDa protein in the insoluble fraction of the *E. coli* cell lysate represented the recombinant (1→3,1→4)-β-glucanase NC-F. The fact that the recombinant (1→3,1→4)-β-glucanase NC-F did not fold correctly in the purification buffer (50 mM sodium phosphate, pH 7.8, containing 300 mM NaCl), indicated that the enzyme was very unstable compared with the corresponding wildtype (1→3,1→4)-β-glucanase. For this reason, no attempts were made to refold the denatured recombinant enzyme NC-F *in vitro*.

5.3.3 Western Blot Analyses of Recombinant (1→3,1→4)-β-Glucanase NC-F

Western blot analyses were performed to help confirm the identity of the expressed 33.0 kDa protein as the recombinant (1→3,1→4)-β-glucanase NC-F. The urea-denatured, Ni-NTA purified protein was separated by SDS-PAGE and transferred to a nylon membrane. The membrane was incubated with monoclonal antibodies specific for either barley (1→3,1→4)-β-glucanase isoenzyme EII or the polyhistidine tag sequence (Figure 5.4). Results show that a single protein with an approximate molecular mass 33.0 kDa gave a positive signal with both monoclonal antibodies (Figure 5.4). This indicated the presence of the barley (1→3,1→4)-β-glucanase isoenzyme EII epitope and the six-histidine tag sequence. Together, these results provided very strong evidence that the purified 33.0 kDa protein was indeed the recombinant (1→3,1→4)-β-glucanase NC-F.

5.3.4 Protein Sequencing of Recombinant (1→3,1→4)-β-Glucanase NC-F

The putative recombinant (1→3,1→4)-β-glucanase NC-F was further analysed by protein sequencing. Proteins must be of relatively high purity (generally greater than 95%) in order to produce reliable NH_2- terminal sequencing data. Thus, the nickel column-purified putative recombinant (1→3,1→4)-β-glucanase NC-F was further purified using reverse-phase HPLC. Protein fractions corresponding to the major peak were pooled and used for protein sequencing. The resulting data revealed two major protein sequences, designated A and B, of approximately equal abundance (Figure 5.4). The first 25 reliable NH_2- terminal

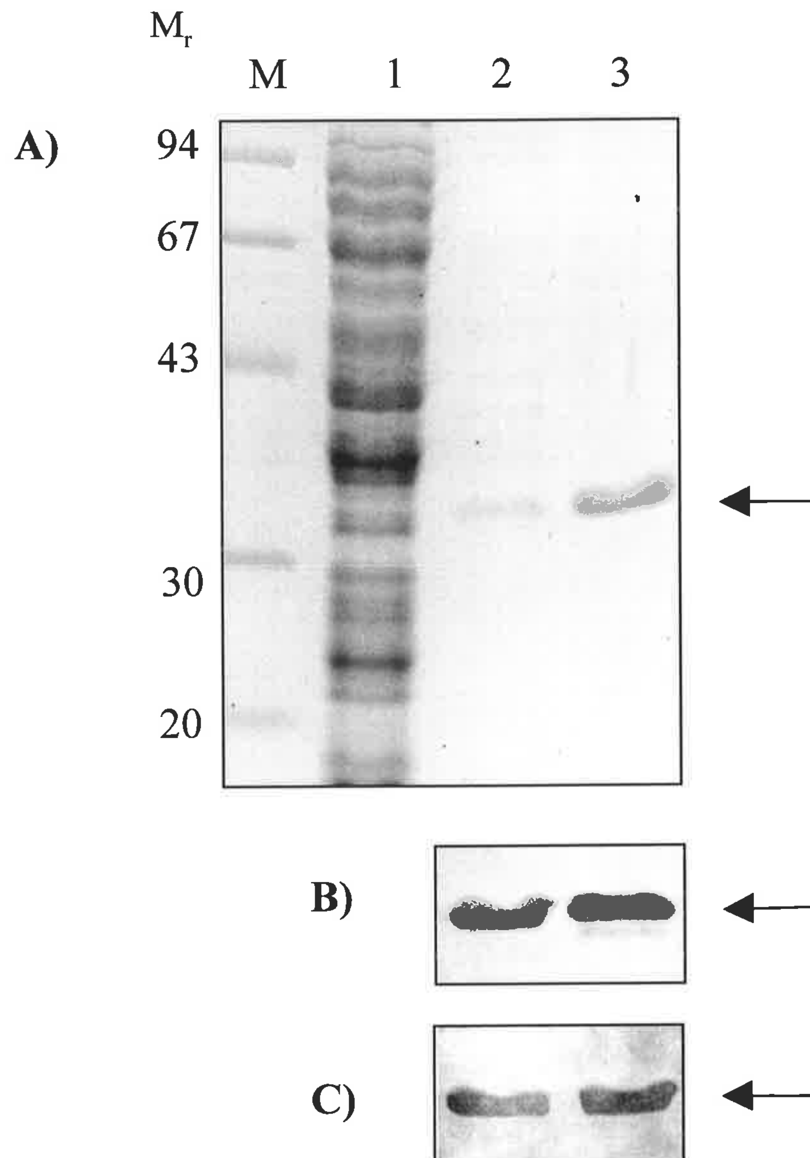


Figure 5.4 SDS-PAGE and Western blot analysis of recombinant (1→3,1→4)-β-glucanase NC-F. The circularly permutated barley (1→3,1→4)-β-glucanase NC-F was expressed in *E. coli* as a fusion protein and purified under denaturing conditions using Ni-NTA resin (see text for details). Lane 1, bacterial cell lysate; Lane 2, urea-purified NC-F fusion protein; Lane 3, urea-purified wildtype barley (1→3,1→4)-β-glucanase isoenzyme EII fusion protein; M, molecular weight markers. A, Coomassie-stained SDS-PAGE gel; B, Western blot using monoclonal antibodies to native barley (1→3,1→4)-β-glucanase isoenzyme EII; C, Western blot using monoclonal antibodies to the polyhistidine tag (see text for details); The arrows indicate fusion proteins of approximately 32 kDa.

	1				25
Predicted Sequence (NC-F):	VKLVV	SESGW	PSGGG	TAATP	ANARF
Sequence 1:	VKLVV	SESGW	PSGGG	TAATP	ANARF
Sequence 2:	MKVAK	DLVVS	LAYQV	RTEDG	VLVDE

Figure 5.5 Sequence analysis of the first 25 NH_2-terminus residues of the circularly permutated mutant NC-F. The circularly permutated barley (1→3,1→4)-β-glucanase NC-F was expressed as a fusion protein in *E. coli* and purified under denaturing conditions using Ni-NTA resin (see text for details). The enzyme was further purified using HPLC and sequenced using a Hewlett Packard Protein Sequencer. The two predominant protein sequences are aligned with the predicted amino acid sequence for the circularly permutated mutant, NC-F.

amino acids of sequence A were identical to the expected sequence for recombinant (1→3,1→4)-β-glucanase NC-F (Figure 5.5).

Protein sequence B was found to match exactly to the NH_2-terminus of *E. coli* peptidyl-prolyl *cis-trans* isomerase (PPI; Figure 5.5), which is an enzyme composed of 196 amino acids and contains the following two histidine-rich regions:

Residues 149-164: HGHVHGAHDHHHDHDH (55.3 % histidine)

Residues 171-179: HGHDHGHEH (55.6 % histidine)

Due to the presence of these histidine-rich proteins, the PPI has a high affinity for nickel and zinc (Hottenrott *et al.*, 1997). Although Ni-NTA resin has the strongest affinity for six consecutive histidines (Janknecht *et al.*, 1991), it also binds relatively strongly to proteins with two or more consecutive histidines (Qiaexpressionist product literature, Qiagen). It would therefore be difficult to completely remove PPI from the Ni-NTA column under the relatively low stringency wash conditions (20 mM imidazole) which were used for the purification of the recombinant (1→3,1→4)-β-glucanase NC-F. This may explain the co-purification of PPI with the recombinant enzyme NC-F. Alternatively, the PPI enzyme may have formed a protein-protein interaction with the recombinant (1→3,1→4)-β-glucanase NC-F, which would also explain the co-purification of the two enzymes. Indeed, it has been shown that PPI can act as a chaperone during the folding of human carbonic anhydrase II (Freskgård *et al.*, 1992), a process which involves relatively strong protein-protein interactions.

5.4 Summary and Conclusions

Protein and DNA sequencing results, together with Western blot analyses, confirmed that the recombinant (1→3,1→4)-β-glucanase NC-F was successfully expressed and purified under denaturing conditions. However, the recombinant (1→3,1→4)-β-glucanase NC-F could not be recovered from *E. coli* in a soluble, active form. This strongly suggests that the recombinant enzyme did not fold correctly, the reasons for which are not immediately apparent from the three-dimensional model of NC-F which was constructed using molecular modelling software (Figure 5.1). One possible explanation is that the major helix α7 which is situated at the 'new' COOH-terminus (Figure 5.1), may not be buried sufficiently during folding. Using alternative loop regions for the relocation of the NH_2- and COOH- termini, such as the surface loop containing glycine 26 (Varghese *et al.*, 1994), may result in the production of an active (1→3,1→4)-β-glucanase with enhanced stability.

CHAPTER SIX:

TRANSFORMATION OF BARLEY WITH DNA ENCODING THE THERMOSTABLE BARLEY (1→3,1→4)-β-GLUCANASE MUTANT H300P

6.1 Introduction

Barley (1→3,1→4)-β-glucanase mutant H300P lasts up to five times longer than the corresponding native barley enzyme during simulated mashing at 65°C (Figure 4.9), and therefore has the potential to reduce the detrimental effects of residual high molecular weight (1→3,1→4)-β-glucans in the brewing industry. However, to measure the performance of the thermostable (1→3,1→4)-β-glucanase mutant H300P under large-scale industrial conditions, it will be necessary to produce transgenic barley that expresses the enzyme. Efforts to transform barley with DNA encoding the thermostable mutant enzyme H300P are described in this Chapter.

6.1.1 Cereal Transformation

In the last decade, there has been increasing interest in the transformation of cereals because of their high commercial potential. Although transformation frequencies are not always high, all of the major cereal crops have now been transformed, including rice (Shimamato *et al.*, 1989; Datta *et al.*, 1990; Christou *et al.*, 1991), maize (Rhodes *et al.*, 1988; Fromm *et al.*, 1990; Gordòn-Kamm *et al.*, 1990), wheat (Vasil *et al.*, 1992, Weeks *et al.*, 1993), oats (Somers *et al.*, 1992), rye (Castillo *et al.*, 1994), sorghum (Casas *et al.*, 1993), and barley (Jähne *et al.*, 1994; Ritala *et al.*, 1994; Wan and Lemaux, 1994; Hagio *et al.*, 1995; Jensen *et al.*, 1996; Tingay *et al.*, 1997). The fundamental steps required for cereal transformation are discussed below, with a particular emphasis on the transformation of barley.

6.1.2 Methods of DNA Delivery

(i) **Agrobacterium***-mediated plant transformation*

The first transgenic plants were produced using the *Agrobacterium*-mediated procedure (Zambryski *et al.*, 1983), which takes advantage of the natural process through which the soil-borne bacterium *Agrobacterium tumefaciens* infects a wounded plant tissue and causes a crown gall tumour (Figure 6.1). The *Agrobacterium* cells contain a large (> 200 kb)

tumour-inducing plasmid (Ti plasmid) which contains a T-DNA (transfer DNA) region and the *vir* (virulence) gene operon (Zambryski *et al.*, 1983). During the natural infection process, the *vir* gene products are responsible for the transfer of the T-DNA region from the bacterial cell to the plant cell, and its subsequent integration into the plant genome. The short left and right border sequences (25 bp) which enclose the T-DNA region are critical for the excision and transfer of this region to the plant cell. The 20-25 kb T-DNA region contains genes that encode plant hormones (auxin and cytokinins) and tumour-specific metabolites (opines) under the control of plant-inducible promoters. The expression of these genes by the plant cell machinery therefore supports the rapid growth of the bacterium and leads to the formation of a crown gall tumour.

There are two major changes which must be made in order to exploit the natural *Agrobacterium* system for genetic engineering purposes. Firstly, the tumor-inducing genes in the T-DNA region must be removed from the Ti-plasmid, otherwise infection may lead to the development of a tumour which will not regenerate into a fertile plant. Secondly, because the natural Ti-plasmid is too large (> 200 kb) to manipulate *in vitro*, the *vir* genes must be removed from the plasmid. Once the tumour-inducing genes and *vir* genes have been removed from the Ti-plasmid, the foreign DNA insert of interest is ligated between the T-DNA borders. In addition, Ti-plasmids designed for transformation studies contain a bacterial selectable marker located outside the T-DNA borders, for the selection of the plasmid in *E. coli*, and a plant selectable marker located within the T-DNA borders, for the selection of plant cells which have incorporated the T-DNA. *Agrobacterium tumefaciens* strains used for transformation experiments contain the *vir* genes on a separate Ti plasmid, and these genes therefore act *in trans* to facilitate T-DNA transfer (Figure 6.1). Because there are two plasmids involved in the transfer, the system is referred to as a "binary vector system". Following integration of the transgene into the plant genome, the resulting cells can be induced to form callus which subsequently can be regenerated into fertile plants.

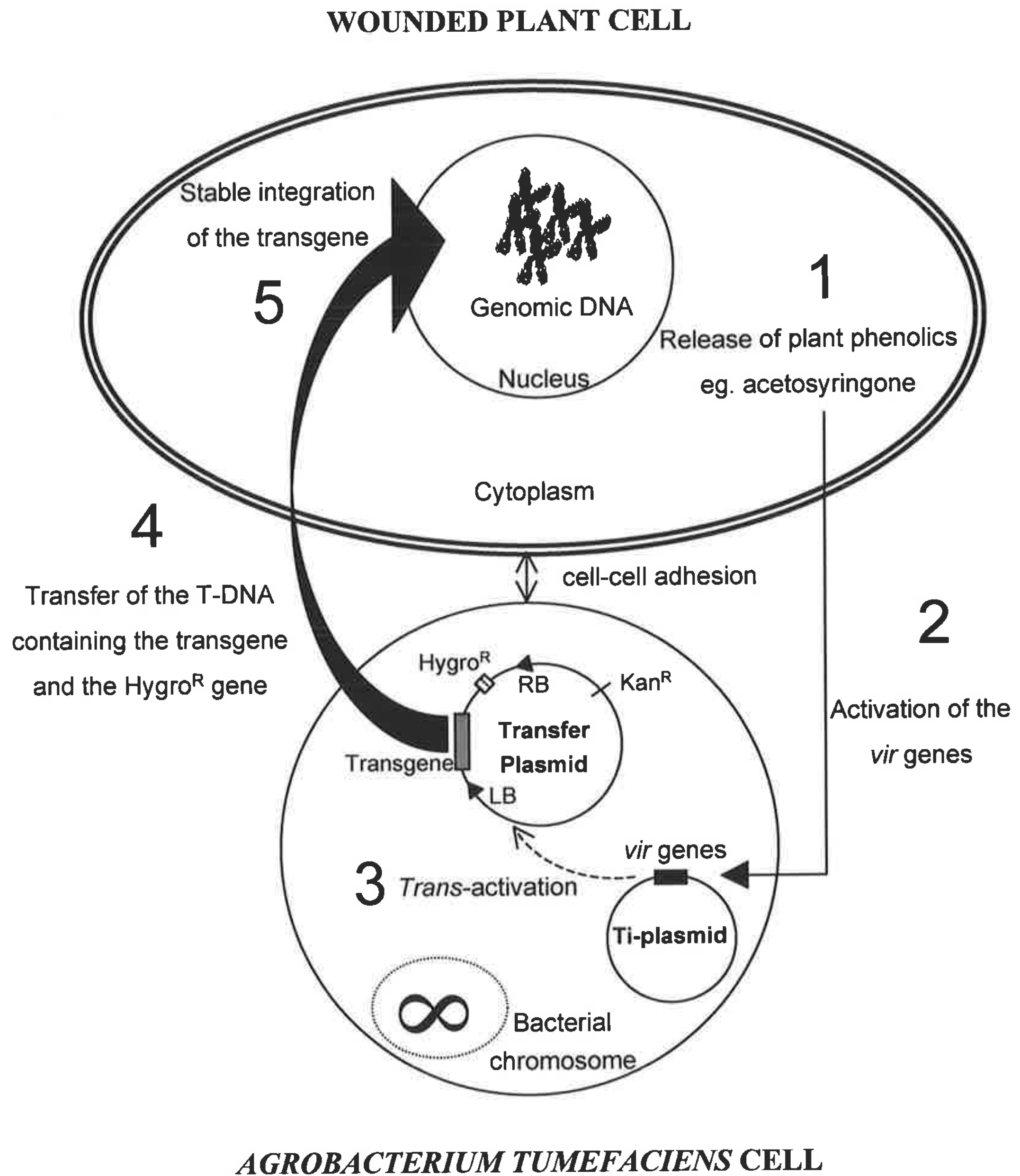


Figure 6.1 A schematic drawing of the binary vector system used for *Agrobacterium tumefaciens*-mediated plant transformation. The T-DNA region is defined by a right border (RB) and a left border (LB) and contains the transgene and a plant selectable marker such as the gene encoding hygromycin resistance ($Hygro^R$). The steps in the transformation process are; 1, production of phenolic compounds by the plant cell in response to wounding; 2, activation of the *vir* genes on the Ti-plasmid; 3) the *in trans* initiation of T-DNA transfer from the transfer plasmid; 4) transfer of the T-DNA containing the transgene and the hygromycin resistance gene ($Hygro^R$) into the plant cell; 5) stable integration of the T-DNA into the plant genome. The transfer plasmid also contains a selectable marker such as the gene encoding kanamycin resistance (Kan^R), which is used during the cloning of the transfer plasmid in *E. coli*.

Traditionally, it was believed that cereals and other monocotyledonous plants could not be readily transformed using the *Agrobacterium*-mediated method because they do not represent natural hosts of the soil-borne bacterium and do not show any obvious wound response to infection (DeCleene, 1985). However, recent technical modifications have led to a resurgence in the use of the *Agrobacterium*-mediated method for cereal transformation, and the method has now been used to produce fertile transgenic maize (Gould *et al.*, 1990; Ishida *et al.*, 1996), rice (Chan *et al.*, 1992; Hiei *et al.*, 1994; Park *et al.*, 1996), wheat (Cheng *et al.*, 1997), and barley (Tingay *et al.*, 1997). The *Agrobacterium*-mediated method has a high frequency of transformation relative to other transformation methods (see below), and has a propensity to integrate single or double copies of the transgene into transcriptionally active regions of the host genome. This can help alleviate problems of co-supression or gene silencing.

(ii) Protoplast-mediated plant transformation

Another method which can be used for plant transformation involves the direct transfer of DNA to plant protoplasts. The pre-treatment of plant protoplasts with polyethylene glycol (PEG) or electroporation creates transient pores in the plasma membrane, which facilitates the uptake of exogenous DNA. Protoplasts which have taken up DNA in this way can be induced to synthesise cell walls and subsequently to form callus that can be regenerated into whole plants (Lazerri *et al.*, 1991; Vasil and Vasil, 1992; Jähne *et al.*, 1994). Protoplast-mediated transformation has been successfully used to transform rice (Shimamato *et al.*, 1989), maize (Rhodes *et al.*, 1988), and barley (Funatsuki *et al.*, 1995; Salmenkallio-Marttila *et al.*, 1995). Despite these successes, the transformation of cereals using protoplast-mediated methods is generally associated with low regeneration frequencies and poor reproducibility, and the preparation of viable protoplasts from embryogenic suspension cultures is laborious and strongly genotype-dependent (Lemaux, 1999). Furthermore, somaclonal variation can arise because of the relatively long culture periods required for protoplast maintenance (Vasil, 1994).

(iii) The microprojectile bombardment method for plant transformation

The most widely-used method for the direct transfer of DNA into cereals is the microprojectile bombardment method (Klein *et al.*, 1987), which involves coating microscopic metal particles with plasmid DNA and accelerating the particles into plant cells using a specialised biolistic gun (Figure 6.2). The most popular biolistic gun is the Biolistic PDS-1000/He Particle Delivery System manufactured by Bio-Rad (Hercules, CA, USA). This system uses a sudden release of helium pressure to accelerate a macroprojectile capsule loaded with DNA-coated gold particles (microprojectiles) into a specialised screen which stops the macroprojectile, but allows the microprojectiles to continue their passage into the target plant cells (Figure 6.2). Despite the destructive nature of this procedure, a relatively small percentage of the bombarded cells may incorporate the plasmid DNA into their genome, and these cells can be subsequently induced to form callus that can be regenerated into plants. The major advantage of the microprojectile bombardment system over other methods for direct DNA delivery is that it allows species-independent transfer of DNA into a wide variety of tissues. This method has been successfully used to produce transgenic wheat (Vasil *et al.*, 1992), maize (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990), oats (Somers *et al.*, 1992), rye (Castillo *et al.*, 1994), sorghum (Casas *et al.*, 1993), rice (Christou *et al.*, 1991), and barley (Wan and Lemaux, 1994; Jensen *et al.*, 1996).

6.1.3 Reporter Genes and Selectable Markers

Reporter genes can be used to analyse the spatial and temporal expression patterns of a particular gene promoter sequence, and generally encode proteins that can are easily detected by a visual or histochemical assay. The most common reporter genes for studying plant gene promoters are the *uid*A gene from *E. coli*, the luciferase gene from the firefly *Vibrio fischeri* (Ow *et al.*, 1986), and the gene encoding green fluorescence protein (GFP) from the jellyfish *Aequorea victoria* (Heim *et al.*, 1994; Dopf and Horiagon, 1996). The *uid*A gene encodes β-glucuronidase (*gus*) which produces a blue reaction product in the presence of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal;

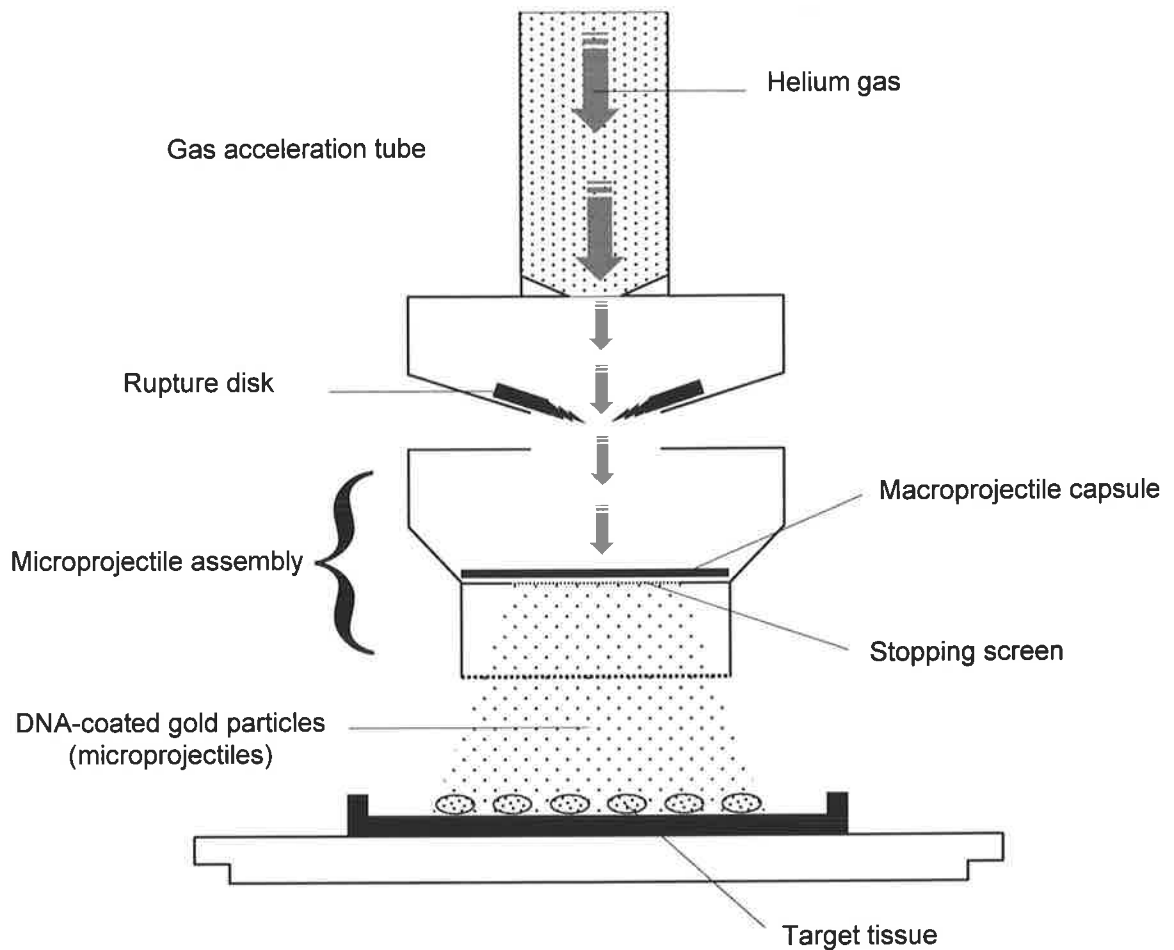


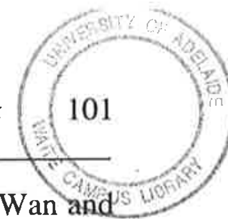
Figure 6.2 The Biolistic PDS-1000/He microprojectile gun for plant transformation. Helium gas is held in the gas acceleration tube until it reaches a critical pressure and ruptures the disk at the base of the tube. This releases a shock wave of helium into the microcarrier assembly which subsequently propels a plastic macroprojectile disk carrying DNA-coated gold particles into the stop screen. The macroprojectile disk is retained in the chamber while the DNA-coated gold particles are propelled into the target tissue at high velocity. This schematic drawing was adapted from the product literature for the Bio-Rad Biolistic PDS-1000/He Microprojectile Gun.

Jefferson, 1987). The luciferase and GFP genes encode proteins which strongly fluoresce under particular wavelengths of light (Heim *et al.*, 1994; Dopf and Horiagon, 1996). The two main limitations of using reporter genes are that analysing their expression products often involves a destructive assay (eg. *gus* assay), and that plant cells sometimes contain background signals which makes data difficult to interpret and to quantify.

Because the occurrence of stable transformation events is so rare (10^{-2} to 10^{-5}), the effective selection of transgenic plant cells is a critical step in the plant transformation process. The selection of stably-transformed plant tissue is facilitated by the use of selectable marker genes, which are either introduced into the target plant cells on the same plasmid as the transgene, or on a separate plasmid ('co-transformation'). The most commonly used selectable marker genes for cereal transformation are the *bar* gene from *E. coli*, the *cat* gene from *E. coli*, and the *hpt*II gene from *Streptomyces hygroscopicus*. The *bar* gene encodes phosphinothrycin acetyltransferase (PAT; Thompson *et al.*, 1987) which confers resistance to herbicides such as Bialophos. The *cat* gene encodes chloramphenicol acetyltransferase, which confers resistance to chloramphenicol, and the *hpt*II gene encodes hygromycin phosphotransferase which confers resistance to the antibiotic hygromycin (Hiei *et al.*, 1994; Ortiz *et al.*, 1996). To ensure that they are expressed at high levels in all cell types, selectable marker genes are normally driven by strong, constitutive promoters such as those which regulate the CaMV 35S gene, the maize ubiquitin gene (Cornejo *et al.*, 1993), or the rice actin gene (McElroy *et al.*, 1990).

6.1.4 Plant Tissue Culture and Regeneration

The final step in the successful production of transgenic plants is the *in vitro* regeneration of vegetative tissues from transformed cells. For barley in particular, the ability of undifferentiated cells to regenerate into plants under *in vitro* conditions appears to be highly cultivar-dependent. For example, the barley variety Golden Promise is far more amenable to *in vitro* regeneration than most other varieties (Lürhs and Lörtz, 1987), and



has therefore been the variety of choice for barley transformation studies to date (Wan and Lemaux, 1994; Jensen *et al.*, 1996; Tingay, *et al.*, 1997).

6.1.5 Producing Transgenic Barley for Industry

Recent successes in the production of fertile transgenic barley (Wan and Lemaux, 1994; Tingay *et al.*, 1997) provides us with the opportunity to engineer barley with superior physiological and biochemical characteristics. From a malting and brewing perspective, the important characteristics of barley are the starch content of the grain, the composition of the endosperm cell wall, and the performance of hydrolytic enzymes that degrade cell wall polysaccharides after germination (Chapter 1). Increasing the starch content of the barley endosperm may lead to a higher malt extract value, which is a major determinant of the commercial value of malt (Stuart *et al.*, 1988). Alternatively, reducing the (1→3,1→4)-β-glucan content of the barley endosperm cell wall may help alleviate the detrimental effects of residual (1→3,1→4)-β-glucans during brewing. However, these approaches will require a more comprehensive understanding of the enzymes involved in starch and (1→3,1→4)-β-glucan synthesis in barley.

In contrast to the enzymes involved in (1→3,1→4)-β-glucan synthesis, the enzymes which hydrolyse barley (1→3,1→4)-β-glucan have been extensively characterised (Section 1.3.1). Increasing the heat stability of these enzymes to ensure they remain active for longer periods of time, or increasing the total activity in the malt, is likely to ensure sufficient degradation of residual barley (1→3,1→4)-β-glucans. Transforming barley with an enhanced ability to hydrolyse residual barley (1→3,1→4)-β-glucans is now a real possibility. Jensen *et al.* (1996) transformed barley with the gene encoding a modified thermostable (1→3,1→4)-β-glucanase of bacterial origin (Olsen *et al.*, 1991; Olsen and Thomsen, 1991), under the control of the barley high-pI α-amylase gene promoter. Although the transgene was inherited in the normal Mendelian fashion (Jensen *et al.*, 1996, 1998), the activity and thermostability of the expressed enzyme was difficult to interpret

from the published data. Efforts to transform barley with the gene encoding thermostable barley (1→3,1→4)-β-glucanase mutant H300P are described in this Chapter.

6.2 Methods and Materials

6.2.1 Materials

Donor barley plants (cv Sloop L.) were grown under glasshouse conditions at the Victorian Institute for Dryland Agriculture (VIDA), Victoria, Australia. The plasmid pCAMBIA-1390 was supplied by Cambia Pty Ltd, Canberra, ACT, Australia. Bialophos was purchased from the Shinyo Sangyo Company Ltd, Tokyo, Japan. DNA restriction enzymes, DNA ligase and T4 DNA polymerase were from New England Biolab. DNA oligonucleotides were synthesised on an ABI DNA synthesiser. Plasmid pBluescript (SK+) was from Stratagene and plasmid pDM302 was kindly supplied by professor R. Wu, Cornell University, Ithaca, NY, USA. Reverse transcriptase (Superscript II), Trizol reagent, and *Taq* DNA polymerase were from GIBCO BRL. The MegaPrime DNA labeling Kit, Hybond$^+$ membranes and [α-^{32}P]-dCTP was purchased from Amersham International Ltd, UK. Whatman 3MM chromatography paper was purchased from Whatman, Maidstone, Kent, UK.

6.2.2 Plasmid pE2HPg: The (1→3,1→4)-β-Glucanase Mutant H300P Gene Construct

The gene encoding barley (1→3,1→4)-β-glucanase isoenzyme EII (Wolf, 1992) was previously cloned in this laboratory as two separate fragments which were ligated into pUC19 to produce plasmids pEII-20 (2.0 kb fragment) and pEII-50 (5.0 kb fragment; Qi, 1994). The 5.0 kb fragment represents the 5' portion of the gene including the promoter region and a single intron, while the 2.0 kb fragment represents the 3' portion of the gene including the 3' untranslated region (UTR). To assemble the entire gene into a single construct, the 5.0 kb fragment was excised from pEII-50 using *Eco*RI and *Pst*I and ligated into the corresponding sites of the plasmid pBluescript (SK$^+$). The 2.0 kb fragment was excised from pEII-20 using *Eco*RI and ligated into the *Eco*RI site of the pBluescript (SK$^+$) plasmid containing the 5.0 kb fragment. The correct orientation of the 2.0 kb fragment, relative to the barley (1→3,1→4)-β-glucanase isoenzyme EII gene, was confirmed by DNA sequencing (as described in Section 2.2.2 vii). The resulting plasmid, designated

pE2g, consisted of the entire gene encoding barley (1→3,1→4)-β-glucanase isoenzyme EII including the 5' promoter region, two exons, a single intron, and the 3' UTR.

To introduce the mutation encoding amino acid substitution H300P (CAC→CCC) into pE2g, the 770 bp *AccI/MluI* fragment of pE2g was replaced with the corresponding fragment from pET3aHT/H300P (see Chapter 3). A 30 bp DNA fragment containing a *Bam*HI site (underlined below) was inserted into the 3' UTR of the gene to help distinguish the endogenous gene from the transgene during subsequent analyses of transgenic tissue by PCR. The DNA 'marker' sequence (5'-CGTGGCGCAAGGTGGATCCGAAGCCCT-GACCC-3') was synthesised as two separate complementary strands, which were subsequently boiled and allowed to anneal at room temperature. The resulting double-stranded DNA fragment was end-filled using T4 DNA polymerase (New England Biolab) as described in the product literature, and blunt-end ligated into the unique *Hinc*II site located in the 3' UTR region of the gene, where it would not interfere with the translation product. Finally, the *nos* 3' transcription terminator sequence from *Agrobacterium tumefaciens* was blunt-end ligated into the plasmid at the unique *Eco*RV site located downstream of the gene insert. The final plasmid, designated pE2/HPg, contained the entire barley (1→3,1→4)-β-glucanase isoenzyme EII gene including the promoter region, the open reading frame, a single intron, the Transgene marker, the *nos* 3' transcription terminator, and sequence encoding the signal peptide and the H300P amino acid substitution (Figure 6.3). Automated DNA sequencing was used to confirm that plasmid pE2HPg contained no errors across the ligation junctions.

6.2.3 Plasmid pE2HPc: The (1→3,1→4)-β-Glucanase Mutant H300P cDNA Construct

The plasmid pDM302, which contains the *bar* gene under the control of the constitutive rice actin promoter (Cao *et al.*, 1992), was used to construct a DNA vector for the transient expression of the cDNA encoding barley (1→3,1→4)-β-glucanase mutant H300P in immature barley embryos. The 2.5 kb *Kpn*I fragment of pE2/HPg was ligated into the *Kpn*I site of a pBluescript (SK^+)-derived plasmid containing the full-length cDNA encoding

barley (1→3,1→4)-β-glucanase isoenzyme EII (Fincher *et al.*, 1986; Slakeski *et al.*, 1990). This transferred the H300P mutation and the Transgene marker to the cDNA insert. The entire cDNA was excised with *Eco*RI and blunt-end ligated into the *Sma*I site of pDM302, which effectively replaced the *bar* gene with the full-length isoenzyme EII cDNA. The resulting plasmid, designated pE2/HPc, contained the entire cDNA encoding barley (1→3,1→4)-β-glucanase isoenzyme EII (including the H300P mutation and the Transgene marker) under the control of the rice actin promoter and the 3' *nos* transcription terminator (Figure 6.3). Automated DNA sequencing was used to confirm that plasmid pE2HPc contained no errors across the ligation junctions.

6.2.4 Plasmid pCAM/E2HPg for Agrobacterium*-Mediated Barley Transformation*

In preparation for the transformation of barley using *Agrobacterium tumefaciens*, the gene encoding barley (1→3,1→4)-β-glucanase isoenzyme EII was inserted into the plasmid pCAMBIA 1390, which contains the gene that confers hygromycin resistance under the control of the constitutive 35S promoter from Cauliflower Mosaic Virus (CaMV 35S). For ligation into plasmid pCAMBIA 1390, the entire barley (1→3,1→4)-β-glucanase isoenzyme EII gene insert was excised as two fragments (a 5.0 kb *Bam*HI/*Eco*RI fragment and a 2.0 kb *Eco*RI fragment) from plasmid pE2/HPg using *Eco*RI and *Bam*HI. The resulting 5 kb fragment, which represents the 5' region of the gene, was ligated into the same restriction sites in the multiple cloning region of plasmid pCAMBIA 1390. The 2.0 kb fragment from pE2/HPg (which represented the 3' region of the gene) was ligated into the *Eco*RI site of the pCAMBIA 1390 plasmid that already contained the 5.0 kb gene fragment. The correct orientation of the 2.0 kb fragment, with respect to the barley (1→3,1→4)-β-glucanase isoenzyme EII gene, was confirmed by DNA sequencing. The resulting vector, designated pCAM/E2HPg, was ready for *Agrobacterium*-mediated transformation of barley.

6.2.5 Microprojectile Bombardment

The Biolistic PDS-1000/He Particle Delivery System (Bio-Rad) was used for all bombardment experiments. Gold particles were prepared as follows: 25 mg gold particles

(1 µm diameter) were washed with 1ml absolute ethanol in a 2 ml plastic tube. The tube was vortexed for 5 min and centrifuged at 13,200 rpm for 1 min. Ethanol was removed, and the washing procedure was repeated twice. The gold particles were washed three times with 1 ml sterile water and finally resuspended in 1 ml sterile water. To coat the gold particles with DNA, 3 µl of a 1:1 mixture of plasmids pE2HPg (1 mg/ml) and pDM302 (1 mg/ml) was added to 50 µl washed gold particles in a fresh 2 ml plastic tube. To precipitate the DNA onto the particles, 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine were sequentially added. The tube was vortexed for 3 min, left on ice for 10 min and centrifuged at 1,000 rpm for 10 sec to pellet the gold particles. The supernatant was removed and the particles were washed with 250 µl absolute ethanol, and finally resuspended in 50 µl of absolute ethanol. For each bombardment, 10 µl coated gold particles suspension (i.e. approximately 250 µg gold particles per bombardment) was placed in the centre of a macroprojectile capsule (Figure 6.2).

In preparation for bombardment, approximately twenty immature scutella (harvested 10-14 days post-anthesis) were placed in the centre of a 55 x 14 mm plastic dish containing callus induction medium MSB (Appendix I; Murashige and Skoog, 1962) supplemented with 0.4 M mannitol for 4 hrs prior to bombardment. The scutella were bombarded under vacuum at 28 inches Hg, as described in the product literature (Biolistic PDS-1000/He Particle Delivery System, Bio-Rad). The distance between the stopping screen and the plant tissue was set at 6 or 9 cm and the bombardment pressure was 900-1300 psi. Following bombardment, scutella were incubated for a further 18-22 hrs on osmotic media, transferred to 90 mm plastic dishes containing fresh callus induction medium, and incubated at 23°C in the dark for 20-25 days.

For transient assays, immature embryos were bombarded with plasmid pE2HPc as described above for plasmid pE2HPg, except no secondary plasmids or reporter genes were used. Following bombardment, embryos were transferred to fresh MSB media and incubated at 23°C in the dark. Embryos were sampled 0, 6, 12 and 24 h after bombardment

and immediately transferred to 2 ml plastic tubes using sterile forceps, frozen in liquid nitrogen, and stored at -80°C.

6.2.6 Tissue Culture and Selection of Transgenic Tissue

Following their post-bombardment treatment and incubation, scutella were divided into 2-3 mm pieces and cultured on MSB media containing 1 µM $CuSO_4$ and 5 mg/ml bialophos at 24°C in the dark for up to 4 weeks. Proliferating cells were excised using forceps and a scalpel, and cultured on fresh medium for a further 3 to 4 weeks. Sub-culturing was repeated every 3 to 4 weeks. Uniformly growing, bialophos-resistant callus lines were established after 10 to 16 weeks of culture, and were thereafter maintained by monthly sub-culturing. In an attempt to regenerate roots and shoots from the resistant callus, embryogenic sectors of the bialophos-resistant callus were transferred to hormone-free MSB medium (without 2,4-D) containing 1 µM $CuSO_4$ and 1 mg/ml bialophos, and incubated under 16-hour daylight at 24°C.

6.2.7 Genomic DNA Isolation and Southern Blot Analysis

For the extraction of genomic DNA from barley callus, approximately 0.2 g tissue was placed in a 2 ml plastic tube and frozen in liquid nitrogen. Frozen tissue was crushed to a fine powder using a metal micropestle and 0.5 ml extraction buffer (100 mM Tris-HCl buffer, pH 9.0, containing 40 mM EDTA) was added. After mixing, 10 µl 10% (w/v) SDS and 0.5 ml benzyl chloride were added and the mixture was thoroughly vortexed. Following incubation at 50°C for 30 min with constant mixing, 300 µl sodium acetate buffer, pH 4.8, was added and the mixture was left on ice for 10 min. After centrifugation for 10 min at 13,200 rpm, the supernatant was removed to a fresh tube and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and mixed for 5 minutes. To precipitate DNA from the aqueous phase, 600 µl isopropanol was added and genomic DNA was collected by centrifugation at 13,200 rpm for 10 minutes at room temperature. The DNA pellet was washed in 70% ethanol, air-dried, and resuspended in 50 µl sterile distilled water containing 40 µg/ml RNase A. The DNA was left at 4°C overnight to completely dissolve.

For Southern blot analysis, genomic DNA (20 μg) was digested with *Hin*dIII using 1.5 enzyme units/ml, 1 mg/ml BSA, and 4 mM spermidine at 37°C (Sambrook *et al.*, 1989). Digested fragments were separated on a 1.2% (w/v) agarose gel at 40 V for 16 h. The gel was stained with 1 mg/ml ethidium bromide for 15 min on a rocking platform, destained with sterile distilled water for 20 min, and photographed under a UV light source. To transfer the DNA to a nylon membrane, the gel was first soaked in 200 ml denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 30 min and neutralised for 30 min in 200 ml 0.5 M Tris-HCl buffer, pH 7.0, containing 1.5 M NaCl and 1 mM EDTA. A piece of sponge was soaked in 20 X SSC (175.3 g NaCl and 88.2 g sodium citrate in 1000 ml water, adjusted to pH 7.0 with NaOH) and placed in a shallow plastic tray, and two sheets of Whatman 3MM filter paper were placed individually on top of the sponge. The edges of the filter paper were sealed with a cellulose/acetate frame and the gel was placed upside down on the paper. Hybond N^+ membrane was placed on top of the gel and air bubbles were removed by rolling a glass pipette over the surface. Two additional sheets of filter paper were soaked in 20 X SSC, placed on top of the membrane and overlaid by a stack of dry paper towels. A glass plate was placed on top of the stack and the bottom tray was filled with 20 X SSC until the sponge was thoroughly wetted. DNA was allowed to transfer overnight. The membrane containing the DNA was fixed by laying it DNA-side up on a piece of Whatman 3 MM filter paper soaked with 0.4 M NaOH. The membrane was now ready for hybridisation with a radiolabelled probe specific for the barley (1→3,1→4)-β-glucanase genes.

The 770 bp *Pst*I/*Acc*I fragment from plasmid pE2/HPg (Figure 6.3) was used to prepare the probe for Southern blot hybridisation, using the Megaprime DNA Labeling Kit (Amersham). The DNA fragment (approximately 100 ng) was mixed with 5 μl random nonanucleotide primers and made up to a final volume of 50 μl with sterile distilled water. The primer-DNA mix was boiled for 5 min and immediately transferred to ice. After cooling, 10 μl labeling buffer, 5 μl [α-^{32}P]-dCTP, and 2 μl Klenow fragment of DNA polymerase were added, and the reaction was incubated for 20 min at 37°C. The labeled

DNA was separated from unincorporated nucleotides using a Sephadex G-100 column. A sterile Pasteur pipette was plugged with glass wool and pre-swollen Sephadex G-100 was added to a height of approximately 8 cm. The column was equilibrated with 1 X TE buffer, pH 8.0. A mixture of 1% (w/v) Blue Dextran and 1% (w/v) Orange G in TE buffer, pH 8.0, was slowly added to the column and allowed to equilibrate. Labelled probe was collected as the blue dye fraction which was eluted from the column using TE buffer, pH 8.0.

The membrane containing digested genomic DNA was prehybridised for 4 h in a solution containing 6 X SSC, 1 X Denhardt's solution (Denhardt, 1966), 1% (w/v) SDS, and 100 μg/ml salmon sperm DNA. The labelled DNA probe (see above) was boiled for 5 min and immediately added to the hybridisation solution, which was subsequently incubated overnight at 65°C with gentle agitation. After hybridisation, the filter was washed in 2 X SSC containing 1% (w/v) SDS for 30 min, followed by 30 min in 1 X SSC containing 1% (w/v) SDS. The washed membrane was air-dried and exposed to X-ray Hyperfilm (Amersham) for up to three days.

6.2.8 PCR Analysis of Genomic DNA

Genomic DNA extracted from barley callus (see Section 6.2.7) was analysed by PCR for the presence of the barley (1→3,1→4)-β-glucanase mutant H300P transgene. The forward primer used for the PCR was E2f (5'-CCA TCC TCG GCG TGT TCA GCC-3') and the reverse primer was either MARKr (5'-GGG TCA GGG CTT CGG ATC CAC CTT GCGCCA CG-3'), or the H300P mutagenic primer (5'- GTT GAT GGG GTA GAC GGG CTG CAT GTT GGG -3'). The primer pair E2f/MARKr was designed to amplify a 690 bp fragment of the transgene only, while the primer pair E2f/H300P was designed to amplify a 585 bp fragment of both the endogenous barley (1→3,1→4)-β-glucanase isoenzyme EII gene and the transgene (Figures 6.3 and 6.5). The PCR reaction contained the following; 1 μl genomic DNA (approximately 100 ng), 1 μl of each primer (5 nmol), 3μl $MgCl_2$ (5 mM), 5 μl DMSO, 2 μl dNTP's, 5 μl 10 X *Taq* buffer (GIBCO BRL), 1 μl *Taq* DNA

polymerase (GIBCO BRL), and 31 μl sterile distilled water. The temperature regime used for the PCR amplification was; 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, followed by a final extension of 72°C for 10 min. The PCR products were analysed by electrophoresis on a 0.8% agarose gel (Sambrook *et al.*, 1989).

6.2.9 Analysis of Transient Expression using RT-PCR

Following the bombardment of immature embryos with plasmid pE2/HPc, transient expression of the cDNA encoding barley (1→3,1→4)-β-glucanase mutant H300P was analysed using reverse transcriptase PCR (RT-PCR). Frozen embryo samples were ground to a fine powder under liquid nitrogen using a mortar and pestle, and total RNA was extracted using Trizol, a commercially available phenol/guanidine isothiocyanate reagent (GIBCO BRL). One ml of Trizol was added to 100 mg frozen tissue in a 2 ml plastic tube and thoroughly vortexed. Next, 200 μl chloroform was added and the mixture was centrifuged for 1 min at 13,200 rpm at room temperature. The aqueous phase was transferred to a fresh tube and total RNA was precipitated by the addition of 0.5 ml isopropanol and recovered by centrifugation at 13,200 rpm for 10 min at room temperature. The RNA pellet was washed with 70% ethanol and air-dried, before being resuspended in 50 μl DEPC-treated water and stored at -80°C.

For cDNA synthesis, 3 μg total RNA was combined with 1μl of T-RACE primer using Reverse Transcriptase (Superscript II, GIBCO BRL) at 50°C. For PCR amplification, the two primers E2f (5'-CCA TCC TCG GCG TGT TCA GCC-3') and MARKr (5'-GGG TCA GGG CTT CGG ATC CAC CTT GCGCCA CG-3') were used as the forward and reverse primers, respectively (Figure 6.3). The reverse primer, MARKr, was complementary to the Transgene marker sequence in the 3' UTR of the mutant H300P gene/cDNA, and therefore would not amplify endogenous barley (1→3,1→4)-β-glucanase genes. The PCR reaction contained 5 μl 10 X PCR buffer (GIBCO BRL), 2 μl 10 mM dNTPs, 3 μl 25 mM $MgCl_2$, 5 μl DMSO, 1 μl (0.4 nmoles) of each primer, 1 μl Taq DNA polymerase (GIBCO BRL) and water to 50 μl. The PCR temperature program was 35

cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 60 s. The resulting fragments were analysed by electrophoresis on a 0.8% agarose gel (Sambrook *et al.*, 1989). To ensure equal amounts of cDNA were used for each amplification, control PCR reactions were performed using the same conditions as described above, except two primers specific for the mRNA encoding the constitutive glycolytic pathway enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from barley were used. The 5' and 3' primers were 5'-CCACCGGGTCTTCACTGACAAGG-3' and 5'-GCCTTAGCATCAAAGTGCTGG-3', respectively. The expected size of the GAPDH PCR fragment was approximately 550 bp.

6.2.10 Western Blot Analysis of Transient Expression

For Western blot analysis, bombarded embryos were ground in 200 µl SDS-PAGE loading buffer (Section 2.2.6) using a metal micropestle in a 2.5 ml plastic tube, and left to extract for 30 min at room temperature. Western blot analysis was performed essentially as described in Section 5.2.5, using monoclonal antibodies raised against barley (1→3,1→4)-β-glucanase isoenzyme EII (Høj *et al.*, 1990).

6.2.11 Viscometric Determination of (1→3,1→4)-β-Glucanase Activity

For (1→3,1→4)-β-glucanase activity assays, bombarded immature embryos were extracted with 50 mM sodium acetate buffer, pH. 5.0, containing 300 mM NaCl and 1 mM PMSF, using a metal micropestle in a 2 ml plastic tube. Insoluble material was removed by centrifugation at 4°C for 5 min at 10,000 rpm, and the supernatant containing soluble proteins was stored at 4°C. Viscometric assays were performed in an Ostwald viscometer (Cannon 100, Cannon Instruments, State College, Pennsylvania, USA) using 0.3% (w/v) barley (1→3,1→4)-β-glucan as a substrate. The substrate was prepared and equilibrated as described in Section 2.2.7. Protein sample (50 µl) was added to 550 µl substrate which had been pre-incubated in the viscometer at 40°C. The flow time was checked at the start and after 5 min of incubation. Enzyme activity against barley (1→3,1→4)-β-glucan was expressed in units, where 1 unit is the change in the reciprocal of the specific viscosity (N_{sp}), where N_{sp} is defined as follows;

$$N_{sp} = 1/[(t_5/t_b)-1] - 1/[(t_0/t_b)-1]$$

where t_b is the flow rate of 600 µl 50 mM sodium acetate buffer, pH 5.5; t_0 is the flow rate of 550 µl substrate and 50 µl 50 mM sodium acetate buffer, pH 5.5; t_5 is the flow rate 5 min after the addition of enzyme to the substrate. Specific activity is expressed as $\Delta (1/N_{sp})$ per min per mg protein (Woodward and Fincher, 1982a).

6.3 Results and Discussion

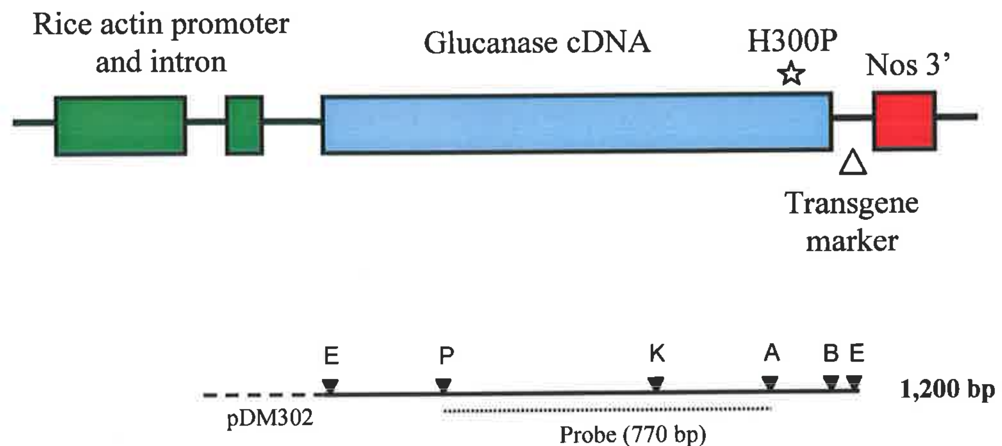
6.3.1 Preparation of DNA Constructs for Barley Transformation

Three DNA constructs, designated pE2/HPg, pE2/HPc, and pCAM/HPg, were prepared for introducing the gene and cDNA encoding (1→3,1→4)-β-glucanase mutant H300P into various barley tissues (Figure 6.3).

Plasmid pE2/HPg consisted of the entire gene sequence encoding barley (1→3,1→4)-β-glucanase isoenzyme EII (Wolf, 1991) including the 5' promoter region, the open reading frame, a 2.2 kb intron, and the 3' UTR (Figure 6.3). The endogenous barley (1→3,1→4)-β-glucanase isoenzyme EII promoter was used for this vector, because we wanted to achieve similar dosage and temporal/spatial expression patterns for the transgene as for the the endogenous gene, so as not to adversely affect cellular metabolism. For example, if the relatively strong barley α-amylase promoter was used, it may result in the production of unnaturally high levels of the (1→3,1→4)-β-glucanase enzyme, which, in turn, may be detrimental to other cellular processes. Plasmid pE2/HPg also contained the mutation CAC→CCC at amino acid position 300 (encoding the amino acid substitution H300P) and a 35 bp transgene marker in the 3' UTR (Figure 6.3). The *nos* 3' sequence from *Agrobacterium tumefaciens* was inserted downstream from the (1→3,1→4)-β-glucanase mutant H300P gene to facilitate the correct transcription of the transgene in barley (Figure 6.3).

A second plasmid, designated pE2/HPc, was prepared to enable the cDNA encoding (1→3,1→4)-β-glucanase mutant H300P to be transiently expressed in immature barley embryos (Figure 6.3). Plasmid pE2/HPc was derived from the vector pDM302, and consisted of the constitutive rice actin promoter driving the cDNA encoding (1→3,1→4)-β-glucanase mutant H300P, followed by the 3' *nos* transcription terminator (Figure 6.3). The rice actin promoter was chosen for the transient expression studies in barley embryos because the vector pDM302 was available in our laboratory, and the promoter had been

A) pE2/HPc



B) pE2/HPg

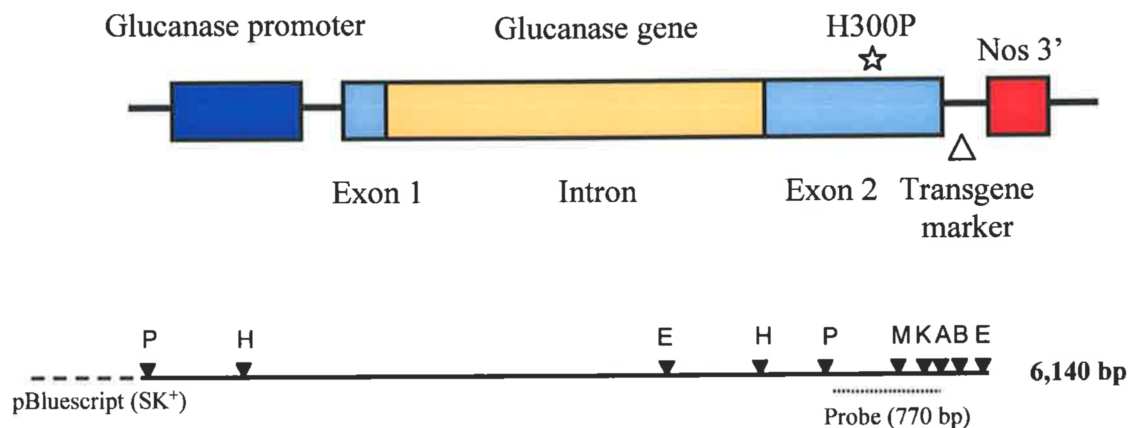


Figure 6.3 Schematic diagram of DNA constructs for barley transformation. A) The cDNA encoding barley (1→3,1→4)-β-glucanase mutant H300P under the control of the rice actin promoter its associated intron. B), Plasmid pE2/HPg, which contains the barley (1→3,1→4)-β-glucanase mutant H300P under the control of the native barley (1→3,1→4)-β-glucanase isoenzyme EII promoter. 'Nos' refers to the nos 3' transcription terminator sequence from rice, and 'transgene marker' represents a 35 bp DNA sequence which was inserted into the 3' untranslated region of the DNA constructs to assist in the analysis of transformation and expression. Restriction maps for both constructs are represented by black lines. Restriction enzyme sites used for the construction of the plasmids are denoted as follows: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; A, *Acc*I; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I

used in a previous study to express the *bar* gene in barley (J. Qureshi, personal communication).

A third DNA construct, designated pCAM/HPg, consisted of the entire (1→3,1→4)-β-glucanase mutant H300P gene fragment from plasmid pE2/HPg (see above) inserted into the commercial plant transformation vector pCAMBIA 1390, which contains the gene encoding hygromycin resistance driven by the constitutive 35S promoter from the CaMV 35S gene. The efficacy of all constructs was confirmed by DNA nucleotide sequence analysis.

6.3.2 Stable Transformation of Barley Cells with Plasmid pE2/HPg

Barley scutella were bombarded with a 1:1 mixture of plasmids pE2/HPg and pDM302 using the microprojectile bombardment transformation procedure. Two independent lines of callus, designated C1 and C2, showed a high level of resistance to bialophos, indicating that they contained the *bar* gene from plasmid pDM302. The two resistant callus lines were analysed by Southern blot analysis to determine whether they contained pE2/HPg, pDM302, or both pE2/HPg and pDM302.

(i) Southern blot and PCR analysis of bialophos-resistant callus lines

Southern blot analyses showed that all callus lines, including the resistant lines C1 and C2 and non-bombarded control callus, contained a 3.8 kb band and a 1.8 kb band (Figure 6.4). The 1.8 kb fragment hybridised to the probe more strongly than the 3.8 kb (Figure 6.4). Because the probe was derived from the gene sequence of (1→3,1→4)-β-glucanase isoenzymes EII, it was concluded that the 1.8 kb and 3.8 kb bands represented the genes of barley (1→3,1→4)-β-glucanase isoenzymes EII and EI, respectively. These results are entirely consistent with those from two previous studies (Loi *et al.*, 1988; Slakeski *et al.*, 1990). A single additional band of approximately 2.5 kb in length, was evident for callus line C1 only (Figure 6.4), indicating the presence of the (1→3,1→4)-β-glucanase mutant H300P transgene. Because no additional bands were evident for callus line C2 or the

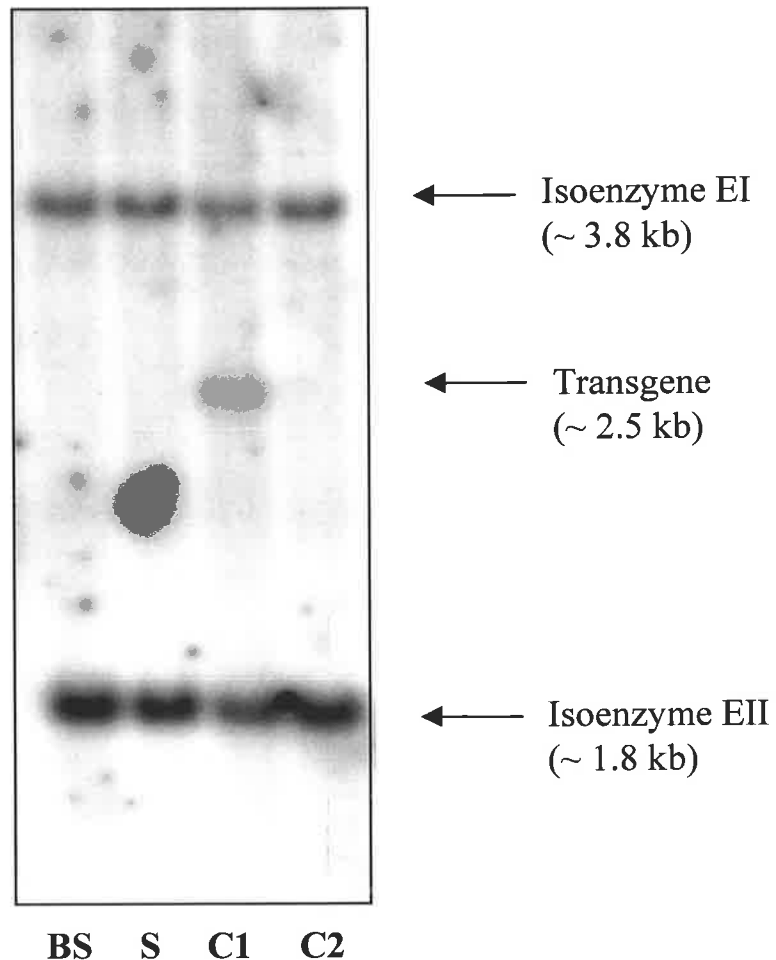
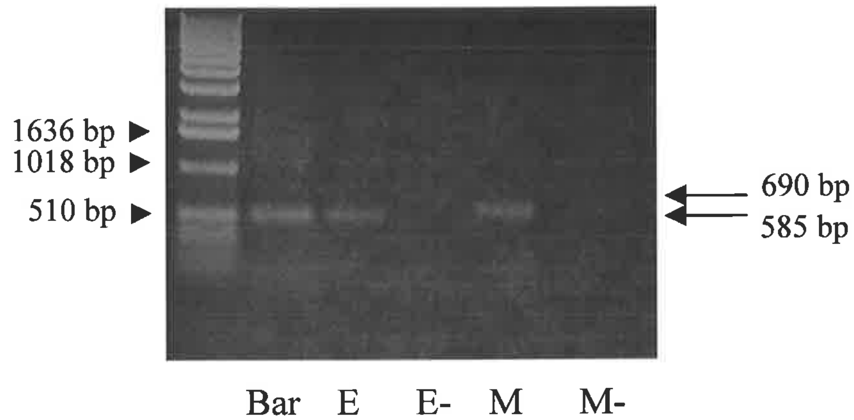


Figure 6.4 Southern blot analysis of genomic DNA extracted from barley callus. Immature scutella were bombarded with the DNA construct encoding the thermostable (1→3,1→4)-β-glucanase mutant H300P under the control of the native barley (1→3,1→4)-β-glucanase isoenzyme EII gene promoter. Genomic DNA isolated from two independent callus lines showing resistance to bialophos, was digested with *Hin*dIII and used for Southern blot analysis. A 770 bp fragment excised from the plasmid pE2g using *Pst*I and *Acc*I was used as a probe. BS, control callus derived from scutella bombarded in the absence of plasmid DNA; S, callus derived from non-bombarded scutella; C1 and C2, independent bialophos-resistant callus lines resulting from scutella bombarded with the H300P transgene.



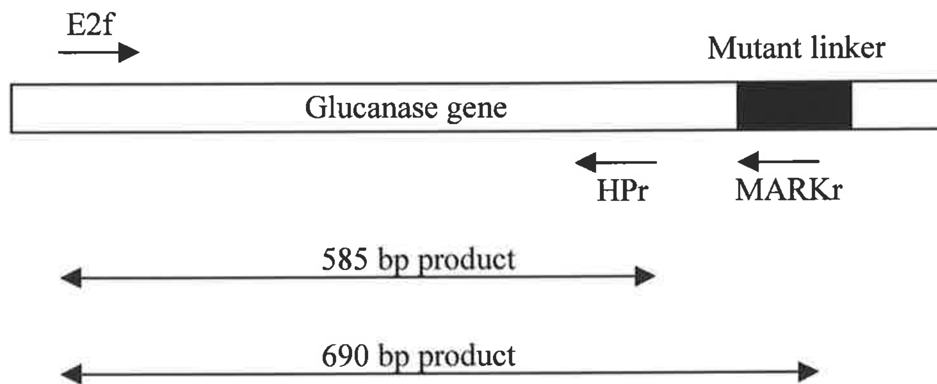


Figure 6.5 PCR analysis of genomic DNA extracted from barley callus line C1. Barley scutella were bombarded with the plasmid pE2/HPg and induced to form callus. Genomic DNA was extracted from the bialophos-resistant callus line C1 and used as a template for PCR. Bar, *bar* gene primers; E, endogenous (1→3,1→4)-β-glucanase gene primers HPr/E2f; M, mutant (1→3,1→4)-β-glucanase gene primers MARKr/E2f; E- and M-, negative controls; DNA molecular weight markers are shown in lane 1.

control callus, it was concluded that the 2.5 kb band for callus line C1 represented a single copy of the barley (1→3,1→4)-β-glucanase mutant H300P transgene.

To confirm the presence of the *bar* selectable marker gene in the bialophos-resistant callus lines, Southern hybridisation filters were stripped and re-probed with a specific probe for the *bar* gene. Results indicated that both cell lines contained at least five copies of the *bar* gene (data not shown), which explains why these cells were resistant to a relatively high concentration (50 mg/ml) of bialophos. Thus, it appeared that callus line C1 contained multiple copies of the *bar* gene and a single copy of the (1→3,1→4)-β-glucanase mutant H300P transgene, whereas callus line C2 contained multiple copies of the *bar* gene only. PCR analysis using the reverse primer MARKr (which is unique to the transgene) confirmed the presence of the transgene in callus line C1 (Figure 6.5) but not in callus line C2 (data not shown). A different reverse primer, HPr, was used in a control PCR reaction to show that the amplified transgene band had not inadvertently originated from the endogenous barley (1→3,1→4)-β-glucanase genes (Figure 6.5). Another pair of PCR primers designed to amplify a 500 bp fragment were used to confirm the presence of the *bar* gene in callus line C1.

(ii) Attempts to regenerate barley plants from resistant callus

Attempts to regenerate fertile green plants from barley callus lines C1 and C2 were unsuccessful. This may have been due to the amount of time they had spent in culture, because it has been shown that *in vitro*-derived cereal tissues often lose the ability to regenerate after prolonged periods of tissue culture (Jiang *et al.*, 1998). Furthermore, it has been shown that the quality of the donor material for bombardment, in this case immature scutella, strongly influences the ability of the resulting callus to regenerate (J.A. Qureshi, K.T. Gatford, R.R. Singh, Z. Basri, personal communication), and this may also have contributed to the inability to regenerate roots or shoots from the bialophos-resistant callus lines C1 and C2.

Two parameters are generally used to compare the success rate of transformation for different plant cultivars under varying transformation conditions. The transformation frequency (TF) is calculated by dividing to the number of independently transformed cell lines by the original number of targeted explants such as single scutella or immature embryos, while the effective transformation frequency (ETF) is calculated by dividing of number of independent fertile plants by the number of targeted explants. For barley, the values of TF and ETF are normally less than 8% and 5%, respectively (Wan and Lemaux, 1994; Ritala *et al.*, 1994; Hagio *et al.*, 1995; Jensen *et al.*, 1996). In the current study, approximately 250 scutella were bombarded to produce the independent callus lines C1 and C2, which represents a TF value of approximately 2/250, or 0.8%. Because no plants were regenerated from the two transgenic callus lines, it was not possible to calculate ETF.

6.3.3 Transient Expression of Plasmid pE2/HPc in Immature Barley Embryos

Immature barley embryos were bombarded with plasmid pE2/HPc, which contained the (1→3,1→4)-β-glucanase mutant enzyme H300P under the constitutive control of the rice actin promoter, to assess the stability and activity of the mutant enzyme in barley tissues. Plasmid pE2/HPg, with or without the 3' *nos* transcription terminator sequence, was also shot into immature barley embryos to assess whether the native barley (1→3,1→4)-β-glucanase isoenzyme EII gene promoter would direct the expression of (1→3,1→4)-β-glucanase mutant enzyme H300P in barley embryos. Furthermore, the transient expression experiments served to check the integrity of plasmids pE2/HPg and pE2/HPc. Immature barley embryos were sampled up to 24 hours after bombardment and transient expression was assessed using RT-PCR, Western blot analysis, and viscometric assays of barley (1→3,1→4)-β-glucanase activity.

(i) RT-PCR analysis of transient expression

To detect the presence of mRNAs encoding (1→3,1→4)-β-glucanase mutant enzyme H300P, total RNA was extracted from bombarded embryos and cDNA was synthesised using reverse transcriptase (Frohmann *et al.*, 1988). The forward primer E2f and the reverse primer MARKr were used to specifically amplify transcripts encoding (1→3,1→4)-

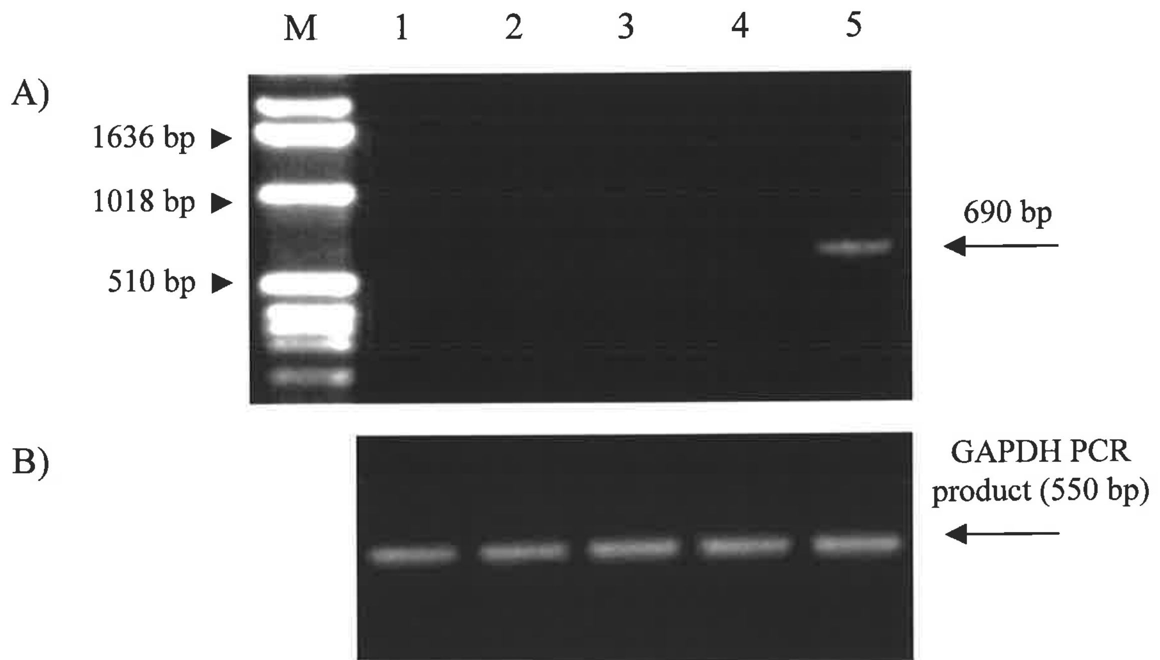


Figure 6.6 RT-PCR analysis of RNA extracted from immature barley immature embryos bombardment with plasmids pE2/HPg and pE2/HPc. A, products amplified with primers specific for the barley (1→3,1→4)-β-glucanase mutant H300P gene or cDNA. B, products amplified using the GAPDH-specific primers. Total RNA for the RT-PCR reactions were extracted from 1, non-bombarded embryos; 2, embryos bombarded in the absence of plasmid DNA; 3 and 4, embryos bombarded with the H300P gene construct with and without the nos 3' sequence, respectively; 5, embryos bombarded with the H300P cDNA construct. M, DNA molecular weight markers.

β-glucanase mutant enzyme H300P (Figure 6.6). A single fragment of the expected size (690 bp) was amplified from cDNA prepared from embryos bombarded with the plasmid pE2/HPc (Figure 6.6). No products were amplified using cDNA prepared from non-bombarded embryos, embryos bombarded in the absence of plasmid DNA, or embryos bombarded with the plasmid pE2/HPg, with or without the *nos* 3' sequence (Figure 6.6). Primers specific for the constitutively expressed barley GAPDH gene resulted in a single 550 bp product of similar abundance for all samples (Figure 6.6), indicating that approximately equal amounts of cDNA were used for each PCR reaction.

Results from the RT-PCR analysis indicate that the constitutive rice actin promoter present in plasmid pE2/HPc is directing the over-expression of the cDNA encoding (1→3,1→4)-β-glucanase mutant H300P. Expression of barley (1→3,1→4)-β-glucanase isoenzyme EII is restricted to the germinated grain (Fincher, 1989), and we would therefore not expect the promoter of the native gene to direct the expression of the (1→3,1→4)-β-glucanase mutant H300P in immature barley embryos. As shown in Figure 6.6, the barley (1→3,1→4)-β-glucanase isoenzyme EII gene promoter was not activated in embryos bombarded with pE2/HPg.

(ii) Analysis of transient expression at the protein level

To analyse the transient expression of (1→3,1→4)-β-glucanase mutant H300P at the post-transcriptional level, total proteins were extracted from immature embryos bombarded with pE2/HPg and pE2/HPg and subjected to Western blot analysis using monoclonal antibodies raised against barley (1→3,1→4)-β-glucanase isoenzyme EII (Figure 6.7). A strong signal indicating a protein of approximately 33.0 kDa was detected for proteins extracted from embryos bombarded with plasmid pE2/HPc, but no signals were detected for the other samples (Figure 6.7). These results are in total agreement with the RT-PCR results (Figure 6.6), indicating that the rice actin promoter is driving the expression of the cDNA encoding (1→3,1→4)-β-glucanase mutant H300P from plasmid pE2/HPc. Because the monoclonal antibodies raised against (1→3,1→4)-β-glucanase isoenzyme EII are highly specific for the

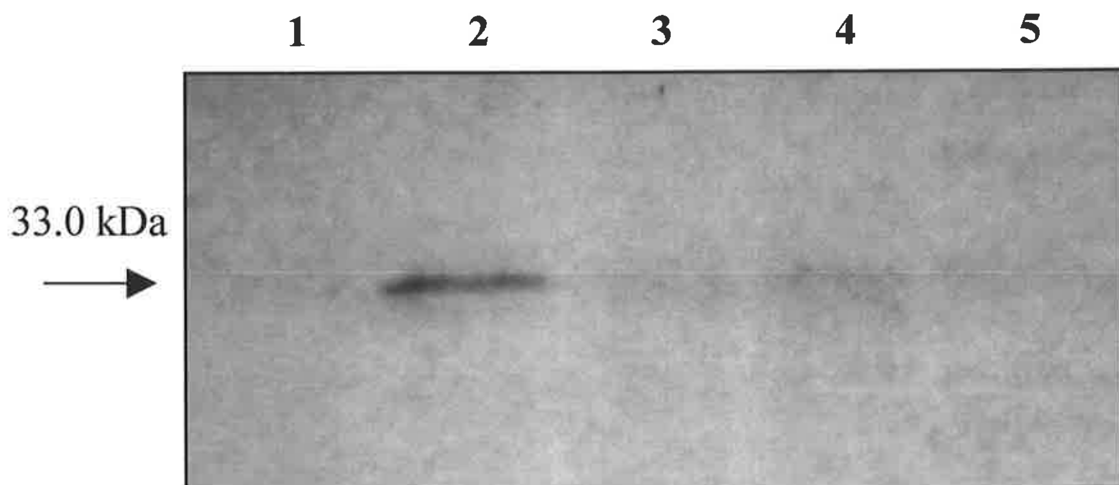


Figure 6.7 Western blot analysis of proteins isolated from barley embryos bombarded with a cDNA construct encoding the thermostable (1→3,1→4)-β-glucanase mutant H300P. Immature barley embryos were bombarded with the cDNA encoding (1→3,1→4)-β-glucanase mutant H300P under the control of the constitutive rice actin promoter (plasmid pE2/HPc), or the gene encoding (1→3,1→4)-β-glucanase mutant H300P under the control of the native isoenzyme EII promoter (plasmid pE2/HPg). Soluble proteins were extracted from embryos 24 h after bombardment and subjected to Western blot analysis using monoclonal antibodies raised against barley (1→3,1→4)-β-glucanase isoenzyme EII. Lane 1, non-bombarded embryos; Lane 2, embryos bombarded with plasmid pE2/HPc; Lanes 3 and 4, embryos bombarded with plasmid pE2/HPg with or without the nos 3' sequence, respectively; Lane 5, embryos bombarded in the absence of plasmid DNA. The arrow indicates a molecular mass of 33.0 kDa.

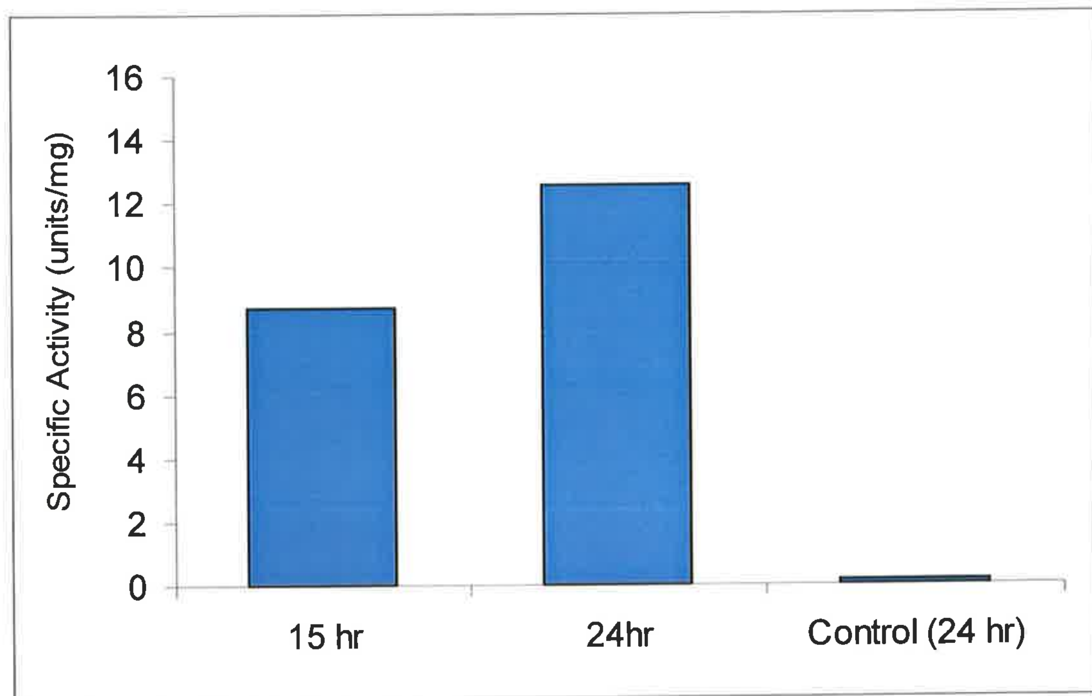


Figure 6.8 (1→3,1→4)-β-Glucanase activity in soluble protein extracts from barley immature embryos bombarded with plasmid pE2/HPc. Total proteins were extracted from immature barley embryos 15 hours and 24 hours after bombardment with plasmid pE2/HPc which encodes barley (1→3,1→4)-β-glucanase mutant H300P under the control of the constitutive rice actin promoter. (1→3,1→4)-β-Glucanase activity was measured viscometrically using barley (1→3,1→4)-β-glucan as a substrate. Control embryos were bombarded in the absence of plasmid DNA.

enzyme, and because the size of the signal indicated the presence of a protein of approximately 33 kDa, it was concluded that the detected protein was the (1→3,1→4)-β-glucanase mutant H300P. The absence of a western blot signal in embryos bombarded with gold particles only, confirmed that there was no endogenous barley (1→3,1→4)-β-glucanase expression in the immature barley embryos.

To assess whether the protein was processed and folded correctly in the immature barley embryo cells, total soluble proteins were extracted from embryos 14 and 24 hours after bombardment with the plasmid pE2/HPc and assayed viscometrically for (1→3,1→4)-β-glucanase activity. Significant levels of (1→3,1→4)-β-glucanase activity were detected in embryos sampled both 15 and 24 hours after bombardment (Figure 6.8), but control embryos that had been bombarded in the absence of plasmid DNA contained no (1→3,1→4)-β-glucanase activity (Figure 6.8). It was not possible to conduct accurate thermostability assays on the transiently-expressed (1→3,1→4)-β-glucanase mutant H300P, because levels of the mutant enzyme in the bombarded embryos were relatively low.

The transient uptake and expression of reporter genes in a variety of barley tissues has been widely reported (Kartha *et al.*, 1989; Lee *et al.*, 1989; Knudsen and Müller, 1991; Lazzeri *et al.*, 1991; Wan and Lemaux, 1994; Hänsch *et al.*, 1996). Transient expression can be used to assess whether foreign proteins will be correctly folded and processed by the cells of the target plant, or to study the expression patterns of foreign gene promoter sequences. For example, the barley (1→3,1→4)-β-glucanase isoenzyme EII gene promoter was used to direct transient expression of the *gus* reporter gene in barley scutella to test the temporal and spatial function of the promoter (R.R. Singh, R.A. Burton, J.F. Kollmorgan and G.B. Fincher, unpublished data). In the current study, the constitutive rice actin promoter was used to direct the transient expression of (1→3,1→4)-β-glucanase mutant H300P in immature barley embryos, which was confirmed at both the mRNA and protein levels. This indicates that if stably transformed barley expressing the gene for (1→3,1→4)-β-

glucanase mutant H300P could be produced, it is likely that the corresponding mutant enzyme will be folded and processed in a similar way to the native enzymes and should therefore be active.

6.3.4 **Agrobacterium-*Mediated Transformation***

A transgenic callus line containing the gene encoding thermostable (1→3,1→4)-β-glucanase mutant H300P was produced using the microprojectile bombardment method, but no plants could be regenerated from this callus. Therefore, it was decided to use the *Agrobacterium*-mediated method in an attempt to produce transgenic barley which expresses the thermostable (1→3,1→4)-β-glucanase mutant H300P. The *Agrobacterium*-mediated method has a higher transformation frequency than the microprojectile bombardment method, and generally inserts single or double copies of the transgene into transcriptionally active regions of the plant genome.

The plasmid pCAM/HPg was constructed to facilitate the introduction of the DNA encoding (1→3,1→4)-β-glucanase mutant H300P into barley using the *Agrobacterium*-mediated transformation method (see Section 6.1.1). It was decided that the gene encoding (1→3,1→4)-β-glucanase mutant H300P should be used in preference to the cDNA, because the gene contains the native (1→3,1→4)-β-glucanase isoenzyme EII promoter which would ensure the correct temporal and spatial expression of the enzyme. Furthermore, it has been shown that the presence of at least one intron is essential for the correct expression of transgenes in plants (McElroy *et al.*, 1990).

Plasmid pCAM/HPg contains the gene encoding (1→3,1→4)-β-glucanase mutant H300P under the control of the native barley (1→3,1→4)-β-glucanase isoenzyme EII promoter, and the gene encoding hygromycin resistance under the control of the constitutive CaMV 35S promoter. The pCAMBIA series of plasmids, from which the plasmid pCAM/HPg was derived, have been used to produce transgenic tobacco and rice (Hajdukiewicz *et al.*, 1994; Hiei *et al.*, 1994). Attempts to introduce plasmid pCAM/HPg into barley using the

Agrobacterium-mediated transformation method are underway in the Department of Plant Science at the University of Adelaide, South Australia.

6.4 Summary and Conclusions

Although attempts to produce transgenic barley that expresses the thermostable (1→3,1→4)-β-glucanase mutant H300P were unsuccessful, the work described in this chapter has provided valuable information about what is required to achieve this task. Results from the transient expression studies show that the gene encoding the (1→3,1→4)-β-glucanase mutant H300P is expressed and processed correctly by barley cells, and that the expressed enzyme is active. Furthermore, it has been demonstrated that the microprojectile bombardment method can be used to stably integrate the gene encoding (1→3,1→4)-β-glucanase mutant H300P into the barley genome, although no plants could be regenerated from the transgenic cells. Producing transgenic barley plants that express the thermostable (1→3,1→4)-β-glucanase mutant H300P will ultimately rely on increasing the transformation frequency of 0.8% which was achieved in the current study using the microprojectile bombardment method. Using an alternative transformation system may be the key to increasing transformation frequencies and hence increasing the chances of producing fertile transgenic barley. The *Agrobacterium*-mediated transformation method generally has a higher transformation frequency than the microprojectile bombardment method, and has recently been used to transform barley with transformation frequencies of up to 8% (Tingay *et al.*, 1997). Thus, the *Agrobacterium*-mediated method represents an attractive alternative to the microprojectile bombardment method for barley transformation. In the current study, plasmid pCAM/HPg was constructed to facilitate the introduction of the gene encoding the thermostable (1→3,1→4)-β-glucanase mutant H300P into barley using the *Agrobacterium*-mediated transformation method.

CHAPTER SEVEN:

SUMMARY AND FUTURE DIRECTIONS

7.1 Summary of the Work Described in this Thesis

The principle aim of the work described in this thesis was to use protein engineering to increase the thermostability of barley (1→3,1→4)-β-glucanases. This required the development of a system for the efficient heterologous expression and purification of barley (1→3,1→4)-β-glucanase isoenzyme EII, barley (1→3)-β-glucanase isoenzyme GII, and their respective mutant forms. Using the pET vector, both barley enzymes were successfully expressed as fusion proteins containing a polyhistidine tag at the COOH-terminus. This tag has a high affinity to a commercially available nickel-based resin and thus enabled the one-step purification of the recombinant enzymes by affinity chromatography. For barley (1→3,1→4)-β-glucanase isoenzyme EII, reducing the expression temperature from 37°C to 22°C dramatically increased the solubility and yield of the expressed protein. However, low expression temperatures were not necessary to produce high yields of pure barley (1→3)-β-glucanase isoenzyme GII, which is probably a reflection of the latter enzyme's inherently higher stability.

Initial attempts to produce a thermostable barley (1→3,1→4)-β-glucanase involved changing the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII to that of a (1→3,1→4)-β-glucanase. The aim of this approach was to produce an enzyme which could hydrolyse (1→3,1→4)-β-glucan but would retain the thermostability characteristics of barley (1→3)-β-glucanase isoenzyme GII. Based on a comparison with the three-dimensional structure of barley (1→3,1→4)-β-glucanase isoenzyme EII, four single amino acid substitutions were introduced into the substrate binding groove of (1→3)-β-glucanase isoenzyme GII. Although the resulting mutant enzymes showed reduced (1→3)-β-glucanase activity, they did not hydrolyse barley (1→3,1→4)-β-glucan.

The second approach that was employed to engineer a barley (1→3,1→4)-β-glucanase with increased heat stability, involved using site-directed mutagenesis to introduce rational amino acid substitutions into the barley (1→3,1→4)-β-glucanase isoenzyme EII itself.

These substitutions were based on structural comparisons of barley (1→3,1→4)-β-glucanase isoenzyme EII and barley (1→3)-β-glucanase isoenzyme GII, together with existing knowledge of the principles governing protein stability. One mutant enzyme, which contained the single amino acid substitution H300P, displayed a significant increase in thermostability when compared with the wildtype recombinant (1→3,1→4)-β-glucanase isoenzyme EII, both under *in vitro* conditions and during simulated mashing at 65°C. The histidine to proline substitution decreases the entropy of the unfolded state of the protein, which effectively increases the stability of the folded state. Residue 300 is located in the COOH-terminal loop of barley (1→3,1→4)-β-glucanase isoenzyme EII, and this region clearly lacks defined secondary structure. Two additional stabilising substitutions, M298K;T17D and N290H, were also located in the COOH-terminal loop, and this region was therefore identified as a potential weak link in the enzyme's structure. In an attempt to strengthen the COOH-terminal loop, the NH_2- and COOH-termini of the enzyme were fused and new termini were created in a more stable region of the enzyme. However, the resulting permutated (1→3,1→4)-β-glucanase displayed no activity.

Finally, attempts were made to transform barley with the gene and cDNA encoding the thermostable barley (1→3,1→4)-β-glucanase mutant H300P. The cDNA encoding the thermostable enzyme was transiently expressed in barley immature embryos under the control of a rice actin promoter, and expression was confirmed at both the mRNA and protein levels. Furthermore, the gene encoding (1→3,1→4)-β-glucanase mutant H300P, under the control of the endogenous barley (1→3,1→4)-β-glucanase isoenzyme EII promoter, was stably integrated into cultured barley cells using the microprojectile bombardment procedure. Attempts to regenerate plants from the transgenic callus were unsuccessful. Finally, a construct designed to facilitate the delivery of the H300P gene into barley using the *Agrobacterium*-mediated transformation procedure was generated and is undergoing evaluation in this laboratory.

7.2 Further Enhancing the Thermostability of Mutant H300P

Although barley (1→3,1→4)-β-glucanase mutant H300P lasts significantly longer than its wildtype counterpart during simulated mashing at 65°C, it is probably not sufficiently thermostable for industrial use. Preliminary data from simulated mashing experiments suggests that the capacity of the thermostable mutant H300P to reduce wort viscosity is minimal when compared to that of commercial endo-β-glucanase from fungi and bacteria (R.J. Stewart, D.C. Stewart, G.B. Fincher, unpublished data). It has been estimated that a thermostable barley (1→3,1→4)-β-glucanase with a ΔT_{50} of 10-15°C would be required to significantly reduce the problems associated with residual (1→3,1→4)-β-glucans in industry (G.B. Fincher, personal communication). Mutant (1→3,1→4)-β-glucanase H300P has a ΔT_{50} of 3.7°C. Therefore, we must achieve further increases in thermostability are required if engineered barley (1→3,1→4)-β-glucanases are to be useful for industrial applications. Several possible strategies for achieving this are presented below.

(i) Implementing additional amino acid changes

A list of more than 75 amino acid substitutions for increasing the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII was compiled by Drs J.N. Varghese and T.P.J. Garrett at the Biomolecular Research Institute in Parkville, Victoria, Australia. As part of the work described in this thesis, eight of these amino acid substitutions were introduced into (1→3,1→4)-β-glucanase isoenzyme EII, and only H300P significantly increased the thermostability of the enzyme. In hindsight, the criteria used in the current study to select the eight amino acid substitutions were biased, because the substitutions were not only selected on their potential to increase thermostability, but also on the type of molecular interaction they represented. Thus, the mutants were selected to include examples of ion pair formation, reduced entropy of unfolding, removal of surface lysines, and the introduction of additional hydrogen bonding. Furthermore, two other promising mutations, G223A and K23R, were discarded because they could not be successfully introduced at the DNA level using the USE mutagenesis procedure used here. Thus, further increases in

thermostability may well be achieved by utilising additional amino acid substitutions from the original list. This would require a site-directed mutagenesis method with a higher mutation frequency and reproducibility than the USE mutagenesis procedure. There is now a PCR-based method available with a mutation frequency of 95-100%. The QikChange Kit from Stratagene involves a direct method for selection of the mutated DNA strand rather than relying on an indirect selection mechanism, as is the case for the USE method (Chapter 2). A mutational frequency of 100% was achieved when the QuikChange kit was used to introduce several mutations into the cDNA encoding barley (1→3)-β-glucanase isoenzyme GII (S.J. Rutten, personal communication).

(ii) The thermal motion of atoms: An indication of protein stability?

An alternative method that can be used to identify relatively unstable regions of a protein involves an examination of the thermal motion associated with each residue in the protein structure. B-values are used to describe the vibrational motion of atoms relative to their resting position in the protein structure (Rhodes, 1993). A plot of the average isotropic B-values for each main chain residue can potentially identify which parts of the protein structure are vibrationally active and which parts are particularly rigid. A plot of the B-values associated with the polypeptide backbone residues of barley (1→3,1→4)-β-glucanase isoenzyme EII (Figure 7.1) reveals a region of relatively high thermal motion at the southern end of the substrate binding groove of the enzyme, as oriented in Figure 4.1. Although time constraints precluded the examination of this region in the current study, it should represent a target for future attempts to increase the thermostability of barley (1→3,1→4)-β-glucanase isoenzyme EII. Indeed, two amino acid substitutions that were specifically designed for this purpose, namely G185N and A191P, are located in this region of high thermal motion.

(iii) Random mutagenesis

If attempts to further increase the stability of barley (1→3,1→4)-β-glucanase isoenzyme EII by the strategic replacement of amino acids are unsuccessful, random mutagenesis may

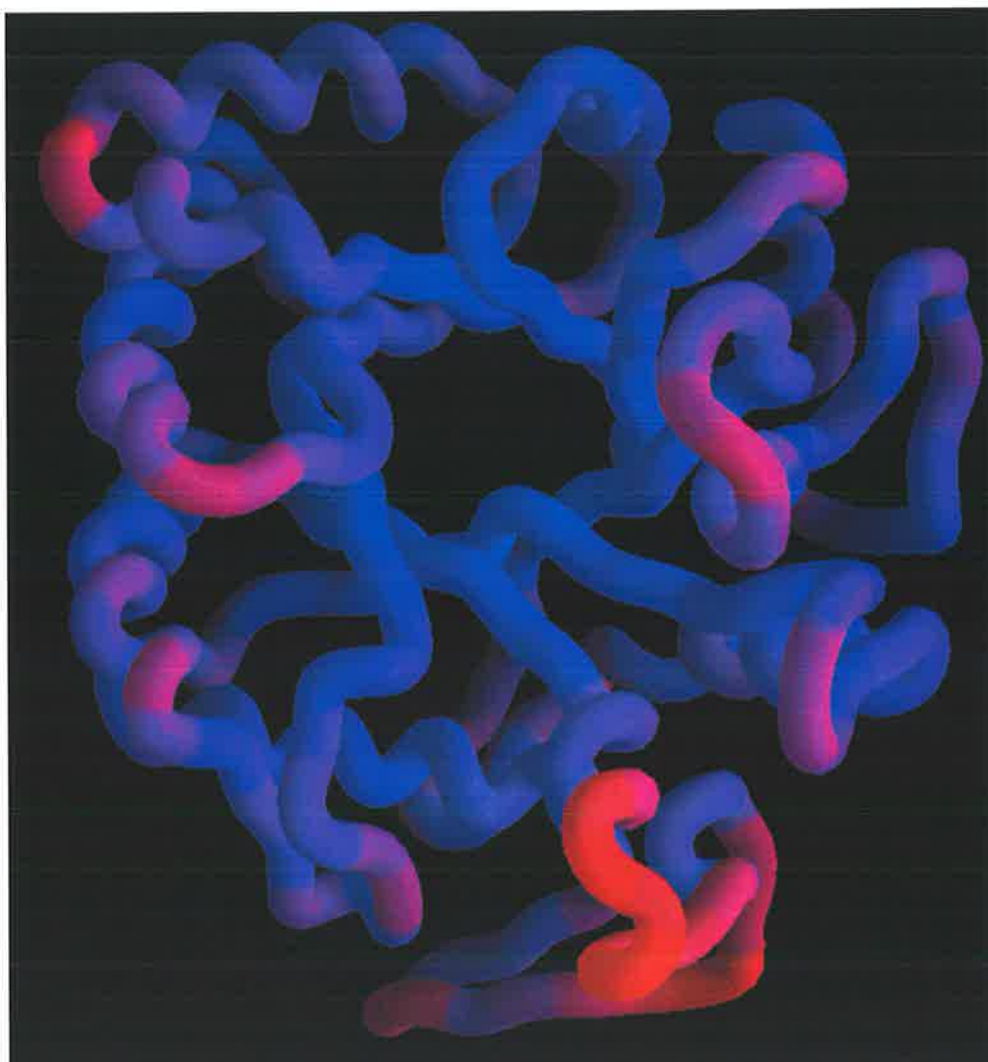


Figure 7.1 A plot of the average isotropic B-values for the polypeptide backbone of barley (1→3,1→4)-β-isoenzyme EII. Polypeptide backbone residues are coloured according to their B-values, with blue and red indicating low and high thermal motion, respectively. This figure was produced by Dr J.N. Varghese, Biomolecular Resaerch Institute, Parkville, Victoria, Australia.

represent a viable alternative. This method involves the use of low fidelity PCR conditions to introduce random nucleotide changes into the cDNA encoding the enzyme of interest. The randomly mutated cDNA sequences are expressed in an appropriate heterologous host and the resulting mutant proteins are screened for desirable characteristics such as enhanced heat stability. This approach has been successfully used to increase the thermostability of barley β-amylase (Okada *et al.*, 1995). Random mutagenesis does not require knowledge of a protein's three-dimensional structure, and amino acid substitutions which increase thermostability, but which would not normally be conceived from our knowledge of the factors affecting protein stability, may be thereby be identified.

The major disadvantage of the random mutagenesis procedure is that it requires a system for screening large numbers (>10,000) of mutant proteins in a quick and efficient manner. The first step in developing a rapid screening method for barley (1→3,1→4)-β-glucanases would involve ligating the mutated cDNAs into a bacterial expression vector which directs the extra-cellular secretion of expressed proteins. Expression could be induced by growing the bacterial colonies on LB agar plates containing IPTG. If the agar plates contained a dyed (1→3,1→4)-β-glucan substrate (Biely *et al.*, 1985) which is solubilized upon hydrolysis, (1→3,1→4)-β-glucanase activity could be correlated with clear zones around each bacterial colony. Furthermore, if replicates of each plate were incubated at increasing temperatures following the induction of expression, the production and size of the clear 'halos' around each colony may reflect the thermostability of the mutant enzyme being secreted from that colony. Finally, plasmid DNA could be isolated from the appropriate cells and sequenced to reveal which mutations are responsible for the apparent increase in thermostability.

(iv) Changing substrate specificity

It is likely that changing the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII to that of barley (1→3,1→4)-β-glucanase isoenzyme EII, which remains a viable option for increasing barely (1→3,1→4)-β-glucanase thermostability, will require a more

comprehensive understanding of the binding and catalytic mechanisms of both enzymes. The substrate-binding groove of barley (1→3)-β-glucanase isoenzyme GII has recently been mapped (Hrmova *et al.*, 1995), and experiments designed to determine the three-dimensional structures of the barley (1→3)- and (1→3,1→4)-β-glucanases in complex with natural or synthetic substrates are underway (Hrmova *et al.*, 1998b). In addition, solving the three-dimensional structures of enzymes with dual (1→3)/(1→3,1→4)-β-glucanase activity such as the β-glucan endohydrolase from *Rhizopus arrhizus* (E.C. 3.2.1.6; Anderson and Stone, 1975; Clark *et al.*, 1978), may help determine the factors responsible for binding β-glucan molecules with different linkage compositions.

7.3 Improving Barley Transformation

Realizing the true industrial potential of barley (1→3,1→4)-β-glucanase mutant H300P will ultimately depend on our ability to produce fertile transgenic plants which express the thermostable enzyme. Stably transformed barley has been successfully produced using *Agrobacterium*-mediated (Tingay *et al.*, 1997) and microprojectile bombardment methods (Wan and Lemaux, 1994), but transformation frequencies remain frustratingly low. In the current study, numerous attempts to produce transgenic barley that expresses the thermostable mutant enzyme H300P were unsuccessful. Possible reasons for this lack of success and some alternative approaches are outlined below.

(i) Selectable markers and reporter genes

The rapid identification of transgenic tissue at an early stage of the tissue culture process is essential for the efficient production of transgenic plants. This requires a selection mechanism that is both rapid and accurate. To date, the most frequently used reporter gene in cereal transformation has been the *gus* gene, while the *bar* gene has been the selectable marker gene of choice. Although these genes are used extensively for the *in vitro* selection of transgenic tissue, they do have some limitations. In the case of herbicide resistance genes, an even exposure of cells to the selective herbicide in the growth media is not always achieved and this can result in the inadvertent selection of non-transgenic plants.

Also, because detection of *gus* gene expression requires the use of a destructive assay, tissue can not be regenerated directly from cells which have been shown to express the gene.

A reporter gene that has the potential to overcome limitations of traditional reporter genes is the Green Fluorescence Protein (GFP) gene from the jelly-fish *Aequorea victoria*. This gene can be used for direct selection of transgenic plant tissue because the corresponding gene product glows with a green fluorescence when viewed under an ultraviolet light of the appropriate wavelength (Heim *et al.*, 1994; Dopf and Horiagon, 1996). GFP represents a non-destructive, non-toxic selective agent which allows for direct visual selection of transgenic tissue, and does not involve the use of antibiotics or herbicides. Although the GFP gene has been used to produce a variety of transgenic plant species (Chiu *et al.*, 1996; Pang *et al.*, 1996; Haseloff *et al.*, 1997; Leffel *et al.*, 1997; Vonarnim *et al.*, 1998; Elliott *et al.*, 1999), a comprehensive suite of GFP-based vectors designed particularly for cereal transformation is not yet available.

(ii) Plant tissue culture and regeneration

In the previous Chapter, barley callus line was stably transformed with the gene encoding barley $(1\rightarrow3,1\rightarrow4)$-β-glucanase mutant H300P but plants could not be regenerated from the callus. Indeed, the problems associated with regenerating barley plants from embryonic callus *in vitro* represent a major obstacle to the widespread production of transgenic barley. The inability of callus to form vegetative tissues can be attributed to the de-differentiation of the transformed cells *via* a callus intermediate. Cell de-differentiation is often associated with loss of genetic fidelity, which can lead to somoclonal variation, chromosomal rearrangements, or the loss of chromosomal material. Because this loss of genetic fidelity is normally progressive, the period of time which callus cells spend in culture should be minimized (Jiang *et al.*, 1998). The de-differentiation of plant cells during the transformation procedure can be avoided by targeting the meristematic tissue of the plant, which can be directly regenerated into plants without the need of a callus

intermediate. *Agrobacterium*-mediated infection of apical meristems is a rapid and well-established procedure which has been used to produce transgenic cereals including maize (Gould *et al.*, 1990; Ishida *et al.*, 1995) and rice (Chan *et al.*, 1993; Hiei *et al.*, 1994). Future attempts to transform barley with the gene encoding the thermostable (1→3,1→4)-β-glucanase mutant H300P should include the infection of meristem tissues with *A. tumefaciens*.

(iii) Transgene integration into the plant genome

Microprojectile bombardment and, to a lesser extent, *Agrobacterium*-mediated plant transformation methods integrate DNA into the plant genome in an essentially random manner. If transgenes could be targeted to specific areas of the plant genome it would alleviate several problems associated with current plant transformation technology. These problems include the insertional inactivation of other functional genes, the placement of transgene into transcriptionally-inactive regions of the genome, and the uncontrollable insertion of multiple copy numbers of the transgene. Although homologous recombination has been used for the site-specific replacement of genes in mammalian, yeast and bacterial cells (Fink, 1998), the plant genome has proven to be particularly recalcitrant to this kind of manipulation probably because of the randomness and extremely low frequency of homologous recombination events.

A technique for the site-specific manipulation of plant genes has recently been developed. This technique, known as 'chimeraplasty', uses chimeric RNA/DNA oligonucleotides to specifically alter 1-2 DNA base pairs in endogenous plant genes (Beetham *et al.*, 1999; Zhu *et al.*, 1999). The mutagenic oligonucleotide consists of the same sequence as the target endogenous gene but contains a 1-2 bp mismatch. Using the microprojectile bombardment method, the chimeric oligonucleotide is delivered to the plant nucleus where it binds to the endogenous target gene. The plant DNA repair enzymes recognize the apparent DNA base pair mismatch and proceed to repair it using the oligonucleotide sequence as a template, thereby introducing the mismatch into the endogenous gene. Zhu *et al.* (1999) used this

technique to target the endogenous acetohydroxyacid synthase (AHAS) gene in maize that encodes an enzyme which is the target of sulfonylurea-based herbicides. A chimeric RNA/DNA oligonucleotide designed to change a single base in the AHAS gene was introduced into cells of herbicide-susceptible maize plants in an attempt to produce herbicide-resistance plants. Transgenic tissue was selected on media containing a sulfonylurea herbicide and resistant plants were produced. Furthermore, these changes to the endogenous maize AHAS gene were inherited in subsequent populations. Similar results have been obtained using chimeric oligonucleotide-directed site-specific gene manipulation in tobacco (Beetham *et al.*, 1999).

For the thermostable barley (1→3,1→4)-β-glucanase H300P, the chimeric oligonucleotide method for site-specific *in vivo* mutagenesis provides an attractive alternative to traditional transformation methods for a number of reasons. Firstly, the histidine to proline change only involves a single DNA base change (CAC to CCC) which has proven to be the preferred arrangement for successful chimeric oligonucleotide-mediated mutagenesis (Zhu *et al.*, 1999). Secondly, because chimeraplasty does not involve the permanent introduction of foreign DNA into the plant genome, plants that are manipulated using this method may be more acceptable to the consumer than transgenic plants produced by traditional plant transformation methods. Finally, this method does not involve altering gene copy number and therefore alleviates the potential for metabolic imbalance and gene silencing effects in the host plant. For this technique to become widely used for the site-specific manipulation of plant genes however, a rigorous method will need to be developed for selecting cells that contain the altered gene. To date, this promising technique has only been used to change easily selectable plant genes that encode herbicide resistance or GFP (Beetham *et al.*, 1999; Zhu *et al.*, 1999).

7.4 Transgenic Barley: The Final Product

Will industry adopt the use of transgenic barley that expresses a thermostable (1→3,1→4)-β-glucanase? In the USA, breweries have indicated that they will use transgenic barley if it adds value to the brewing process and is accepted by the consumer (D.C. Stewart, personal communication). The general consensus from consumer groups in the USA is that they will not oppose the use of transgenic plants for the production of human food or beverages if it is beneficial in terms of health and cost, and if the appropriate health regulations are implemented (Hoban, 1997). In contrast, European consumers strongly oppose the use of transgenic plants and related products in the food and beverage industries. In fact, British brewing authorities have done their utmost to ensure that their industry does not use genetically modified barley or maize for beer production (Anon., 1999a). This fear of genetically modified organisms (GMOs) is primarily driven by a perceived lack of trust for the regulatory authorities which may be well justified by the recent outbreak of Bovine Spongiform Encephalopathy (BSE) which has devastated the European beef market (Dickson, 1997). However, the reluctance of some sectors of the European public to accept the use of genetically modified plants may also be engendered by a general lack of understanding of biotechnology. For example, it seems ironic that while the use of thermostable fungal and bacterial β-glucanases is permitted in European breweries, the use of transgenic barley expressing these same enzymes is strongly opposed. Similarly, the two major Japanese breweries, Sapporo and Kirin, have stated that they will not use genetically modified cereals for beer production (Anon., 1999b). Australian breweries have taken an intermediate stance on the issues surrounding the use of biotechnology in brewing, in that they are prepared to adopt the use of superior transgenic malting barley, but not for at least 4-5 years (D.C. Stewart, personal communication). This should allow time for the development of appropriate food safety regulations and enable consumer attitudes to be unequivocally determined.

APPENDIX I

MSB callus induction media contained the following chemicals:

Macrosalts (mg/ml):	
NH_4NO_3	1520
KNO_3	2224
$CaCl_2.2H_2O$	441
KH_2PO_4	370
$MgSO_4.7H_20$	170
Microsalts (mg/ml):	
H_3BO_3	6.2
$MnSO_4.H_20$	16.9
$ZnSO_4.7H_2O$	8.6
KI	0.83
$CoCl_2.6H_2O$	0.024
$Na_2MoO_4.2H_2O$	0.24
Iron Solution (mg/ml):	
Na_2EDTA	37
$FeSO_4.7H_2O$	28
Vitamins (mg/ml):	
Thiamine	1.0
Inositol	250
Sugar (gm/ml):	
Sucrose	30
Other (mg/ml):	
2,4-D	3.0
Casein hydrolysate	1000
pH.	5.8

REFERENCES

Aastrup, S. (1983). Selection and characterization of low β-glucan mutants from barley. *Carlsberg Res Comm* **48**, 307-316.

Aisien, A., Palmer, G., and Stark, J. (1986). The ultrastructure of germinating sorghum and millet (*Pennisetum americanum*) grains. *J Inst Brew* **92**, 162-167.

Albersheim, P., An, J. H., Freshour, G., Fuller, M. S., Guillen, R., Ham, K.-S., Hahn, M. G., Huang, J., O'Neill, M., Whitcombe, A., Williams, M. V., York, W. S., and Darvill, A. (1994). Structure and function studies of plant cell wall polysaccharides. *Biochem Soc Trans* **22**, 374-378.

Anderson, D. E., Becktel, W. J., and Dahlquist, F. W. (1990). pH-induced denaturation of proteins: A single salt bridge contributes 3-5 kcal/mol to the free energy of folding of T4-lysozyme. *Biochemistry* **29**, 2403-2408.

Anderson, M. A., and Stone, B. A. (1975). A new substrate for investigating the specificity of β-glucan hydrolases. *FEBS Lett* **52**, 202-207.

Andrewartha, K., Phillips, D. R., and Stone, B. A. (1979). Solution properties of wheat flour and enzymically modified arabinoxylans. *Carbohyd Res* **77**, 191-204.

Anonymous (1999a). UK brewers say no to GM maize in British beer. Reuters Press Release, London, UK.

Anonymous (1999b). Asahi breweries to stop using GM corn by April 2000. Dow Jones Press Release, Tokyo, Japan.

Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y., and Okada, H. (1993). Stabilization of xylanase by random mutagenesis. *FEBS Lett* **316**, 123-127.

Aspegren, K., Mannonen, L., Ritala, A., Puupponenepimia, R., Kurten, U., Salmenkalliomarttila, M., Kauppinen, V., and Teeri, T. H. (1995). Secretion of a heat-stable fungal β-glucanase from transgenic, suspension-cultured barley cells. *Mol Breed* **1**, 91-99.

Ay, J., Hahn, M., Decanniere, K., Piotukh, K., Borriss, R., and Heinemann, U. (1998). Crystal structures and properties of *de novo* circularly permuted (1→3,1→4)-β-glucanases. *Proteins* **30**, 155-167.

Bacic, A. and Stone, B. (1981a). Isolation and ultrastructure of aleurone cell walls from wheat (*Triticum aestivum* cultivar Insignia) and barley (*Hordeum vulgare* cultivar Clipper). *Aust J Plant Physiol* **8**, 453-474.

Bacic, A. and Stone, B. A. (1981b). Chemistry and organization of aleurone cell wall components from wheat (*Triticum aestivum* cultivar Insignia) and barley (*Hordeum vulgare* cultivar Clipper). *Aust J Plant Physiol* **8**, 475-495.

Ballance, G. M., Hall, R. S., and Manners, D. J. (1986). Studies of some arabinoxylans from barley endosperm walls. *Carbohyd Res* **150**, 290-294.

Ballance, G. M. and Manners, D. J. (1978). Structural analysis and enzymic solubilization of barley endosperm cell walls. *Carbohyd Res* **61**, 107-118.

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

Bamforth, C. W. (1998). Beer: tap into the art and science of brewing. Plenum Press, New York.

Bamforth, C. W. (1994). β-Glucans and β-glucanases in malting and brewing: practical aspects. *Brew Dig* **69**, 12-16.

Bamforth, C. W. (1985). Biochemical approaches to beer quality. *J Inst Brew* **91**, 154-160.

Bamforth, C. W. and Barclay, A. H. P. (1993) Malting technology and the uses of malt. In: Barley; Chemistry and Technology (A. W. MacGregor and R. S. Bhatty, eds.) American Association of Cereal Chemists, St Paul, Minnesota, USA. pp. 297-355

Banik, M. (1996). Structure, hormonal regulation and chromosomal location of genes encoding barley (1→4)-β-xylan endohydrolases. Thesis (Ph.D.), Department of Plant Science, University of Adelaide, Adelaide, South Australia.

Banik, M., Garrett, T. P. J., and Fincher, G. B. (1996). Molecular cloning of cDNAs encoding (1→4)-β-xylan endohydrolases from the aleurone layer of germinated barley (*Hordeum vulgare*). *Plant Mol Biol* **31**, 1163-1172.

Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. I., Wilson, I. A., Corran, P. H., Furth, A. J., Milman, J. D., Offord, R. E., Priddle, J. D., and Waley, S. G. (1975). Structure of chicken muscle triose phosphate isomerase determined crystallographically at 2.5 angstrom resolution using amino acid sequence data. *Nature* **255**, 609-614.

Bathgate, G. N. and Palmer, G. H. (1975). The prediction of brewing behaviour of malts from bench scale trials. In: Proceedings of the European Brewing Convention Congress, pp. 61-80.

Baulcombe, D. C. (1999). Fast forward genetics based on virus-induced gene silencing. *Curr Opin Plant Biol* **2**, 109-133.

Beetham, P. R., Kipp, P. B., Sawycky, X. L., Arntzen, C. J., and May, G. D. (1999). A tool for functional plant genomics: Chimeric RNA/DNA oligonucleotides cause *in vivo* gene-specific mutations. *Proc Natl Acad Sci USA* **96**, 8774-8778.

Benjavongkulchai, E. and Spencer, M. S. (1986). Purification and characterization of barley aleurone xylanase. *Planta* **169**, 415-419.

Bennet, P. A. Chrispeels, M. J. (1972). *De novo* synthesis of ribonuclease and (1→3)-β-glucanase by aleurone cells of barley. *Plant Physiol* **49**, 455-457.

Bertrand, D., Brillouet, J. M., Rasper, V. F., Bouchet, B., and Mercier, C. (1981). Effects of rat digestion upon native, enzymically or chemically modified wheat brans and native oat bran. *Cereal Chem* **58**, 375-380.

Bewley, J. and Black, M. (1994). Seeds: physiology of development and germination. Plenum Press, New York, USA.

Bhatty, R. (1993a). Physicochemical properties of roller-milled barley bran and flour. *Cereal Chem* **70**, 397-402.

Bhatty, R. S. (1993b). Non-malting uses of barley. In: Barley; Chemistry and Technology (A. W. MacGregor and R. S. Bhatty, eds.) American Association of Cereal Chemists, St Paul, Minnesota, USA. pp. 355-417

Biely, P., Mislovicová, D., and Toman, R. (1985). Soluble chromogenic substrates for the assay of endo-(1→4)-β-xylanases and endo-(1→4)-β-glucanases. *Anal Biochem* **144**, 142-146.

Boller, T. (1987). Hydrolytic enzymes in plant disease resistance. In: Plant-Microbe Interactions; Molecular and Genetic Perspectives (T. Kosuge and E. W. Nester, eds.) MacMillan, New York, USA, pp. 385-413.

Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**, 248-254.

Branden, C. and Tooze, J. (1991). Introduction to protein structure. Garland Publishing Inc., New York, USA.

Brandt, A. (1976). Endosperm protein formation during kernel development of wild type and a high-lysine barley mutant. *Cereal Chem* **53**, 890-901.

Breddam, K., Sorensen, S. B., and Ottesen, M. (1983). Isolation of a carboxypeptidase from malted barley by affinity chromatography. *Carlsberg Res Comm* **48**, 217-230.

Breddam, K. and Sorensen, S. B. (1987). Isolation of carboxypeptidase-III from malted barley by affinity chromatography. *Carlsberg Res Comm* **52**, 275-283.

Briggs, D. (1992). Barley germination: biochemical changes and hormonal control. In: Barley; Genetics, Biochemistry, Molecular Biology and Biotechnology, (P. Shewry, ed.) CAB International, pp. 369-401.

Briggs, D. E., Hough, J. S., Stevens, R., and Young, T. W. (1981). Malt and Sweet Wort, Volume 1. Chapman and Hall, London, UK.

Brown, P. H., and Ho, T.-H. D. (1986). Barley aleurone layers secrete a nuclease in response to gibberellic acid. *Plant Physiol* **82**, 801-806.

Brunswick, P., Manners, D. J., and Stark, J. R. (1988). Degradation of isolated barley endosperm cell walls by purified endo-(1→3,1→4)-β-glucanase and malt. *J Cereal Sci* **7**, 153-168.

Buliga, G., Brant, D., and Fincher, G. (1986). The sequence statistics and solution configuration of barley (1→3,1→4)-β-D-glucan. *Carbohyd Res* **157**, 139-156.

Burley, S.K. and Petsko, G.A. (1985). Aromatic-aromatic interaction: A mechanism of protein structure stabilization. *Science* **229**, 23-28.

Burnett, G. S. (1966). Studies of viscosity as the probable factor involved in the improvement of certain barleys for chickens by enzyme supplementation. *Brit Poult Sci* **7**, 55-75.

Burton, R. A., Zhang, X.-Q., Hrmova, M., and Fincher, G. B. (1999a). A single limit dextrinase gene is expressed both in the developing endosperm and in germinated grains of barley. *Plant Physiol* **119**, 859-871.

Burton, R. A., Gibeaut, D. M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D. C., and Fincher, G. B. (1999b). Virus-induced silencing of a plant cellulose synthase gene. *Submitted.*

Campbell, G. L. and Bedford, M. R. (1992). Enzyme applications for monogastric feeds: a review. *Can J Animal Sci* **72**, 449-466.

Carpita, N. (1996). Structure and biogenesis of the cell walls of grasses. *Annu Rev Plant Physiol Plant Mol Biol* **47**, 445-476.

Casas, A. M., Kononowicz, A. K., Zehr, U. B., Tomes, D. T., Axtell, J. D., Butler, L. G., Bressan, R. A., and Hasegawa, P. M. (1993). Transgenic sorghum plants *via* microprojectile bombardment. *Proc Natl Acad Sci USA* **90**, 11212-11216.

Castillo, A. M., Vasil, V., and Vasil, I. K. (1994). Rapid production of fertile transgenic plants of rye (*Secale cereale* L.). *BIO/TECH* **12**, 1366-1371.

Chan, M., Lee, M. T., and Chang, H. (1992). Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium*. *Plant Cell Phys* **33**, 577-583.

Chen, L., Fincher, G. B., and Høj, P. B. (1993). Evolution of polysaccharide hydrolase substrate specificity: catalytic amino acids are conserved in barley (1→3,1→4)-β-glucanase and (1→3)-β-glucanase. *J Biol Chem* **268**, 13318-13326.

Chen, L., Garrett, T. P. J., Fincher, G. B., and Høj, P. B. (1995). A tetrad of ionizable amino acids is important for catalysis in barley β-glucanases. *J Biol Chem* **270**, 8093-8101.

Chen, W.-J. L., Anderson, J. W., and Gould, M. R. (1981). Effects of oat bran, oat gum and pectin on lipid metabolism of cholesterol-fed rats. *Nutr Rep Int* **24**, 1093-1098.

Cheng, M., Fry, J. E., Pang, S. Z., Zhou, H. P., Hironaka, C. M., Duncan, D. R., Conner, T. W., and Wan, Y. C. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol* **115**, 971-980.

Chiu, W. L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996). Engineered GFP as a vital reporter in plants. *Curr Biol* **6**, 325-330.

Chrispeels, M. and Varner, J. (1967). Gibberellic acid enhances synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. *Plant Physiol* **42**, 398-406.

Christou, P., Ford, T. L., and Kofron, M. (1991). Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties *via* electric discharge particle acceleration of exogenous DNA inot immature zygotic embryos. *BIO/TECH* **9**, 957-962.

Clark, D. R., Johnson Jr, J., Chung, K. H., and Kirkwood, S. (1978). Purification, characterization, and action-pattern studies on the endo-(1→3)-β-D-glucanase from *Rhizopus arrhizus* QM 1032. *Carbohyd Res* **61**, 457-477.

Colonna, P. and Mercier, C. (1985). Gelatinization and melting of maize and pea starches with normal and high amylose genotypes. *Phytochemistry* **24**, 1667-1674.

Conrad, U. and Fiedler, U. (1998). Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. *Plant Mol Biol* **38**, 101-109.

Coote, N. and Kirsop, B. H. (1976). A haze consisting largely of pentosan. *J Inst Brew* **82**, 34.

Cornejo, M. J., Luth, D., Blankenship, K. M., Anderson, O. D., and Blechl, A. E. (1993). Activity of a maize ubiquitin promoter in transgenic rice. *Plant Mol Biol* **23**, 567-581.

Coutinho, P. M., Dowd, M. K., and Reilly, P. J. (1997). Automated docking of glucosyl disaccharides in the glucoamylase active site. *Proteins: Struct Funct Genet* **28**, 162-173.

Crawford, I. P., Niermann, T., and Kirschner, K. (1987). Prediction of secondary structure by evolutionary comparison: application to the a subunit of tryptophan synthase. *Proteins: Struct Funct Genet* **2**, 118-129.

Dao-Pin, S., Sauer, U., Nicholson, H., and Matthews, B. W. (1991). Contributions of engineered surface salt bridges to the stability of T4 lysozyme determined by directed mutagenesis. *Biochemistry* **30**, 7142-7153.

Dashek, W. and Chrispeels, M. (1977). Gibberellic-acid-induced synthesis and release of cell wall degrading endoxylanase by isolated aleurone layers of barley. *Planta* **134**, 251-256.

Datta, S. K., Peterhans, A., Datta, K., and Potrykus, I. (1990). Genetically engineered fertile Indica-rice recovered from protoplasts. *BIO/TECH* **8**, 736-740.

Daussant, J. and Corvazier, P. (1970). Biosynthesis and modifications of α- and β-amylases in germinating wheat seeds. *FEBS Lett* **7**, 191-194.

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

Davies, G. J., Dodson, G. G., Hubbard, R. E., Tolley, S. P., Dauter, Z., Wilson, K. S., Hjort, C., Mikkelsen, J. M., Rasmussen, G., and Schülein, M. (1993). Structure and function of endoglucanase V. *Nature* **365**, 362-364.

DeCleene, M. (1985). The susceptibility of monocotyledons to *Agrobacterium tumefaciens*. *Phytopathol Z* **113**, 81-89.

Dekker, R. F. H. (1989). Biodegradation of the hetero-(1→4)-linked xylans. *Amer Chem Soc* **399**, 619-629.

Dekker, R. F. J. and Richards, G. N. (1976). Hemicellulases: Their occurrence, purification properties, and mode of action. *Adv Carbohyd Chem Biochem* **32**, 278-352.

Deng, W. P. and Nickoloff, J. A. (1992). Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal Biochem* **200**, 81-88.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387-395.

Di Guan, C., Li, P., Riggs, P. D., and Inouye, H. (1988). Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. *Gene* **67**, 21-30.

Dickson, D. (1997). UK policy learns about risk the hard way. *Nature* **385**, 8-9.

Dill, K. A. (1990). Dominant forces in protein folding. *Biochemistry* **29**, 7133-7155.

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

Dopf, J. and Horiagon, T. M. (1996). Deletion mapping of the *Aequorea victoria* green fluorescent protein. *Gene* **173**, 39-44.

Ducroo, P. and Frelon, P. G. (1989). Improvement of beer production by the use of β-glucanase-pentose from *Disporotrichum dimorphosporum*. In: Proceedings of the 21st Congress of the European Brewing Convention, Zürich, Switzerland, pp. 445-452.

Dunn, C. A., Bonnici, M. J., Logue, S. J., Long, N. R., Allan, G. R., and Stuart, I. M. (1996). An assesment of the physical and chemical properties of barley starch to predict malt quality. In: Proceedings of the 24th Convention of the Institute of Brewing (Asia Pacific Section), Singapore, pp. 120-128.

Edwards, A. C. (1999). Feed processing and feeding management to enhance nutrient utilization in commercial livestock production. *Rec Adv Animal Nutr Aust* **12**, 137-143.

Eijsink, V. G. H., Van der Zee, R., Van den Burg, B., Vriend, G., and Venema, G. (1991). Improving the thermostability of the neutral protease of *Bacillus stearothermophilus* by replacing a buried asparagine by leucine. *FEBS Lett* **282**, 13-16.

Eijinsk, V. G. H., Dijkstra, B. W., Vriend, G., Van der Zee, J. R., Veltman, O. R., Van der Vinne, B., Van den Burg, B., Kempe, S., and Venema, G. (1992). The effect of cavity-filling mutations on the thermostability of *Bacillus stearothermophilus* neutral protease. *Protein Eng* **5**, 421-426.

Eijinsk, V. G. H., Veltman, O. R., Aukema, W., Vriend, G., and Venema, G. (1995). Structural determinants of the stability of thermolysin-like proteinases. *Nature Struct Biol* **2**, 374-379.

Elliott, A. R., Campbell, J. A., Brettell, R. I. S., and Grof, C. P. L. (1998). *Agrobacterium*-mediated transformation of sugarcane using GFP as a screenable marker. *Aust J Plant Physiol* **25**, 739-743.

Ellis, R. J. (1991). Chaperone function: cracking the second half of the genetic code. *Plant J* **1**, 9-13.

Evans, D. E., Wallace, W., Lance, R. C. M., and MacLeod, L. C. (1997). Measurement of β-amylase in malting barley (*Hordeum vulgare* L.). II. The effect of germination and kilning. *J Cereal Sci* **26**, 241-250.

Fágáin, C. Ó. (1995). Understanding and increasing protein stability. *Biochim Biophys Acta* **1252**, 1-14.

Fersht, A. R. and Serrano, L. (1993). Principles of protein stability derived from protein engineering experiments. *Curr Opin Struct Biol* **3**, 75-83.

Fincher, G. (1976). Ferulic acid in barley cell walls: a fluorescence study. *J Inst Brew* **82**, 347-349.

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

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

Fincher, G. B. (1989). Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annu Rev Plant Physiol Plant Mol Biol* **40**, 305-346.

Fincher, G. B. and Stone, B. A. (1986). Cell walls and their components in cereal grain technology. In: Advances in Cereal Science and Technology (P. Pomeranz, ed.) American Association of Cereal Chemists, St Paul, Minnesota, USA, **8**, 207-295.

Fink, G. R. (1998). Anatomy of a revolution. *Genetics* **149**, 473-477.

Fresgård, P. -O., Bergenhem, N., Bengt-Harald, J., Svensson, M., and Carlsson, U. (1992). Isomerase and chaperone activity of prolyl isomerase in the folding of carbonic anhydrase. *Science* **258**, 466-468.

Frohman, M. A., Dush, M. K., and Martin, R. G. (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* **85**, 8998-9002.

Fromm, M. E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., and Klein, T. M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *BIO/TECH* **8**, 833-839.

Frommer, W. B. and Ninnemann, O. (1995). Heterologous expression of genes in bacterial, fungal, animal, and plant cells. *Annu Rev Plant Physiol Plant Mol Biol* **46**, 419-444.

Fry, S. (1995). Polysaccharide-modifying enzymes in the plant cell wall. *Annu Rev Plant Physiol Plant Mol Biol* **46**, 497-520.

Fulcher, R. G., O'Brien, T. P., and Simmonds, D. H. (1972). Localization of arginine-rich proteins in mature seeds of some members of the Graminae. *Aust J Biol Sci* **25**, 487-497.

Fulcher, R. G., Setterfield, G., McCully, M. E., and Wood, P. J. (1977). Observations on the aleurone layer. II. Fluorescence microscopy of the aleurone-sub-aleurone function with emphasis on possible (1→3)-β-glucan deposits in barley. *Aust J Plant Physiol* **4**, 917-928.

Funatuski, H., Kuroda, M., Lazzeri, P. A., Müller, E., Lörz, H., and Kishinami, I. (1995). Fertile transgenic barley generated by direct DNA transfer to protoplasts. *Theor Appl Genet* **91**, 707-712.

Gatenby, A. A., Boccara, M., Baulcombe, D. C., and Rothstein, S. J. (1986). Expression of a wheat α-amylase gene in *Escherichia coli*: Recognition of the translational initiation site and the signal peptide. *Gene* **45**, 11-18.

Gibbons, G. (1981). The relative role of the scutellum and aleurone in production of hydrolases during germination of barley (*Hordeum vulgare*). *Carlsberg Res Comm* **46**, 215-225.

Gibbons, G. C., Gibbons, G. C., and Gibbons, G. C. (1979). Localization and transport of α-amylase during germination and early seedling growth of *Hordeum vulgare* cultivar Nordal. *Carlsberg Res Comm* **44**, 353-366.

Gibeaut, D. M. and Carpita, N. C. (1994). Biosynthesis of plant cell wall polysaccharides. *FASEB J* **8**, 904-915.

Gold, L. (1990). Expression of heterologous proteins in *E. coli*. *Methods Enzymol* **185**, 11-14.

Goldenberg, D. P. (1989). Circularly permuted proteins. *Protein Eng* **2**, 493-495.

Goodsell, D. S., and Olson, A. J. (1990). Automated docking of substrates to proteins by simulated annealing. *Proteins: Struct Funct Genet* **8**, 195-202.

Gordon-Kamm, W. J., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., Adams, W. R., Willets, N. G., Rice, T. B., Mackey,

C. J., Krueger, R. W., Kausch, A. P., and Lemaux, R. G. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* **2**, 603-618.

Gould, J., Devey, M., Hasegawa, O., Ulian, E. C., Peterson, G., and Smith, R. H. (1990). Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. *Plant Physiol* **95**, 426-434.

Graham, H., Fadel, J. G., Newman, C. W., and Newman, R. K. (1989). Effect of pelleting and β-glucanase supplementation on the ileal and fecal digestibility of a barley-based diet in the pig. *J Animal Sci* **67**, 1293-1298.

Grodberg, J., and Dunn, J. J. (1988). OmpT encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J Bacteriol* **170**, 1245-1253.

Grütter, M. G., Hawkes, R. B., and Matthews, B. W. (1979). Molecular basis of thermostability in the lysozyme from bacteriophage T4. *Nature* **177**, 667-669.

Guan, H. P., Baba, T., and Preiss, J. (1994). Expression of branching enzyme I of maize endosperm in *Escherichia coli. Plant Physiol* **104**, 1449-1453.

Gubler, F., Ashford, A., Bacic, A., Blakeney, A., and Stone, B. (1985). Release of ferulic acid esters from barley aleurone. II. Characterization of feruloyl compounds released in response to GA_3. *Aust J Plant Physiol* **12**, 307-317.

Gubler, F., and Jacobsen, J. V. (1992). Gibberellin-responsive elements in the promoter of a barley high-pI α-amylase gene. *Plant Cell* **4**, 1435-1441.

Haase-Pettingell, C. A., and King, J. (1988). Formation of aggregates from a thermolabile *in vivo* folding intermediate in P22 tailspike maturation. A model for inclusion body formation. *J Biol Chem* **263**, 4977-4983.

Hagio, T., Hirabayashi, T., Machii, H., and Tomotsune, H. (1995). Production of fertile transgenic barley (*Hordeum vulgare* L.) plant using the hygromycin-resistance marker. *Plant Cell Rep* **14**, 329-334.

Hahn, M., Olsen, O., Politz, O., Borriss, R., and Heinemann, U. (1995). Crystal structure and site-directed mutagenesis of *Bacillus macerans* endo-(1→3,1→4)-β-glucanase. *J Biol Chem* **270**, 3081-3088.

Hammerton, R. W., and Ho, T. H. D. (1986). Hormonal regulation of the development of protease and carboxypeptidase activities in barley (*Hordeum vulgare* cultivar Himalaya) aleurone layers. *Plant Physiol* **80**, 692-697.

Han, F., Ullrich, S., Chirat, S., Menteur, S., Jestin, L., Sarrafi, A., Hayes, P., Jones, B., Blake, T., Wesenberg, D., Kleinhofs, A., and Kilian, A. (1995). Mapping of β-glucan content and β-glucanase activity loci in barley grain and malt. *Theor Appl Genet* **91**, 921-927.

Hancock, J. D., Wondra, K. J., Traylor, S. L., and Mavromichalis, I. (1997). Grinding, mixing, pelleting and formulation strategies to improve growth performance and decrease cost of gain in pigs. In Proceedings 58th Minnesota Nutrition Conference, pp. 185-204.

Hänsch, R., Korprek, T., Heydemann, H., Mendel, R. R., and Schulze, J. (1996). Electroporation-mediated transient gene expression in isolated scutella of *Hordeum vulgare*. *Physiol Plant* **98**, 20-27.

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

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

Hardy, F., Vriend, G., Veltman, O. R., Van der Vinne, B., Venema, G., and Eijsink, V. G. H. (1993). Stabilization of *Bacillus stearothermophilus* neutral protease by introduction of prolines. *FEBS Lett* **317**, 89-92.

Harvey, A. J., Hrmova, M., and Fincher, G. B. (1999). Regulation of genes encoding β-D-glucan exohydrolases in barley (*Hordeum vulgare* L.). *submitted.*

Haseloff, J., Siemering, K. R., Prahser, D. C., and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc Natl Acad Sci USA* **94**, 2122-2127.

Heim, U., Manteuffel, R., Bäumlein, H., Steinbiss, H.-H., and Wobus, U. (1995). Transient expression of a lysine-rich vicilin gene of *Vicia faba* in barley endosperm detected by immunological tissue printing after particle bombardment. *Plant Cell Rep* **15**, 125-128.

Hennig, J., Dewey, R. E., Cutt, J. R., and Klessig, D. F. (1993). Pathogen, salicyclic acid and developmental dependent expression of a β-(1→3)-glucanase/GUS gene fusion in transgenic tobacco plants. *Plant J* **4**, 481-493.

Henrissat, B., Callebaut, I., Fabrega, S., Lehn, P., Mornon, J. P., and Davies, G. (1995). Conserved catalytic machineray and the prediction of a common fold for several families of glycosyl hydrolases. *Proc Natl Acad Sci USA* **92**, 7090-7094.

Henry, R. J. (1986). Genetic and environmental variation in the pentosan and β-glucan contents of barley, and their relation to malting quality. *J Cereal Sci* **4**, 269-277.

Henry, R. J. (1988). Changes in β-glucan and other carbohydrate components of barley during malting. *J Sci Food Agric* **42**, 333-341.

Hesselman, K., and Åman, P. (1986). The effect of β-glucanase on the utilization of starch and nitrogen by broiler chickens fed on barley of high or low viscosity. *Animal Feed Sci Tech* **15**, 83-93.

Hesselman, K. and Thomke, S. (1982). Influence of some factors on development of viscosity in the water-extract of barley. *Swedish J Agric Res* **12**, 17-22.

Hesselman, K., Elwinger, R. K., and Thomke, S. (1982). Influence of increasing levels of β-glucanase on the productive value of barley diets for broiler chickens. *Animal Feed Sci Tech* **7**, 351-358.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* **6**, 271-282.

Higgins, C. and Payne, J. (1981). The peptide pools of germinating barley grains: relation to hydrolysis and transport of storage proteins. *Plant Physiol* **67**, 785-792.

Hoban, T. J. (1997). Consumer acceptance of biotechnology - an international perspective. *Nature Biotech* **15**, 232-234.

Hockney, R. C. (1994). Recent developments in heterologous protein production in *Escherichia coli*. *TIBTECH* **12**, 456-463.

Høj, P. B. and Fincher, G. B. (1995). Molecular evolution of plant β-glucan endohydrolases. *Plant J* **7**, 367-379.

Høj, P. B., Hartman, D. J., Morrice, N. A., Doan, D. N. P., and Fincher, G. B. (1989). Purification of (1→3)-β-glucan endohydrolase isoenzyme II from germinated barley and determination of its primary structure from a cDNA clone. *Plant Mol Biol* **13**, 31-42.

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

Hottenrott, S., Schumann, T., Pluckthun, A., G., F., and Rahfeld, J. U. (1997). The *Escherichia coli* slyd is a metal ion-regulated peptidyl-prolyl cis/trans-isomerase. *J Biol Chem* **272**, 15697-15701.

Hrmova, M. and Fincher, G. B. (1993). Purification and properties of three (1→3)-β-glucanase isoenzymes from young leaves of barley (*Hordeum vulgare*). *Biochem J* **289**, 453-461.

Hrmova, M., and Fincher, G. B. (1998). Barley β-D-glucan exohydrolases; substrate specificity and kinetic properties. *Carbohyd Res* **305**, 209-221.

Hrmova, M., Garrett, T. P. J., Fincher, G. B. (1995). Subsite affinities and disposition of catalytic amino acids in the substrate-binding region of barley (1→3)-β-glucanases; implications in plant-pathogen interactions. *J Biol Chem* **270**, 14556-14563.

Hrmova, M., Harvey, A. J., Wang, J., Shirley, N. J., Jones, G. P., Stone, B. A., Høj, P. B., and Fincher, G. B. (1996). Barley β-D-glucan exohydrolases with β-D-glucosidase activity; Purification, characterization, and determination of primary structure from a cDNA clone. *J Biol Chem* **271**, 5277-5286.

Hrmova, M., MacGregor, E. A., Biely, P., Stewart, R. J., and Fincher, G. B. (1998a). Substrate binding and catalytic mechanism of a barley β-D-glucosidase/(1→4)-β-D-glucan exohydrolase. *J Biol Chem* **273**, 11134-11143.

Hrmova, M., Fincher, G. B., Viladot, J. L., Planas, A., and Driguez, H. (1998b). Chemo-enzymatic synthesis of (1→3,1→4)-β-D-glucooligosaccharides for subsite mapping of (1→3,1→4)-β-D-glucan endohydrolases. *J Chem Soc Perkin Trans 1* **1998**, 3571-3576.

Hughes, P. S. and Simpson, W. J. (1993). Production and composition of hop products. *MBAA Tech Quarterly* **30**, 146-154.

Inouye, M., Arnheim, N., and Sternglanz, R. (1973). Bacteriophage T7 lysozyme is an N-acetylmuramyl-L-alanine amidase. *J Biol Chem* **248**, 7247-7252.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T., and Kumashiro, T. (1996). High efficiency transformation of maize (*Zea mays* L) mediated by *Agrobacterium tumefaciens. Nature Biotech* **14**, 745-750.

Ishikawa, K., Okumura, M., Katayanagi, K., Kimura, S., Kanaya, S., Nakamura, H., and Morikawa, K. (1993). Crystal structure of ribonuclease H form *Thermus thermophilus* HB8 refined at 2·8 Å resolution. *J Mol Biol* **230**, 529-542.

Jackson, E. A., Ballance, B. M., and Thomsen, K. K. (1986). Construction of a yeast vector directing the synthesis and release of barley (1→3,1→4)-β-glucanase. *Carlsberg Res Comm* **51**, 445-458.

Jacobsen, J. and Chandler, P. (1987). Gibberellin and abscisic acid in germinating cereals. In: Plant Hormones and Their Role in Plant Growth and Development (P. Davies, ed.) Martinus Nijhoff, Dordrecht, pp. 164-193.

Jähne, A., Becker, D., Brettschneider, R., and Lörz, H. (1994). Regeneration of transgenic, microspore-derived, fertile barley. *Theor Appl Genet* **89**, 525-533.

Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R. A., Nordheim, A., and Stunnenberg, H. G. (1991). Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proc Natl Acad Sci USA* **88**, 8972-8976.

Jarvis, D. L., Kawar, Z. S., and Hollister, J. R. (1998). Engineering *N*-glycosylation pathways in the baculovirus-insect cell system. *Curr Opin Biotech* **9**, 528-533.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol Biol Rep* **5**, 387-405.

Jenkins, J., Leggio, L. L., Harris, G., and Pickersgill, R. (1995). β-Glucosidase, β-galactosidase, family A cellulases, family F xylanases and two barley glycanases form a superfamily of enzymes with 8-fold β/α architecture and with two conserved glutamates near the carboxy-terminal ends of β-strands four and seven. *FEBS Lett* **362**, 281-285.

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

Jensen, L. G., Politz, O., Olsen, O., Thomsen, K. K., and von Wettstein, D. (1998). Inheritance of a codon-optimized transgene expressing heat stable (1→3,1→4)-β-glucanase in scutellum and aleurone of germinating barley. *Hereditas* **129**, 215-225.

Jiang, W., Cho, M. J., and Lemaux, P. G. (1998). Improved callus quality and prolonged regenerability in model and recalcitrant barley (*Hordeum vulgare* L.) genotypes. *Plant Biotech* **15**, 63-69.

Jones, T., Zou, J., Cowan, S., and Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Cryst* **A47**, 110-119.

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

Kaji, A. (1984). L-Arabinosidases. *Adv Carbohydr Chem Biochem* **42**, 383-394.

Karpusas, M., Baase, W. A., Matsumura, M., and Matthews, B. W. (1989). Hydrophobic packing in T4 lysozyme probed by cavity-filling mutants. *Proc Natl Acad Sci USA* **86**, 8237-8241.

Kartha, K. K., Chibbar, R. N., Georges, F., Leung, N., Caswell, K., Kendall, E., and Qureshi, J. (1989). Transient expression of chloramphenicol actyltransferase (CAT) gene inbarley cell cultures and immature embryos through microprojectile bombardment. *Plant Cell Rep* **8**, 429-432.

Keitel, T., Simon, O., Borriss, R., and Heinemann, U. (1993)., Molecular and active-site structure of a *Bacillus* (1→3,1→4)-β-glucanase. *Proc Natl Acad Sci USA* **90**, 5287-5291.

Kelly, C. A., Nishiyama, M., Ohnishi, Y., Beppu, T., and Birktoft, J. J. (1993). Determinants of protein thermostability observed in the 1.9 A crystal structure of malate dehydrogenase from the thermophilic bacterium *Thermus flavus. Biochemistry* **32**, 3913-3922.

Kenn, D. A., Dagg, A. H. S., and Stuart, I. M. (1993). Effect of environment and genotype on the fermentability of malt produced from four Australian barley varieties. *J Amer Soc Brew Chem* **51**, 119-122.

Klein, T. M., Wolf, E. D., Wu, R., and Sandford, J. C. (1987). High velocity microprojectiles for delivery of nucleic acids into living cells. *Nature* **327**, 70-73.

Knudsen, S. and Müller, M. (1991). Transformation of the developing barley endosperm by particle bombardment. *Planta* **185**, 330-336.

Korth, K. L., and Levings III, C. S. (1993). Baculovirus expression of the maize mitochondrial protein URF13 confers insecticidal activity in cell cultures and larvae. *Proc Natl Acad Sci USA* **90**, 3388-3392.

Kotake, T., Nakagawa, N., Takeda, K., and Sakurai, N. (1997). Purification and characterization of wall-bound exo-(1→3)-β-D-glucanase from barley (*Hordeum vulgare* L) seedlings. *Plant Cell Physiol* **38**, 194-200.

Kumria, R., Verma, R., and Rajam, M. V. (1998). Potential applications of antisense RNA technology in plants. *Curr Sci* **74**, 35-41.

Kunze, W. (1996). Technology Brewing and Malting. VLB Berlin, Verlagsabteilung, Berlin, Germany.

Labrador, E. and Nevins, D. (1989). Selected cell wall proteins from *Zea mays*: Assessment of their role in wall hydrolysis. *Physiol Plant* **77**, 487-492.

Lance R. C. M., Chevalier, P., Bogyo, T.P., and Nilan, R.A. Genetic studies of the β-glucan content of barley. In: Proceedings of the 5th International Barley Genetics Symposium, Okoyama, Japan. pp. 216

Laurière, C., Laurière, M., and Daussant, J. (1986). Immunohistochemical localization of β-amylase in resting barley seeds. *Physiol Plant* **67**, 383-388.

Lazzeri, P. A., Brettschneider, R., Lührs, R., and Lörz, H. (1991). Stable transformation of barley *via* PEG-induced direct DNA uptake into protoplasts. *Theor Appl Genet* **81**, 437-444.

Leah, R., Kigel, J., Svendsen, I., and Mundy, J. (1995). Biochemical and molecular characterization of a barley seed β-glucosidase. *J Biol Chem* **270**, 15789-15797.

Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. (1991). Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J Biol Chem* **266**, 1564-1573.

Lee, B. T., Murdock, K., Topping, J., Kreis, M., and Jones, M. G. K. (1989). Transient gene expression in aleurone protoplasts isolated from developing caryopses of barley wheat. *Plant Mol Biol* **13**, 21-29.

Lee, E. Y., Marshall, J. J., and Whelan, W. J. (1971). The substrate specificity of amylopectin-debranching enzymes from sweet corn. *Arch Biochem Biophys* **143**, 365-374.

Leffel, S. M., Mabon, S. A., and Stewart, C. N. (1997). Applications of green fluorescent protein in plants. *Biotechniques* **23**, 912ff.

Lemaux, P. G., Cho, M.-J., Zhang, S., and Bregitzer, P. (1999). Transgenic cereals: *Hordeum vulgare* (barley). In: Molecular Improvement of Cereal Crops (I. K. Vasil, ed.). Kluwer, Dordrecht, pp. 255-316.

Lewis, M. J. and Young, T. W. (1995). Brewing. Chapman and Hall, London, UK.

Li, C. D., Langridge, P., Lance, R. C. M., Xu, P., and Fincher, G. B. (1996). Seven Members of the (1→3)-β-glucanase gene family in barley (*Hordeum Vulgare*) are clustered on the long arm of chromosome 3 (3HL). *Theor Appl Genet* **92**, 791-796.

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

Loi, L., Barton, P. A., and Fincher, G. B. (1987). Survival of barley β-glucanase isoenzyme during kilning and mashing. *J Cereal Sci* **5**, 45-50.

Lührs, R. and Lörz, H. (1987). Plant regeneration *in vitro* from embryogenic cultures of spring- and winter-type barley (*Hordeum vulgare* L.) varieties. *Theor Appl Genet* **75**, 16-25.

Ma, J. K. C. and Hein, M. B. (1995). Immunotherapeutic potential of antibodies produced in plants. *TIBTECH* **13**, 522-527.

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

MacGregor, A. W. and Fincher, G. B. (1993). Carbohydrates of the barley grain. In: Barley; Chemistry and Technology (A. W. MacGregor and R. S. Bhatty eds.) American Association of Cereal Chemists, pp. 73-130.

MacGregor, A. W., Macri, L. J., Schroeder, S. W., and Bazin, S. L. (1994). Limit dextrinase from malted barley: extraction, purification and characterization. *Cereal Chem* **71**, 610-617.

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

MacLeod, A. and Palmer, G. (1967). Gibberellin from barley embryos. *Nature* **216**, 1342-1343.

Maeda, I., Kiribushi, S., and Nakamura, M. (1978). Digestion of barley starch granules by the combined action of α- and β-amylases purified from barley and barley malt. *Agric Biol Chem* **42**, 259-267.

Malehorn, D. E., Scott, K. J., and Shah, D. M. (1993). Structure and expression of a barley acidic β-glucanase gene. *Plant Mol Biol* **22**, 347-360.

Mannonen, L., Kurten, U., Ritala, A., Salmenkallio-Marttila, M., Hannus, R., Aspegren, K., Teeri, T., and Kauppinen, V. (1993). Biotechnology for improvement of malting barley. In: Proceedings of the 23rd EBC Congress, Lisbon. IRL Press, New York, USA, pp. 85-93.

Mansfeld, J., Vriend, G., Dijkstra, B. W., Veltman, O. R., Vandenburg, B., Venema, G., Ulbrichhofmann, R., and Eijsinsk, V. G. H. (1997). Extreme stabilization of a thermolysin-like protease by an engineered disulfide bond. *J Biol Chem* **272**, 11152-11156.

Marqusee, S., and Baldwin, R. L. (1987). Helix stability by Glu^-·····Lys^+ salt bridges in short peptides of *de novo* design. *Proc Natl Acad Sci USA* **84**, 8898-8902.

Matsumura, M. and Aiba, S. (1985). Screening for the thermostable mutant of kanamycin nucleotidyltransferase by the use of a transformation system for a thermophile, *Bacillus stearothermophilus*. *J Biol Chem* **260**, 15298-15303.

Matsumara, M., Becktel, W. J., and Matthews, B. W. (1988). Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. *Nature* **334**, 406-410.

Matthews, B. (1996). Structural and genetic analysis of the folding and function of T4 lysozyme. *FASEB J* **10**, 35-41.

Matthews, B. H., Nicholson, H., and Becktel, W. J. (1987). Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc Natl Acad Sci USA* **84**, 6663-6667.

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

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and (1→3)-β-glucanase. *Plant Physiol* **88**, 936-942.

McCleary, B. V. and Shameer, I. (1987). Assay of malt β-glucanase using Azo-barley glucan: An improved precipitant. *J Inst Brew* **93**, 87-90.

McElroy, D., Zhang, W., Cao, J., and Wu, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* **2**, 163-171.

McFadden, G., Ahluwalia, B., Clarke, A., and Fincher, G. (1988). Expression sites and developmental regulation of genes encoding (1→3,1→4)-β-glucanase in germinated barley. *Planta* **173**, 500-508.

Meldgaard, M. and Svendsen, I. (1994). Different effects of *N*-glycosylation on the thermostability of highly homologous bacterial (1→3,1→4)-β-glucanases secreted from yeast. *Microbiology* **140**, 159-166.

Menendez-Arias, L. and Argos, P. (1989). Engineering protein thermal stability sequence statistics points to residue substitutions in α-helices. *J Mol Biol* **206**, 397-406.

Mikola, J. (1983). Germinating barley grains contain five acid carboxypeptidases with complimentary substrate specificities. *Biochim Biophys Acta* **747**, 241-252.

Mikola, J. (1987). Proteinases and peptidases in germinating cereal grains. In: 4th International Symposium on Pre-Harvest Sprouting in Cereals (D. Mares, ed.) Westview, pp. 295-302.

Mikola, J. and Kolehmainen, L. (1972). Localization and activity of various peptidases in germinating barley. *Planta* **104**, 167-177.

Mikolo, L. and Mikolo, J. (1980). Mobilization of proline in the starchy endosperm of germinating barley grain. *Planta* **149**, 149-154.

Morgan, A. G., Gill, A. A., and Smith, D. B. (1983). Some barley grain and green malt properties and their influence on malt hot-water extract. II. Protein, proteinase and moisture. *J Inst Brew* **89**, 283-291.

Morrison, I. N., Kuo, J., and O'Brien, T. P. (1975). Histochemistry and fine structure of developing wheat aleurone cells. *Planta* **123**, 105-116.

Mrabet, N. T., Van den Broeck, A., Van den Brande, I., Stanssens, P., Laroche, Y., Lambeir, A.-M., Matthijssens, G., Jenkins, J., Chiadmi, M., Van Tilbeurgh, H., Rey, F.,

Janin, J., Quax, W. J., Lasters, I., de Maeyer, M., and Wodak, S. J. (1992). Arginine residues as stabilizing elements in proteins. *Biochemistry* **31**, 2239-2253.

Mundy, J., and Fincher, G. B. (1986). Effects of gibberellic acid and abscisic acid on levels of translatable mRNA for (1→3,1→4)-β-glucanase in barley aleurone. *FEBS Lett* **198**, 349-352.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**, 473-497.

Nelson, N. (1944). A photometric adaptation of the somogyi method for the determination of glucose. *J Biol Chem* **153**, 375-380.

Newman, R. K. and Newman, C. W. (1987). β-Glucanase effect on the performance of broiler chicks fed covered and hulless barley isotypes having normal and waxy starch. *Nutr Rep Int* **36**, 693-699.

Nieuwdorp, P. (1963). Electron microscopic structure of the epithelial cells of the scutellum of barley. I. The structure of the epithelial cells before germination. *Acta Bot Neerl* **12**, 295-301.

Nosoh, Y. and Sekiguchi, T. (1990). Protein engineering for thermostability. *TIBTECH* **8**, 16-20.

Okada, T., Yoshogi, N., Sahara, H., and Koshino, S. (1995). Increase in thermostability of recombinant barley β-amylase by random mutagenesis. *Biosci Biotech Biochem* **59**, 1152-1153.

Olsen, D. B. and Eckstein, F. (1990). High efficiency oligonucleotide-directed plasmid mutagenesis. *Proc Natl Acad Sci USA* **87**, 1451-1455.

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

Olsen, O. and Thomsen, K. K. (1991). Improvement of bacterial β-glucanase thermostability by glycosylation. *J Gen Microbiol* **137**, 579-585.

Ortiz, J. P. A., Reggiardo, M. I., Ravizzini, R. A., Altabe, S. G., Cervigni, G. D. L., Spitteler, M. A., Morata, M. M., Elias, F. E., and Vallejos, R. H. (1996). Hygromycin resistance as an efficient selectable marker for wheat stable transformation. *Plant Cell Rep* **15**, 877-881.

Overbeeke, N. (1989). Synthesis and processing of thaumatin in yeast. In: Yeast Biotechnology (P. J. Barr, A. J. Brake and P. Valenzuela, eds.) Butterworths, Stoneham, MA, USA.

Ow, D. W., Wood, K. V., and DeLuca, M. (1986). Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **234**, 856-859.

Pace, C. N., Shirley, B. A., Mcnutt, M., and Gajiwala, K. (1996). Forces contributing to the conformational stability of proteins. *FASEB J* **10**, 75-83.

Pakula, A. A. and Sauer, R. T. (1989). Genetic analysis of protein stability and function. *Annu Rev Genet* **23**, 289-310.

Paleg, L. (1960). Physiological effect of gibberellic acid. II. On starch hydrolyzing enzymes of barley endosperm. *Plant Physiol* **35**, 902-906.

Palmer, G. H., and Bathgate, G. N. (1976). Malting and brewing. In: Advances in Cereal Science and Technology (P. Pomeranz, ed.) American Association of Cereal Chemists, St Paul, Minnesota, USA, pp. 237-324.

Pang, S. Z., Deboer, D. L., Wan, Y., Ye, G. B., Layton, J. G., Neher, M. K., Armstrong, C. L., Fry, J. E., Hinchee, M. A. W., and Fromm, M. E. (1996). An improved green fluorescent protein gene as a vital marker in plants. *Plant Physiol* **112**, 893-900.

Pantoliano, M. W., Ladner, R. C., Bryan, P. N., Rollence, M. L., Wood, J. F., and Poulos, T. L. (1987). Protein engineering of subtilisin BPN': Enhanced stabilization through the introduction of two cysteines to form a disulfide bond. *Biochemistry* **26**, 2077-2082.

Park, S. H., Pinson, S. R. M., and Smith, R. H. (1996). T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. *Plant Mol Biol* **32**, 1135-1148.

Parrish, F. W., Perlin, A. S., and Reese, R. T. (1960) Selective enzymolysis of poly-β-D-glucans, and the structure of the polymers. *Can J Chem* **38**, 2094-2104

Patterson, R. (1995). Perlite and Diatomite - sources and uses. *The Brewer* (Feb.), 52-55.

Perutz, M. F. (1978). Electrostatic effects in proteins. *Science* **201**, 1187-1191.

Phillipson, B. (1993). Expression of a hybrid (1→3,1→4)-β-glucanase in barley protoplasts. *Plant Sci* **91**, 195-206.

Piatak, M., Lane, J. A., Laird, W., Bjorn, M. J., Wang, A., and Williams, M. (1988). Expression of soluble and fully functional ricin A chain in *Escherichia coli* is temperature-sensitive. *J Biol Chem* **263**, 4837-4843.

Pitz, W. J. (1990). An analysis of malting research. *J Amer Soc Brew Chem* **48**, 33-44.

Plavnik, I. and Sklan, D. (1995). Nutritional effects of expansion and short time extrusion on feeds for broilers. *Animal Feed Sci Tech* **55**, 247-251.

Politz, O., Simon, O., Olsen, O., and Borriss, R. (1993). Determinants for the enhanced thermostability of hybrid (1→3,1→4)-β-glucanases. *Eur J Biochem* **216**, 829-834.

Ponting, C. P. and Russell, R. B. (1995). Swaposins: circular permutations within genes encoding saposin homologues. *TIBS* **20**, 179-190.

Possee, R. D. (1997). Baculoviruses as expression vectors. *Curr Opin Biotech* **8**, 569-572.

Preece, I. and MacDougall, M. (1958). Enzymic degradation of cereal hemicelluloses. II. Patterns of pentosan degradation. *J Inst Brew* **64**, 489-500.

Priestle, J. P., Grutter, M. G., White, J. L., Vincent, M. G., Kania, M., Wilson, E., Jardetzky, T. S., Kirschner, K., and Jansonius, J. (1987). Three-dimensional structure of the bifunctional enzyme N-(5'-phosphoribosyl) anthranilate isomerase-indole-3-glycerol-phosphate synthase from *Escherichia coli*. *Proc Natl Acad Sci USA* **84**, 5690-5694

Querol, E., Perez-Pons, J. A., and Mozo-Villarias, A. (1996). Analysis of protein conformational characteristics related to thermostability. *Protein Eng* **9**, 265-271.

Radley, M. (1967). Site of production of gibberellin-like substances in germinating barley embryos. *Planta* **75**, 164-171.

Rashin, A. A., Iofin, M., and Honig, B. (1986). Internal cavities and buried waters in globular proteins. *Biochemistry* **25**, 3619-3625.

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

Resenberg, A. H., Lade, B. N., Chui, D.-S., Lin, S.-W., Dunn, J. J., and Studier, F. W. (1987). Vectors for selective expression of clones DNAs by T7 RNA polymerase. *Gene* **56**, 125-135.

Rhodes, C. A., Pierce, D. A., Mettler, I. J., Mascarenhas, D., and Detmer, J. J. (1988). Genetically transformed maize plants from protoplasts. *Science* **240**, 204-206.

Rhodes, G. (1993). Crystallography made crystal clear: a guide for users of macromolecular models. Academic Press, London, UK.

Ritala, A., Aspegren, K., Kurtén, U., Salmenkallio-Marttila, M., Mannonen, L., Hannus, R., Kauppinen, V., Teeri, R. H., and Enari, T. (1994). Fertile transgenic barley by particle bombardment of immature embryos. *Plant Mol Biol* **24**, 317-325.

Romanos, M. A., Scorer, C. A., and Clare, J. J. (1992). Foreign gene expression in yeast: A review. *Yeast* **8**, 423-488.

Rose, G. D. and Wolfenden, R. (1993). Hydrogen bonding, hydrophobicity, packing and protein folding. *Annu Rev Biophys Biomol Struct* **22**, 381-415.

Rothstein, S. G., Lazarus, C. M., Smith, W. E., Baulcombe, D. C., and Gatenby, A. A. (1984). Secretion of a wheat α-amylase expressed in yeast. *Nature* **308**, 662-665.

Roulin, S., Xu, P., Brown, A. H. D., and Fincher, G. B. (1997). Expression of specific (1→3)-β-glucanase genes in leaves of near-isogenic resistant and susceptible barley lines infected with the leaf scald fungus (*Rhynchosporium secalis*). *Physiol Mol Plant Pathol* **50**, 245-261.

Rudolph, R. and Lilie, H. (1996). *In vitro* folding of inclusion body proteins. *FASEB J* **10**, 49-56.

Rutten, S. J. (1997). Altering the substrate specificity of barley (1→3)-β-glucanase isoenzyme GII. Thesis (B.Sc. Honours), Department of Plant Science, University of Adelaide, Adelaide, South Australia.

Salisbury, F. B. and Ross C. W. (1985). Plant Physiology. Wadsworth, Belmont, California, USA.

Salmenkallio-Marttila, M., Aspergen, K., Åkerman, S., Kurtén, U., Mannonen, L., Ritala, A., Teeri, T. H., and Kauppinen, V. (1995). Transgenic barley (*Hordeum vulgare* L.) by electroporation of protoplasts. *Plant Cell Rep* **15**, 301-304.

Sambrook, J., Firtsch, E. F., and Maniatis, T. (1989). Molecular cloning: A laboratory manual, 2nd Edition. Cold Spring Harbour Press, New York, USA.

Sandberg, W. C. and Terwilliger, T. C. (1989). Influence of interior packing and hydrophobicity on the stability of a protein. *Science* **245**, 54-57.

Sanger, F., Nicklen, S. and Coulsen, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463-5467.

Sarkar, G. and Sommer, S. S. (1990). The "megaprimer" method of site-directed mutagenesis. *Biotechniques* **4**, 404-407.

Schein, C. H. (1989). Production of soluble recombinant proteins in bacteria. *BIO/TECH* **7**, 1141-1149.

Schein, C. H. and Noteborn, M. H. M. (1988). Production of soluble recombinant mammalian proteins in *Escherichia coli* is favored by lower growth temperature. *BIO/TECH* **6**, 291-294.

Schimming, S., Schwarz, W. H., and Staudenbauer, W. L. (1992). Structure of the *Clostridium thermocellum* gene licB and the encoded (1→3,1→4)-β-glucanase. A catalytic region homologous to *Bacillus lichenases* joined to the reiterated domain of clostridial cellulases. *Eur J Biochem* **204**, 13-19.

Serrano, L., Kellis Jr, J. T., Cann, P., Matouschek, A., and Fersht, A. R. (1992). The folding of an enzyme. II. Substructure of barnase and the contribution of different interactions to protein stability. *J Mol Biol* **224**, 783-804.

Shen-Miller, J., Kreis, M., Shewry, P. R., and Springall, D. R. (1991). Spatial distribution of β-amylase in germinating barley seeds based on the avidin-biotin-peroxidase complex method. *Protoplasma* **163**, 1620173.

Shimamoto, K., Terada, R., Izawa, T., and Fumimoto, H. (1989). Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* **338**, 274-276.

Shortle, D., Stites, W. E., and Meeker, A. K. (1990). Contributions of the large hydrophobic amino acids to the stability of staphylococcal nuclease. *Biochemistry* **29**, 8033-8041.

Simmons, C. R., Litts, J. C., Huang, N., and Rodriguez, R.L. (1992) Structure of a rice β-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elicitors. *Plant Mol Biol* **18**, 33-45

Simos, G., Panagiotidis, C. A., Skoumbas, A., Choli, D., Ouzounis, C., and Georgatsos, J. G. (1994). Barley β-glucosidase; Expression during seed germination and maturation and partial amino acid sequences. *Biochim Biophys Acta* **1199**, 52-58.

Skriver, K., Olsen, F. L., Rogers, J. C., and Mundy, J. (1991). *Cis*-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. *Proc Natl Acad Sci USA* **88**, 7266-7270.

Slade, A., Høj, P., Morrice, N., and Fincher, G. (1989). The purification and characterization of three (1→4)-β-xylan endohydrolases from germinated barley. *Eur J Biochem* **185**, 533-539.

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

Slakeski, N. and Fincher, G. (1992a). Developmental regulation of (1→3,1→4)-β-glucanase gene expression in barley. I. Tissue specific expression of individual isoenzymes. *Plant Physiol* **99**, 1226-1231.

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

Smart, M., and O'Brien, T. (1979a). Observation on the scutellum. I. Overall development during germination in four grasses. *Aust J Bot* **27**, 391-401.

Smart, M., and O'Brien, T. (1979b). Observations on the scutellum. II. Histochemistry and autofluorescence of the cell wall in mature grain and during germination of wheat, barley, oats and rye grass. *Aust J Bot* **27**, 403-411.

Søgaard, M. and Svensson, B. (1990). Expression of cDNAs encoding barley α-amylase 1 and 2 in yeast and characterization of the secreted proteins. *Gene* **94**, 173-179.

Somers, D. A., Rines, H. W., Hu, W. N., Kaeppler, H. F., and Bushnell, W. R. (1992). Fertile, transgenic oat plants. *BIO/TECH* **10**, 1589-1594.

Somogyi, M. (1952). Notes on sugar determination. *J Biol Chem* **195**, 19-23.

Sopanen, T. (1979). Development of peptide transport activity in barley scutellum during germination. *Plant Physiol* **64**, 570-574.

Staudte, R., Woodward, J., Fincher, G., and Stone, B. (1983). Water-soluble (1→3,1→4)-β-D-glucans in barley (*Hordeum vulgare*) endosperm. III. Distribution of cellotriosyl and cellotetraosyl residues. *Carbohyd Polymers* **3**, 299-312.

Stewart, D. C., Hawthorne, D., and Evans, D. E. (1998). Cold sterile filtration: A small scale filtration test and investigation of membrane plugging. *J Inst Brew* **104**, 321-326.

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M., and Fritig, B. (1993). Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie* **75**, 687-706.

Stone, B. A. and Clarke, A. E. (1993). The chemistry and biology of (1→3)–β-glucans. La Trobe University Press, Bundoora, Victoria, Australia.

Stryer, L. (1988). Biochemistry. WH Freeman and Company, New York, USA.

Stuart, I. M., Loi, L., and Fincher, G. B. (1986). Development of (1→3,1→4)-β-glucan endohydrolase isoenzymes in isolated scutella and aleurone layers of barley (*Hordeum vulgare*). *Plant Physiol* **80**, 310-314.

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

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**, 60-89.

Summers, M. D. and Smith, G. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin No. 1555.

Sundblom, N.-O. and Mikolo, J. (1972). On the nature of the proteinases secreted by the aleurone layer of barley grain. *Physiol Plant* **27**, 281-284.

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

Taiz, L. and Honigman, W. (1976). Production of cell wall hydrolyzing enzymes in barley aleurone layers in response to gibberellic acid. *Plant Physiol* **58**, 380-386.

Taiz, L. and Jones, R. (1970). Gibberellic acid, (1→3)-β-glucanase and the cell wall of barley aleurone layers. *Planta* **92**, 73-84.

Taiz, L. and Jones, R. (1973). Plasmodesmata and an associated cell wall component in barley aleurone tissue. *Plant Physiol* **60**, 182-189.

Thompson, C. J., Novva, N. R., Tizard, T., Crameri, R., Davies, J. E., Lauwereys, M., and Botterman, J. (1987). Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *EMBO J* **6**, 2519-2523.

Thomsen, K. K., Jackson, E. A., and Brenner, K. (1988). Genetic engineering of yeast: construction of strains that degrade β-glucans with the aid of a barley gene. *J Amer Soc Brew Chem* **46**, 31-36.

Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S., and Brettel, R. (1997). *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J* **6**, 1369-1376.

Tormakangas, K., Kervinen, J., Ostman, A., and Teeri, T. (1994). Tissue-specific localization of aspartic proteinase in developing and germinating barley grains. *Planta* **195**, 116-125.

Van den Burg, B., Dijkstra, B. W., Vandervinne, B., Stulp, B. K., Eijsink, V. G. H., and Venema, G. (1993). Introduction of disulfide bonds into *Bacillus subtilis* neutral proteinase. *Protein Eng* **6**, 521-527.

Van Loon, L. C. (1985). Pathogenesis-related proteins. *Plant Mol Biol* **4**, 111-116.

Vance, V. B., and Huang, A. H. C. (1988). Expression of lipid body protein gene during maize seed development. Spatial, temporal, and hormonal regulation. *J Biol Chem* **263**, 1476-1481.

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

Vasil, I. K. (1994). Molecular improvement of cereals. *Plant Mol Biol* **25**, 925-937.

Vasil, I. K. and Vasil, V. (1992). Advances in cereal protoplast research. *Physiol Plant* **85**, 279-283.

Vasil, V., Castillo, A. M., Fromm, M. E., and Vasil, I. K. (1992). Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *BIO/TECH* **10**, 667-674.

Vieille, C. and Zeikus, J. G. (1996). Thermozymes: Identifying molecular determinants of protein structural and functional stability. *TIBTECH* **14**, 183-190.

Viëtor, R. J., Angelino, S. A. G. F., and Voragen, A. G. J. (1991). Arabinoxylans in barley, malt and wort. In: Proceedings of the 23rd Congress of the European Brewing Convention, Lisbon, pp. 139-146.

Viëtor, R. J., Angelino, S. A. G. F., and Voragen, A. G. J. Structural features of arabinoxylans from barley and malt cell wall material. (1992). *J Cereal Sci* **15**, 213-222

Viëtor, R. J., Voragen, A. G. J., and Angelino, S. A. G. F. (1993). Composition of non-starch polysaccharides in wort and spent grain from brewing trials with malt from a good malting quality barley and a feed barley. *J Inst Brew* **99**, 243-248.

Vonarnim, A. G., Deng, X. W., and Stacey, M. G. (1998). Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. *Gene* **221**, 35-43.

Vriend, G. and Eijsink, V. (1993). Prediction and analysis of structure, stability and unfolding of *Bacillus* neutral proteases. *J Comp-Aided Mol Design* **7**, 1-28.

Wakarchuk, W. W., Sung, W. L., Campbell, R. L., Cunningham, A., Watson, D. C., and Yaguchi, M. (1994). Thermostabilization of the *Bacillus circulans* xylanase by the introduction of disulfide bonds. *Protein Eng* **7**, 1379-1386.

Wan, Y. and Lemaux, P. G. (1994). Generation of large numbers of independently transformed fertile barley plants. *Plant Physiol* **104**, 37-48.

Watanabe, K. (1996). Studies of protein thermostabilization by proline substitutions. *J Jap Soc Biosci Biotech Agrochem* **70**, 1095-1101.

Watanabe, K., Chishoro, K., Kitamura, K., and Suzuki, Y. (1991). Proline residues responsible for thermostability occur with high frequency in the loop regions of an extremely thermostable oligo-1,6-glucosidase from *Bacillus thermoglucosidasius* KP1006. *J Biol Chem* **266**, 24287-24294.

Watanabe, K., Masuda, T., Ohashi, H., Mihara, H., and Suzuki, Y. (1994). Multiple proline substitutions cumulatively thermostabilize *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase. Irrefragable proof supporting the proline rule. *Eur J Biochem* **226**, 277-283.

Watanabe, K. and Suzuki, Y. (1998). Protein thermostabilization by proline substitutions. *J Mol Catalysis B-Enymatic* **4**, 167-180.

Weeks, J. T., Anderson, O. D., and Blechl, A. E. (1993). Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). *Plant Physiol* **102**, 1077-1084.

Welfle, K., Misselwitz, R., Politz, O., Borriss, R., and Welfle, H. (1996). Individual amino acids in the N-terminal loop region determine the thermostability and unfolding characteristics of bacterial glucanases. *Protein Sci* **5**, 2255-2265.

White, W. B., Bird, H. R., Sunde, M. L., Prentice, N., Burger, W. C., and Marlett, J. A. (1981). The viscosity interaction of barley β-glucan with *Trichoderma viride* cellulase in the chick intestine. *Poultry Sci* **62**, 853-862.

Whitelam, G. C. and Cockburn, W. (1998). Production of recombinant antibodies in transgenic plants (K. Lindsey, ed.). Harwood Academic Publishers.

Wigley, D. B., Clarke, A. R., Dunn, C. R., Barstow, D. A., Atkinson, T., Chia, W. N., Muirhead, H., and Holbrook, J. J. (1987). The engineering of a more thermally stable lactate dehydrogenase by reduction of the area of a water-accessible hydrophobic surface. *Biochem Biophys Acta* **916**, 145-148.

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

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

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

Woodward, J. and Fincher, G. (1982b). Substrate specificities and kinetic properties of two (1→3,1→4)-β-glucan endohydrolases from germinating barley (*Hordeum vulgare*). *Carbohyd Res* **106**, 111-122.

Woodward, J. and Fincher, G. (1983). Water soluble barley β-glucans. Fine structure, solution behaviour and organization in the cell wall. *Brew Dig* **58**, 28-32.

Xu, P., Wang, J., and Fincher, G. B. (1992). Evolution and differential expression of the (1→3)-β-glucan endo-hydrolase-encoding gene family in barley, *Hordeum vulgare*. *Gene* **120**, 157-165.

Xu, P. L., Harvey, A. J., and Fincher, G. B. (1994). Heterologous expression of cDNAs encoding barley (*Hordeum vulgare*) (1→3)-β-glucanase isoenzyme GV. *FEBS Lett* **348**, 206-210.

Yomo, H. (1960). Studies on the amylase activating substance. IV. Amylase-activating activity of gibberellin. *Hakko Kyokaishi* **18**, 600-603.

Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M., and Schell, J. (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO Journal* **2**, 2143-2150.

Zhang, J., Tiwari, V. K., Golds, T. J., Blackhall, N. W., Cocking, E. C., Mulligan, B. J., Power, J. B., and Davey, M. R. (1995). Parameters influencing transient and stable transformation of barley (*Hordeum vulgare* L.) protoplasts. *Plant Cell Tiss Org Cult* **41**, 125-138.

Zhang, N. Y. and Jones, B. L. (1996). Purification and partial characterization of a 31-kDa cysteine endopeptidase from germinated barley. *Planta* **199**, 565-572.

Zhang, N. Y. and Jones, B. L. (1999). Polymorphism of aspartic proteinases in resting and germinating barley seeds. *Cereal Chem* **76**, 134-138.

Zhang, X.-J., Baase, W. A., and Matthews, B. W. (1991). Toward a simplification of the protein folding problem: a stabilizing polyalanine α-helix engineered in T4 lysozyme. *Biochemistry* **30**, 2012-2017.

Zhang, Y., Olsen, D. R., Nguyen, K. B., Olson, P. S., Rhodes, E. T., and Mascarenhas, D. (1998). Expression of eukaryotic proteins in soluble form in *Escherichi coli*. *Protein Exp Purif* **12**, 159-165.

Zhu, T., Peterson, D. J., Tagliani, L., St. Clair, G., Baszcaynski, C. L., and Bowen, B. (1999). Targeted manipulation of maize genes *in vivo* using chimeric RNA/DNA oligonucleotides. *Proc Natl Acad Sci USA* **96**, 8768-8773.