An investigation of a barley protein (SE/BTI-CMe) and

its influence on beer haze stability

by

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ii

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List of Abbreviations and Acronyms

α	alpha
β	beta
γ	gamma
%	percentage
2-D	two-dimensional
AAL	apparent attenuation limit
AFLP	amplified fragment length polymorphism
bp	base pair
BASI	barley α-amylase/subtilisin inhibitor
BBBI	barley Bowman-Birk type trypsin inhibitor
BSA	bovine serum albumin
BTI	barley trypsin inhibitor
BU	bitterness units
°C	degrees centigrade
CHAPS	(3[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid)
cM	centimorgan
cm	centimetre
CMe proteins	chloroform-methanol soluble proteins
cv.	cultivar
DE	diatomaceous earth
DH	double haploid
dNTP	2'deoxynucleoside 5'-triphosphate
DNA	deoxyribonucleic acid
DP	diastatic power
DTT	dithiothreitol
EBC	European Brewing Convention
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EtOH	ethanol
F.A.N.	Free Amino Nitrogen
FU	formazin units
g	gram
h	hour
HRP	horseradish peroxidase
HWE	hot water extract
IEF	isoelectric focusing
IPTG	isopropyl β-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
KI	Kolbach Index
kJ	kilo Joule
L	litre
LB	Luria bertani
LSD	least significant difference
LTP	lipid transfer protein
M	molar

mM	millimolar
MAS	marker assisted selection
mg	milligram
min	minute
mL	millilitre
mg/g	milligrams per gram
mg/L	milligrams per litre
mg/mL	milligrams per millilitre
mm	millimetre
mPa.s	millipascal second
MW	molecular weight
N	nitrogen
NCBI	National Centre for Biotechnology Information
ng	nanogram
Ni-NTA	Nitrilotriacetic acid
nm	nanometre
nmole	nanomole
NTU	nephelos turbidity units
O	oxygen
OD	optical Density
°P	degrees Plato
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
pmol	picomole
ppm	parts per million
PT	protein/tannin
PTH	phenylthiohydantoin
PU	pasteurisation units
PVPP	polyvinylpolypyrolidone
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
RNase A	ribonuclease
RP-HPLC	reversed phase-high pressure liquid chromatography
rpm	revolutions per minute
sarkosyl	N-lauroylsarcosine
SE	silica eluent
sec	second
SDS	sodium dodecyl sulphate
SG	specific gravity
SP	soluble protein
SNPs	single-nucleotide polymorphisms
SSB	small scale brewing
SSR	simple sequence repeats
TA	tannic acid
TAE	Tris-Acetate-EDTA
Taq	thermus aquaticus DNA Polymerase
TBS	tris buffered saline

TE	Tris/EDTA
TEMED	N,N,N',N'-Tetra-Methyl-Ethylenediamine
TFA	trifluoroacetic acid
TIA	trypsin inhibitor activity
TM	trade mark
TPBS	tween phosphate buffed saline
TP	total protein
Tris-HCl	tris(hydroxymethyl)amino methane hydrochloride
Tween 20	polyoxyethylenesorbitan monolaurate
μL	microlitre
μg	microgram
μg/mL	microgram per millilitre
μm	micrometre
U	units
UV	ultraviolet
V	volt
Vh	volt hour
v/v	volume to volume ratio
W	watt
WK	Windisch-Kolbach
w/v	weight to volume ratio
X	times

Publications arising from this thesis

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Robinson, L.H., Ford, C.F., Healy, P., Gibson, C.E., Barr, A.R., and Evans, D.E. (2004). The genetic basis of improved beer haze stability. *Proc. International Barley Genetics Symp., 9th, Brno*, pp. 493-498.

[a]Evans, D.E., Robinson, L.H., Sheehan, M.C., Tolhurst, R.L., Hill, A., Skerritt, J.S., and Barr, A.R. (2003). Application of immunological methods to differentiate between foam-positive and haze active proteins originating from malt. *J. Am. Soc. Brew. Chem.*, **61**(2):55-62. A copy of this paper is included at the back of this thesis.

[a]This publication does not constitute a major part of the work presented in this thesis.

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Table of Contents

Abstract

In bright beers, the formation of haze is a serious quality problem, which places limitations on the storage life of the product. To the consumer haze often represents a sign of ageing or contamination of the product. In this study, SDS-PAGE immunoblot analysis using an antiserum that was raised against a silica eluent (SE) protein fraction (obtained from silica gel, used for the colloidal stabilisation of beer), detected a range of protein bands in barley, malt, beer and haze. A polymorphism was observed in which some barley varieties contained a MW ~12000 band (SE +ve) while in other varieties this band was absent (SE -ve). A survey of 219 Australian and international barley varieties, including a comprehensive selection of current and past malting varieties, identified 181 varieties as SE +ve, and 38 varieties as SE -ve. The genetic basis for the presence or absence of the SE protein was determined by interval mapping analysis which found that the MW ~12000 band mapped to the short arm of chromosome 3H.

Pilot brewing trials (100 L and 300 L) found that beer brewed from SE –ve malt varieties formed less haze in haze force testing trials (5 days at 55°C, 1 day at 0°C) and in natural ageing testing trials, than beer produced from SE +ve malt varieties. The interaction between the presence or absence of the SE protein and controlled atmosphere brewing was investigated by brewing under nitrogen, oxygen or air. Controlled atmosphere pilot brewing trials (10 L) indicated that both oxygen and nitrogen rich atmospheres produced beers with poorer colloidal stability compared to brewing under a normal atmosphere. Filtration trials showed that the haze stability of beer could be influenced by the filtration process. Filtration trials showed that the material used to filter the beer (cellulose sheets impregnated with DE) was capable of

removing some haze protein from the beer, thus improving the haze stability of SE +ve beers. The removal of the SE protein and other proteins during filtration from beer brewed with a SE +ve malt variety, along with a reduction in the level of total protein as measured by Commassie blue dye binding, resulted in improved haze stability.

The SE protein was characterised using comparative two-dimensional (2-D) gel electrophoresis immunoblots of barley seed extracts from both SE +ve and SE -ve varieties. The SE protein spot identified was excised and its partial sequence determined, after in-gel cleavage using trypsin and separation of the resulting fragments by reversed-phase HPLC. N-terminal sequence analysis of the tryptic peptides from SE +ve and SE -ve varieties identified the SE protein as the barley trypsin inhibitor CMe precursor (BTI-CMe). The mature BTI-CMe protein is 13.3 kDa and the functional gene is located on chromosome 3H, consistent with the information presented on the SE protein. Cloning of the BTI-CMe protein demonstrated that both SE -ve and SE +ve barley varieties contain a BTI-CMe protein family member that is similar but consistently different, primarily in the last 30 amino acid residues of their C-termini. Specific primers were designed to amplify the full-length BTI-CMe protein as well as a truncated protein (C-terminal region) in both BTI-CMe1 (SE +ve) and BTI-CMe3.1 (SE –ve) variants and four constructs were made. BTI-CMe was expressed in *E.coli*, purified and polyclonal antibodies raised to the recombinant protein. The recombinant BTI-CMe proteins proved to be poorly immunogenic and thus this experiment was not conclusive.

These results of this study suggest that the selection of SE -ve malt varieties for brewing combined with optimised stabilisation and filtration treatments, has the potential to improve beer haze stability. This would reduce the need or requirement for traditional colloidal stabilisation treatments, reducing brewery costs and environmental wastes.

Chapter 1

Literature Review

1.1 Introduction

In bright beers, the formation of permanent haze is a serious quality problem which places limitations on the storage life of the product. The problem of haze stability has become increasingly important to brewers because of a number of developments in the brewing industry. Firstly, the increasing dominance of beer sold in glass bottles, particularly those that are not packaged in the traditional brown glass (i.e. green or clear), has increased the consumers' ability to identify haze problems in their beer. In addition, the appearance of haze is a visual clue to the reduced flavour stability of beer as the haze and flavour stability are both directly influenced by oxidative processes during storage (Bamforth, 1999a,b). This problem is exacerbated by the modern distribution system for beer, where packaged beer is first warehoused at the brewery and then stored at the retail outlet and finally stored at home before consumption. All three of these places have potential for sub-optimal storage (i.e. temperature >4°C) thus shortening the expected shelf life. The increasing market penetration of "premium" brands has increased haze stability problems of these beers, as there are longer time intervals between these brews due to lower turnover, they are potentially stored for longer periods before consumption, while consumers expect a superior product. Where beer is produced in, or exported to developing world beer markets such as in parts of Asia, these problems may be increased and compounded by inferior transportation systems that expose the beer to higher temperatures during transport, which promotes the formation of haze.

There are a number of factors that can influence the colloidal stability of beer (Buckee, 1985; Bamforth, 1999a; Wainwright, 1974 and the references therein). An important cause of colloidal instability in beer is the interaction of proteins (or their fragments) that contain high levels of proline with polyphenols (Bamforth, 1999a; Siebert, 1999). The protein-polyphenol hazes of beer have recently been extensively reviewed (Bamforth, 1999a; Siebert, 1999). The major source of both of these components is malt, although some polyphenols are also extracted from hops. Haze intensity in beer primarily depends on temperature, with haze formed at 0°C defined as chill haze, any haze remaining at 20°C or higher defined as permanent haze. Haze formation in beer can also be as a result of contamination, biological hazes or non-protein (i.e. oxalate), however, for the purposes of this thesis only the non-biological protein-polyphenol hazes will be discussed.

1.2 The brewing process and its impact on the potential haze stability of beer

There are a number of time points and components of the brewing process from the raw materials right through to packaging and storage of the finished beer that can influence its colloidal stability. This section aims to provide a brief overview of the brewing process and in particular, identify how and where in the process colloidal stability can be affected. Polyphenolic constituents from both the malt and hops can interact with proteins during wort boiling to form the hot break or trub, during wort cooling in the development of the cold break or trub, and during conditioning and subsequent storage in which chill (0°C) and permanent (20°C and higher) hazes can be formed.

1.2.1 Raw materials

A number of studies have investigated the effect of the raw material, (barley, malt and hops) on the overall colloidal stability of beer (see Moll, 1987 for a thorough review). In particular, the influence on haze formation of specific barley varieties, factors such as place of growth or season, and protein content and modification have been investigated. To date, the data is not conclusive in confirming that any of these factors play a significant role in affecting the colloidal stability. Apart from low-polyphenol barleys (Fukuda *et al.*, 1999; Jende-Strid, 1997; von Wettstein *et al.*, 1977 and 1980), which will be discussed later in further detail, and differences in hop preparations, no particular barley variety or growing conditions have been shown to have any substantial or indeed significant effect on overall beer colloidal stability.

1.2.1.1 Malting

The biochemical compounds present within the barley grain are not suitable substrates for yeast growth and metabolism. Without prior modification the sugar molecules are generally unavailable as they are in the form of carbohydrates, the amino acids as proteins and the nucleotides as nucleic acids. For effective growth and yeast metabolism these compounds must be partially degraded and this is achieved during the malting process. Hydrolysis of these biochemical compounds results primarily from the hydrolytic enzymes that form during germination (Briggs, 1992). In particular, protein degradation in barley begins with the insoluble storage proteins hydrolysed by the endopeptidases (proteases) that then generate 'soluble protein' and peptides, upon which the exopeptidases (carboxypeptidases) act to release amino acids (Bamforth and Barclay, 1993). By this mechanism, it has been estimated that around 50% of the total grain protein may be mobilised during the malting of barley (Barrett and Kirsop, 1971).

1.2.2 Brewing

After malting, the grain is milled to yield the grist. This process exposes the enzymes which can then freely interact with their substrates to produce a good extract, but also leaves the husks reasonably intact to allow good filtration during lautering. Mashing of the grain by mixing with a predetermined volume of water (35°C-65°C) (Lewis and Young, 2001), enables the diastase enzymes (α-amylase, β-amylase, α-glucosidase, and limit dextrinase) (Bamforth and Barclay, 1993), which hydrolyse the starch into fermentable sugars.

The duration, temperature, mash concentration and pH of the mash, along with dissolved oxygen throughout the mashing process can have an affect on final beer colloidal stability (Moll, 1987). The chloride and sulphate of calcium ($CaCl_2$ or $CaSO_4$), aid in reducing the pH of the mash and the wort (Moll, 1987). Calcium in the grist is also important to ensure precipitation of oxalate, which if allowed to remain in the beer, can form crystals resulting in the formation of haze (Burger *et al.*, 1956) and can also lead to problems with gushing (violent over-foaming) in the finished beer (Schur, 2001). After mashing and wort separation (lautering) the sugar-rich solution (wort) is transferred to the kettle where it is boiled. Boiling is important for sterilisation of the wort, removing undesirable volatiles (i.e. dimethyl sulfide), concentrating the wort, increasing its colour and also extracting the hops to provide bitterness (Lewis and Young, 2001). The kettle boil is one of the most significant factors in terms of colloidal stability. As the wort is boiled a hot break or trub develops in which materials including haze precursors in proteins and polyphenolics are precipitated. A good rolling boil is important in order for the readily precipitable materials to collect at localised surfaces,

i.e. on the top of the wort (Bamforth, 1999a). This precipitate, or trub, is removed by clarifying the wort, most often using a whirlpool tank.

1.2.3 Fermentation and Maturation

The hot wort is then passed through a heat exchanger to lower its temperature, after which it is oxygenated to create optimal conditions for the yeast during fermentation. The process of fermentation has not been shown to be associated with haze formation (Bamforth, 1999a). After fermentation, the beer is conditioned at 0°C to -1°C for 7 to 21 days depending on the beer style (Lewis and Young, 2001). With some beer styles however, maturation can be for up to 3 months. This can be dependent on the wort gravity however, as higher gravity beers are able to survive lower temperatures (Hough *et al.*, 1982). High-gravity fermentations involve worts of up to 18°P and above (Munroe, 1995). Following fermentation and maturation, the beer is diluted with water to achieve a final gravity or to a prescribed alcohol concentration. High gravity brewing results in beers that are more consistent (i.e. % alcohol, original gravity) and therefore are more physically stable since the compounds responsible for haze formation are more easily precipitated at these higher concentrations due to their inherent hydrophobicity and precipitability (Munroe, 1995). Cold conditioning not only allows for flavour modification but also allows the yeast to settle out and for a secondary cold break or trub (containing haze active material) to form. As with the roiling boil which produces the hot break or trub, cold conditioning of the beer is another important step in the removal of haze active material.

1.2.4 Filtration and Packaging

Before packaging, cold filtration of the beer, usually through diatomaceous earth (DE) sometimes followed by micro-filters removes the solids (yeast and cold break material) as well as the colloids (particles responsible for haze formation). There are a number of components present in beer which can reduce its filterability including non-starch polysaccharides such as β-glucan (which tends to increase the viscosity of beer by forming large gel complexes) (Krüger et al., 1989; Sudarmana et al., 1996), arabinoxylans (which may behave similarly to β-glucans) (Stewart et al., 1998) and α-glucan (starch), and protein-polyphenol complexes (Bamforth, 1999a; Siebert, 1999), which may potentially block or clog the filter. The removal of haze active protein and polyphenol by chill proofing treatments such as proteases, silica gels and polyvinylpolypyrrolidone (PVPP) has been shown to improve beer micro-filtration efficiency (Schumacher, 2000, Honours Thesis, University of Adelaide). Temperature during filtration is also well known to affect the stability of the finished product (Hardwick, 1978). *En route* to and through the filter the temperature of the beer should be maintained as cold as possible to ensure that the precipitated material (haze precursors) does not return back into solution and re-enter the bright beer (Bamforth, 1999a). Oxygen at this stage must not be allowed to enter the beer, as this promotes the polymerisation of polyphenols that can lead to greater colloidal instability problems as well as more rapid deterioration of flavour (Bamforth, 1999a,b; 1988).

1.2.5 Storage and Transport

The formation of haze in bright beer can be symptomatic of problems encountered during production and packaging, but what occurs during storage is equally critical. During the storage of beer, the oxidation of polyphenol monomers such as catechin,

epicatechin and gallocatechin results in the formation of higher polymers. As these compounds are polymerised their ability to cross-link to proteins to form haze increases (Asano *et al.*, 1984). The temperature at which packaged beer is stored at, or in transit from the brewery to the consumer may influence the haze and flavour stability of the product upon consumption. High-temperature storage usually results in accelerated staling/haze formation and is often accompanied by the development of paper, cardboard, and toffee flavour notes (Dalgliesh, 1977). The shaking of beer during transport can also lead to decreased colloidal stability. When Glenister (1979), subjected seven commercial beers to a shaking test (125 to 130 rpm, for 6 hours a day, 6 days total) all the beers had lost their brilliance, with proteinaceous flakes being the predominate particles deposited as a result of the treatment. High temperature and mechanical shaking of beer have been found to increase the deposition of larger particulate matter, with this particulate matter found to be minimal in beer at the time of packaging (Glenister, 1975; Wenn *et al.*, 1989). In another study, agitation of beer was shown to rapidly increase the number of particles present in beer two to three weeks post packaging and they remained at that level for up to 19 weeks (Walters *et al.*, 1996). The amount of protein in this particulate matter was found to be only 0.7% of the total protein available in beer (Walters *et al.*, 1996). The authors have suggested that the particulate matter was likely to be present in the beer within one to two weeks of packaging, and was not attributable to longer storage or elevated temperature.

1.2.6 Beer Styles

Not all beers are intended to be bright or clear. There are exceptions such as "bottle or cask conditioned" beers (i.e. locally Adelaide's Cooper's Pale Ale) where yeast added to the product to enable carbonation, is present to give a hazy impression. With

traditional Belgian white beers, both the intensity and stability of the haze are quality characteristics that the consumer expects. To obtain and maintain a stable haze is often more difficult then it is to achieve a bright or clear product. An investigation into the composition of colloidal haze in Belgian white beers showed that like in bright beers (Pilsner beers), proteins (detected in the range of 7 up to 74%) and polyphenols (1.1 to 7.7%) were the major components (Delvaux *et al.*, 2000).

1.3 Protein-polyphenol haze measurement

The European Brewing Convention (EBC) (see Scriban, 1959) has defined the non-biological hazes of beer as follows: Chill haze can be defined as any haze which is formed when beer is chilled to 0°C but which redissolves when the beer is warmed again to 20°C or more. Any haze which remains in beer at 20°C or higher is defined as a permanent haze.

Haze intensity in beer can be measured using the EBC method (9.29) (Analytica-EBC, 1998), in which the amount of light scattered at 90 degrees to an incident beam is measured. The reading is a ratio of the amount of scatter measured by the 90° sensor to the sum of the forward scatter and the transmitted light sensors (Analytica-EBC, 1998). There are a number of scales used to measure the amount of haze present in beer, but most commonly haze is described using the EBC scale. A value of <0.5 is described as 'brilliant', between 0.5 and 1 as 'almost brilliant', between 1 and 2 as 'very slightly hazy', between 2 and 4 described as 'slightly hazy', and a value ≥ 4 as 'hazy' (Analytica-EBC, 1998).

1.4 Haze Active (HA) proteins

It was shown by Pollock and colleagues in 1959 that barley prolamins, a class of alcohol soluble, proline-rich proteins called the hordeins, were associated with polyphenols from malt to form haze (Pollock *et al.*, 1959). Haze active (HA) proteins isolated from beer have been demonstrated, based on their amino acid compositions (high in proline and glutamine), to be derived from the barley hordeins, as they are relatively rich in proline and are composed of fragments of several different molecular weights (Asano *et al.*, 1982).

Asano *et al.*, (1982) isolated four proteinaceous fractions (designated as I, II, III and IV, combined = 1.759 mg/L) from beer, with fraction II found to have high affinity for polyphenols. These haze forming fractions, comprised roughly one-third of the total nitrogenous substances of beer, had molecular weights ranging between 1 kDa and 40 kDa, and contained high levels of proline and glutamine (Asano *et al.*, 1982). This is indicative that either several of the known barley hordeins (Shewry, 1993) are involved in haze formation, or that one or more of the hordein group or family are degraded by proteolysis, during malting and mashing to provide haze active proteins to beer (Smith, 1990).

It has been shown using catechin (400 mg/L), in a model buffer system that the amount of haze formed was essentially linearly related to the mole percentage of proline in a polypeptide (Asano *et al.*, 1982). The distribution and mole percentage of proline within HA proteins (hordeins) has also been shown to be directly related to haze forming potential (Outtrup *et al.*, 1987; Outtrup, 1989). In contrast, amino acid homopolymers including (Poly-L-glutaminic acid, Poly-L-lysine and Poly-L-serine)

that do not contain proline, did not produce haze (Asano *et al.*, 1982). This was confirmed using additional homopolymers and in showing that even the closely related polyhydroxyproline did not form any notable haze (Siebert *et al.*, 1996b).

The isoelectric point of polypeptides has been linked to increased haze activity. Acidic proteins have been found to be predominant in beer hazes (Belleau and Dadic, 1981). Polypeptides with isoelectric values between 3 and 5 have been suggested to be particularly haze active (Matsuzawa and Nagashima, 1990). On the basis of isoelectric point, Mussche (1990) also claimed that haze and foam forming proteins can be differentiated, with haze forming proteins possessing isoelectric points in the acidic pH range and proteins associated with foaming possessing alkaline isoelectric points.

The investigations of Asano and co-workers into haze formation demonstrated that haze formation was not exclusive to hordein derived polypeptides. Albumin and globulin derived polypeptides were able to come out of solution as chill haze, but only after precipitation of the hordeins (Asano *et al.*, 1982). Antibodies that have been raised to both foaming and haze polypeptides have shown that the foam antibody was specific, however, the haze antibody cross-reacted with both foam and haze polypeptides suggesting that a number of polypeptides can non-specifically enter into haze (Ishibashi *et al.*, 1996).

1.4.1 Hordein

The hordeins are a complex polymorphic mix, which when separated by electrophoresis, can be classified into two major groups of polypeptides, B and C and two minor groups D and γ according to their molecular size, sulphur content and amino acid compositions (Shewry, 1993) (Table 1). The hordein storage proteins are made up of 20-30 proteins that account for 50-60% of the total protein fraction of the barley endosperm (Shewry *et al.*, 1981). Genetic analysis of the hordeins has shown that they are encoded by families of genes at single, linked loci on chromosome 5 (1H) (Shewry, 1993). The N-terminal sequences of the hordeins contain amino acid repeats rich in glutamine and proline (Shewry, 1993). When an absorbed fraction of protein bound to silica gel following its use for the colloidal stabilisation of beer was analysed the mole percentage of proline was found to be between 33.2 and 38.0, and of glutamate/glutamine between 32.7 and 33.0 (Evans *et al.*, 2003; Sheehan *et al.*, 1999), which is consistent with the amino acid composition of the hordeins. In another study, the correlations between the various amino acids bound in non-biological haze were calculated. It was found that the correlation co-efficient for hordein (Pro-Glu r= +0.87) was highly significant (Lontie *et al.*, 1963).

Table 1: Barley hordein characteristics

Characteristics		B hordeins	C hordeins	D hordeins	γ hordeins	
MW (kDa)		35-46[1]	55-75[2]	~105[3]	Similar to B's[4]	
Properties		Sulphur (S) rich[5]	Sulphur (S) poor[5]	HMW[5]	Sulphur (S) rich[5]	
%		70-80	10-20	2-4	Not yet precisely determined	
Amino Acid Composition (mol %)	Pro	20.6[6]	30.6[6]	11.6[7]	γ3 16.5[8]	γ1 + γ2 22.1[8]
	Glu[a]	35.4[6]	41.2[6]	29.6[7]	γ3 32.4[8]	γ1 + γ2 29.9[8]

References: (1.) (Faulks *et al.*, 1981), (2.) (Shewry *et al.*, 1985), (3.) (Shewry and Miflin 1982), (4.) (Rechinger *et al.*, 1993), (5.) (Shewry, 1993) (6.) Total B hordein and C hordein from cv. Julia (Shewry *et al.*, 1980), (7.) D hordein from Risø mutant 1508 (Kreis *et al.*, 1984), and (8.) γ hordein bands from Risø 56 (Kreis *et al.*, 1983). Pro = Proline, Glu = Glutamine, HMW = High molecular weight. [a]The Glu values listed also include Gln.

An important feature of the hordeins in terms of their haze forming potential is their rod-shape, their repetitive domain rich in β-turns, with a loose spiral configuration based on the repetitive β-turns (described in Shewry *et al.*, 1994; Shewry, 1993), which increases both hydrophobic interactions and hydrogen bonding. The high proline content of the hordeins along with the configuration of the proline-rich sequences and their spatial distribution in the three-dimensional conformation governs the efficiency of the interaction with polyphenols, thus the likelihood for the formation of haze in beer (Hagerman and Butler, 1981; Williamson, 1994).

The B, C and D groups of hordeins display differences in their biochemical properties and amino acid compositions and this is reflected in differences in polymer and disulphide bond formation and stability (Shewry *et al.*, 1994). The B hordeins (sulphur rich) are likely to be present as monomers, disulphide-linked polymers and aggregated with D hordein (Forde *et al.*, 1985). C hordein (sulphur poor) lacks cysteine and thus cannot form polymers stabilised by disulphide bonds while D hordein is probably only present in disulphide stabilised polymers (Shewry, 1993; Smith, 1990). Hordeins or their fragments, particularly the hydrophobic parts that survive into finished beer, probably have an effect on the haze forming potential (Smith, 1990). The hordeins (B, C and D) have some important common structural features including at least two regions which differ in amino acid composition and their structural domains. There are the non-repetitive domains of the B and D hordeins that are hydrophilic and are highly soluble in water and salt solutions (Smith, 1990). Their hydrophilic nature makes them highly susceptible to protease digestion and therefore they are unlikely to be found in finished beer (Smith, 1990). In contrast, the repetitive domains of the hydrophobic B, C and D hordeins are rich in proline, and as a result are resistant to the protease activity. Therefore peptides derived from the repetitive domains of B, C and D hordeins are most likely those involved in haze formation.

Such is the degree of heterogeneity in hordein composition between barley varieties that they are often used for varietal identification (Shewry, 1993). The distribution and mole percentage of proline within HA proteins (hordeins) has also been shown to be directly related to haze forming potential (Outtrup *et al.*, 1987; Outtrup, 1989). Taking these two observations together it is likely that the genetic variation in barley hordeins can be

utilised to identify or develop barley varieties in which the levels of haze active proteins

are genetically minimised.

1.5 HA polyphenols

Naturally occurring beer HA polyphenols are members of the proanthocyanidin or

flavonoid family (Siebert, 1999). They consist of monomers, dimers, trimers and higher

polymers of catechin, epicatechin and gallocatechin (Bamforth, 1999a; Siebert, 1999)

(Figure 1).
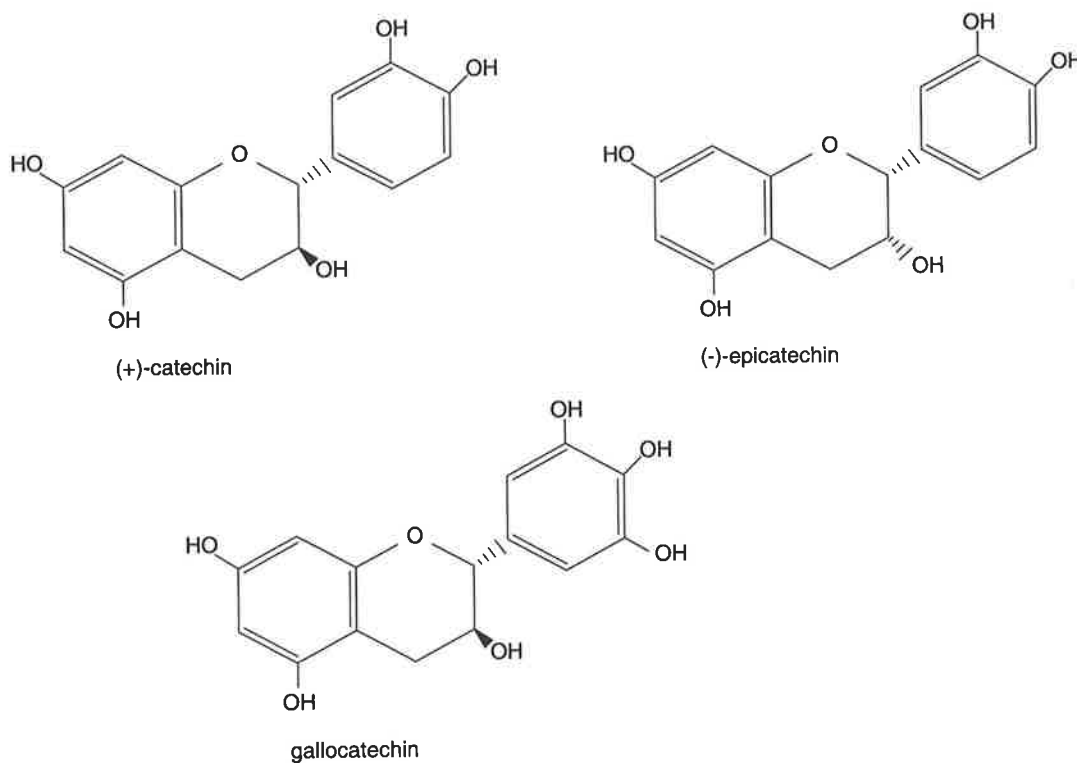
Figure 1: Structures of typical beer proanthocyanidin monomers.

In model systems it has been shown that proanthocyanidins, (dimers and trimers of

catechin, epicatechin, and gallocatechin) are active in haze formation with peptides

(Outtrup *et al.*, 1987). As these compounds are polymerised their ability to form haze

increases (Asano *et al.*, 1984). Oxidation of these monomers and dimers may be

expected to result in the formation of polymers and the subsequent formation of haze. McMurrough *et al.* (1996) have shown that monomeric polyphenols are involved in haze formation once they have been oxidatively polymerised. One would expect to find the haze forming potential in finished beer quite high. Investigations indicate however, that this is not the case, with most tetramers and higher polymers either not extracted from the malt or lost during processing (into the trub during wort boiling and maturation) (Jerumanis, 1979; McMurrough and Baert, 1994), leaving only monomers or dimers. Proanthocyanidin B3 has been shown to be at concentrations of >25 ppm in malt, but after wort boiling the recoverable amount has dropped to <15 ppm and after maturation <10 ppm (Fonknechten *et al.*, 1983; Moll *et al.*, 1984). Most haze forming activity has been associated with two prominent beer proanthocyanidin dimers, procyanidin B3 (catechin-catechin) and prodelphinidin B3 (gallocatechin-catechin). McMurrough *et al.* (1992), demonstrated that the rate of haze formation is closely related ($r = 0.965$) to the product of the sensitive proteins (HA proteins) (as measured by inducing haze formation with tannic acid) and the concentration of these dimeric proanthocyanidins as measured by HPLC.

The haze inducing capacities of prodelphinidins are greater than their procyanidin counterparts due to the occurrence of 3 adjacent hydroxyl groups on the gallotannins moiety of prodelphinidin B3. This highly reactive configuration promotes greater interactions with HA proteins than are seen with procyanidin B3, in which only two hydroxyl groups are found (McMurrough *et al.*, 1996; Mulkay and Jerumanis, 1983).

To bind proteins, polyphenols require an aromatic ring with at least two hydroxyl groups (McManus *et al.*, 1985). In a study by McManus *et al.* (1985) in which the

energy (kJ/mol) produced when several polyphenols were bound to bovine serum albumin (BSA) was measured, the differences found were based on the configuration of the hydroxyl groups. For those polyphenols with vicinal hydroxyl groups such as *o*-diphenol (where the hydroxyl groups are adjacent to the ring), binding was much stronger then with *m*-diphenol in which the hydroxyl groups were separated (McManus *et al.*, 1985). By studying the precipitation of β-glucosidase by gallotannins, Haslam (1974), concluded that for polyphenols to bind to protein an *o*-dihydroxybenzene group is required. Polyphenols with one *o*-diphenol group are able to bind to the protein but are unable to cross-link. As the number of *o*-diphenol groups increases so does the precipitating ability of the polyphenol. The degree to which a proanthocyanidin is polymerised has been shown to influence the colloidal stability of beer more than the number of hydroxyl groups on the aromatic ring (Mulkay and Jerumanis, 1983).

1.6 Protein:Polyphenol interactions in colloidal haze formation

Factors that determine the relative affinities of proteins for polyphenols are not fully understood. Protein-polyphenol interactions are thought to result from a intricate mix of the proline content of the protein (Asano *et al.*, 1982; Hagerman and Butler, 1980 and 1981; Ottrupp *et al.*, 1987; Ottrupp, 1989), its molecular weight (Asano *et al.*, 1982; Hagerman and Butler, 1981) and the protein's conformation (Hagerman and Butler, 1980; Haslam, 1998; Ottrupp *et al.*, 1987; Ottrupp, 1989; Williamson, 1994). The formation of protein-polyphenol complexes has been attributed to the combination of the hydrophobic association between proline and the hydrophobic ring structure of polyphenols, possibly in combination with hydrogen bonding between oxygen atoms of peptide bonds and hydroxyl groups of polyphenols (Asano *et al.*, 1982; Hagerman and Butler, 1981; Oh *et al.*, 1980). The hydrophobic nature of proline, along with its

pyrrolidine ring structure, which confers an unfolded molecular confirmation, allows for the entry of polyphenols into proteins rich in this amino acid (Asano *et al.*, 1982). Also, the proline residues of the protein keep it extended and in doing so, maximise the binding surface available to the polyphenols.

Covalent bonding, at least in the initial reaction between protein and polyphenol, has been excluded, as most hazes are chill hazes and when the beer is warmed the haze is partially or totally dissolved (Chapon, 1968), indicating reversibility of the reaction not commensurate with the formation of covalent bonds. Ionic bonding has been shown to not be involved in haze formation, with salt (NaCl) shown not to inhibit the formation of haze in a model system (500 mg/L haze forming proteins and catechin 400 mg/L in 0.02M sodium phosphate buffer, pH 4.2, ethanol concentration 3.6%) (Asano *et al.*, 1982), nor was it able to dissolve freshly formed haze in a model system (haze was developed using catechin and gliadin combined in a 0.02M (pH 4.2) sodium phosphate buffer, held at 80°C for 30 min) (Siebert *et al.*, 1996b). Dioxane (a non-polar solvent) and *N, N*-dimethyl formamide (a hydrogen bond acceptor) were found to prohibit haze formation in the aforementioned model system (Asano *et al.*, 1982) and were able to dissolve freshly formed haze (Siebert *et al.*, 1996b). The acidity of beer can also inhibit ionic bonding between haze forming proteins and polyphenols since the hydroxyl groups of the polyphenols under acidic conditions have no net charge (Asano *et al.*, 1982).

In a caffeine-polyphenol model system where caffeine was considered to be an analog of peptidically linked proline, the electrophilic (caffeine) and nucleophilic (polyphenol) planar rings were found to be overlaid and held together by π-bonding (Bianco *et al.*,

1997). Siebert and colleagues have also suggested that the absence of haze formation with polyhydroxyproline, and the observation of higher levels of haze formation when proteins are heated (100°C), suggests that hydrogen bonding is not as important as hydrophobic bonding in protein-polyphenol interactions (Siebert *et al.*, 1996b).

1.6.1 A model for protein-polyphenol haze formation

To examine the interaction of HA protein with HA polyphenol, haze formation was measured in model systems in which gelatin or gliadin (protein) and tannic acid (polyphenol) or gliadin (protein) and catechin (polyphenol) were combined in various proportions (concentrations of these components ranged from 0 to 1000 mg/L) in a potassium phosphate buffer (pH 4.02 or 4.2) (Siebert *et al.*, 1996a,b). In both experiments the results revealed that if the polyphenol concentration was increased while still maintaining a fixed concentration of the protein or vice versa, at first the amount of haze increased, then plateaued before declining. These data suggested that not only is the concentration of HA proteins and HA polyphenols important but also their ratios in determining the amount of haze produced (Siebert *et al.*, 1996a,b).

[Polyphenol] ~ [Protein]

[Polyphenol] << [Protein]

[Polyphenol] >> [Protein]

⟵⟶ Polyphenol molecule

Protein molecule with fixed
number of polyphenol binding
sites (i.e. haze-active)
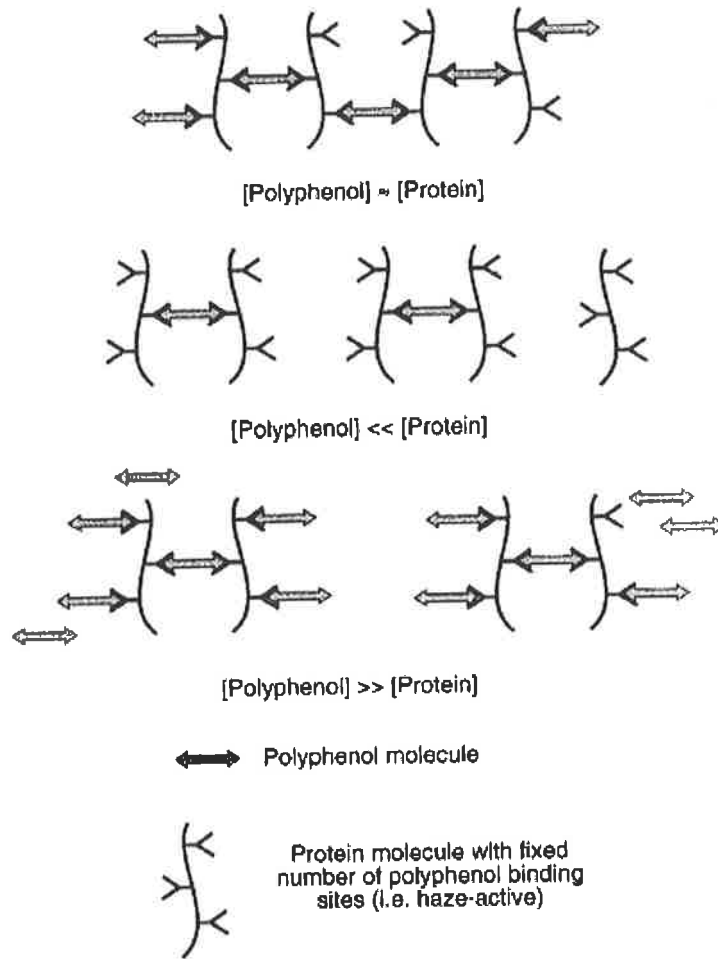
Figure 2: A conceptual mechanism to describe protein-polyphenol interactions in beer

(Siebert *et al.,* 1996b).

A conceptual mechanism to describe this protein-polyphenol haze formation has

recently been developed (Figure 2) by Siebert *et al.,* (1996b). The model makes three

assumptions: (1.) Only proline-containing proteins are haze active; (2.) There is only a

fixed number of polyphenol binding sites, presumably the proline residues, and (3.) The

HA polyphenol has two or more ends that can specifically interact with the binding sites

on the protein, thus allowing a single polyphenol molecule to form a bridge between

two protein molecules (Siebert *et al.*, 1996b). In the situation where the concentration of polyphenol binding ends is roughly equal to the number of protein binding sites available, a large network results, producing large colloidal particles and maximum light scattering (Siebert *et al.*, 1996b).

In beer, however, there is a considerable excess of HA protein to HA polyphenol (Siebert *et al.*, 1996a). Although each HA polyphenol has the ability to bridge two proteins together there are not enough free polyphenols to bridge dimers and form larger complexes. Equally, if there was an excess of HA polyphenol to HA protein there will be a shortage of free proline sites making it difficult for a polyphenol attached to one protein to find an available site on another to bridge to (Siebert, 1999). When the levels of protein and polyphenol are disproportionate the result is smaller particles and therefore less haze.

1.6.2 Single-ended binding polyphenols

In addition to the two or more ended HA polyphenols, there is a class of polyphenols which can only bind to a HA protein at one end (Siebert and Lynn, 1998). Single ended polyphenols (i.e. methyl gallate and gallic acid) are able to bind to a HA protein but are unable to cross-link to another protein and thus would not produce a haze. Single ended polyphenols would be expected to interrupt haze formation by competing for proline residues (Siebert and Lynn, 1998). The role of single ended polyphenols in haze formation will be further discussed in section 1.7.3.

1.6.3 The effect of pH and ethanol concentration on haze formation

The effect of pH and alcohol concentration on protein-polyphenol haze formation has also been investigated in a model system (Siebert *et al.*, 1996a). The effect of pH saw 7 times as much haze produced with the same amounts of protein and polyphenol between pH 4.0 and 4.2 than at pH 3.0 (Siebert *et al.*, 1996a). At pH values greater then 4.2 haze formation declined. This effect may be due to the increased charge on the protein at pH values above or below its isoelectric point and thus a decrease in the inherent hydrophobicity of the molecules, or because as the protein:polyphenol interaction appears to be non-ionic, greater charge may result in the repulsion of protein molecules from one another (Siebert *et al.*, 1996a; Siebert, 1999). At the pH of beer, which is approximately 4.0, higher ethanol concentrations can lead to an increase in haze formation. The higher ethanol concentrations may cause proteins to be precipitated from beer (Siebert *et al.*, 1996a) due to the reduced dielectric constant of the alcohol:water solution. The pH and alcohol content of beer can also influence the size of the colloidal particles formed, thus influencing visual turbidity, sedimentation of the beer during storage, and the removal of these particles during filtration (Siebert and Lynn, 2003).

1.7 Removal of HA proteins and HA polyphenols: stabilisation for colloidal stability

Even with the filtration of beer prior to packaging, which results in a bright and clear product, haze inevitably develops over time. Haze has been shown to form in beer at a rate that is equal to the function of the product of the concentration of the HA proteins and HA polyphenols at the time of packaging (McMurrough *et al.*, 1992). Breweries generally treat their beer in a number of ways to reduce or overcome this problem, to

30

effectively prolong the shelf-life of beer. The removal of HA material while still maintaining foam active protein components is the desired outcome. Currently, a number of stabilisation procedures are utilised in breweries to reduce or delay the onset of haze formation. These may involve additional processing steps, or the use of specific additives aimed at reduced the likelihood of later haze formation. Additions are generally made during the cold conditioning of beer and are removed during filtration. These treatments include the use of prolonged cold stabilisation (precipitates both protein and polyphenol), fining with gelatin, isinglass, or tannic acid (TA) (protein), the addition of proteolytic enzymes (protein) and treatments with adsorbents such as PVPP (polyphenols) or silica (protein) (Coors, 1977).

Traditional practices have involved placing the fermented beer into a tank and holding it for between 7 days to 10 days at –2 to 4°C (Lewis and Young, 2001). Low temperature and storage will eventually produce bright beer in accordance to Stoke's law. Stoke's law predicts that larger denser particles will settle faster than smaller lighter ones, and any particle will settle out better in a liquid of low density and low viscosity (Lewis and Young, 2001). Although sound in principal, this method is disadvantageous in that few breweries can afford the time or space needed to achieve an acceptable degree of clarity. Fining with gelatin, isinglass, or TA is often utilised to speed up the process and remove protein and polyphenol implicated in haze formation, followed by cold filtration through DE in order to remove particulate matter (Siebert, 1999).

1.7.1 Fining agents

Fining agents are substances which, when added to beer speed up the process of clarification and the removal of sediment. Clarifying agents or fining agents are

included in the brewing process in the kettle, to help remove proteins that are barely in solution at the boil (101°C) but which will otherwise become insoluble as the wort cools (O'Neill, 1996) and contribute to cloudiness in the wort. These very fine particles (<2 μm) can be removed with finings agents such as carrageenan which collect these particles to form larger ones. Isinglass and gelatin are used to precipitate proteins and yeast from both wort and beer to aid filtration efficiency. In this process at the pH of beer, both isinglass and gelatin are positively charged molecules which can react with the negatively charged yeast and proteins to promote their sedimentation (Hough *et al.*, 1982). Although the hydroxyproline residues of gelatin are inactive when it comes to combining with polyphenols, they do impair the formation of helical structures in other proteins thus presumably making the proline residues more accessible to the polyphenols (Siebert *et al.*, 1996b). In part, the role of gelatin or isinglass in beer is also combining with polyphenols.

TA is added in excess to beer during cold conditioning, and as a precipitant as opposed to an adsorbent like PVPP or silica, when it reacts with haze forming proteins a sizeable precipitate is formed (Bamforth, 1999a). The effect of TA addition to beer is a more balanced ratio of HA protein to HA polyphenol, which in turn leads to more haze and precipitation (Siebert and Lynn, 1997a). This haze will settle out at low temperatures and can be removed during the filtration process.

1.7.2. Stabilising agents - Protein

The use of proteolytic enzymes such as papain (from *Carica papaya*) was the first haze treatment used by the brewing industry to treat HA protein and delay the onset of haze formation (de Clerk, 1969). Although very effective, papain also removes foam active

proteins from beer, resulting in decreased foam quality. Bentonite as a protein absorbent is widely used to stabilise fruit juices and wines but has been found to not be as useful with beer. Bentonite is non-specific in its activity, and removes both HA and foam active proteins from beer (Siebert and Lynn, 1997a). Silica gels (hydrogels and xerogels) are far more specific for HA proteins and with the use of polypeptide model systems, silica gels have been found to have high specificity for HA protein because the silica gel binds to the same HA proline residues as do HA polyphenols (Siebert and Lynn, 1997a).

1.7.2.1 Measuring HA protein levels

Previous studies have shown that only a small proportion of total beer protein is actually involved in the formation of haze (Siebert and Lynn, 1997b), with as little as 2 mg/L of protein sufficient to induce a haze of 1 EBC unit (Chapon, 1994). As has been discussed previously (section 1.6.1) the proportion of HA protein to HA polyphenol exerts a strong influence on the amount of haze formed. This effect of the proportion of HA protein to HA polyphenol combined with the unique composition of the hordeins means that there are difficulties in accurately measuring HA protein levels. A number of methods are currently used, including, Coomassie blue dye binding (Bradford, 1976), determination of protein by measuring absorption at 280 nm, the Kjeldahl method, the bichinchonic acid method (Siebert, 1999), and turbidimetric methods, such as the 'sensitive proteins' assay (Thompson and Forward, 1969) and turbidimetric titration using a Tannometer (Chapon, 1993). All of these methods have their shortcomings, but I will only discuss in further detail that of the Bradford method, which is most commonly used.

A major limitation of the Bradford (1976), assay is that the quantity of protein which can be measured is primarily determined by the amount of arginine in the molecule and the relative size of the protein, >5 kDa (Compton and Jones, 1985; Hii and Herwig, 1982; Lewis *et al.*, 1980). The Coomassie response is highly biased toward homopolymers of the basic and aromatic amino acids and gives little response to other homopolymers (Compton and Jones, 1985). Thus the significant homopolymers of hordein, proline (~20 mol %) and glutamine (~30 mol %) do not produce any Coomassie blue response (Siebert and Lynn, 1997a). As such the Coomassie blue method has been promoted as being useful in preferentially determining/measuring the level of foam active proteins in finished beer (Evans *et al.*, 2003; Evans and Sheehan, 2002).

1.7.3 Stabilising Agents - Polyphenol

Neutral polyamides such as nylon and polyvinylpyrrolidone (PVP) (which includes PVPP the high molecular weight, insoluble material used as an absorbent) have a great affinity for polyphenols in beer (Siebert and Lynn, 1998). Of interest is that PVPP and polyproline (a known HA peptide) share considerable structural similarities in that both have five-member, saturated, nitrogen-containing rings, amide bonds, and no other functional groups (Siebert and Lynn, 1998). It has been suggested that PVPP is specific for HA polyphenol because it binds to the same part of the polyphenols as does the HA protein (Siebert and Lynn, 1997a).

However, when the authors took into account the concept of single-ended binding polyphenols which can only bind to HA protein at one end this theory was altered. Gallic acid (GA) and methyl gallate (MG) polyphenols which only contain a single

binding location were shown in model systems to be able to bind to HA protein but were unable to cross-link to another HA protein and subsequently develop haze (Siebert and Lynn, 1998). For example when GA was combined with gliadin, haze formation observed was less then 2 NTU or 0.49 EBC, demonstrating the GA is not a HA component (Siebert and Lynn, 1998). Single ended polyphenols were also shown to be able to bind to HA protein and be removed with PVPP, suggesting that PVPP has the ability to absorb both HA and non-HA polyphenols. Using polyproline (a model HA protein) it was shown that at 25°C, catechin produced more haze than epicatechin, but at 80°C the amounts were similar, while with soluble PVP at both temperatures, catechin produced much more haze (Siebert and Lynn, 1998). These observations suggest that the mechanisms by which HA polyphenols attach to PVPP and to HA protein are similar, but not identical (Siebert and Lynn, 1998).

The non-specific nature of PVPP (in that it can absorb both HA and non-HA polyphenols) has the negative consequence that non-HA polyphenols, among which may be those possessing valuable antioxidant activity (i.e. catechin), will be removed from the beer along with HA polyphenols. In investigating PVPP absorption of polyphenols from beer, McMurrough and Baert (1994), found that catechin was readily removed but epicatechin was barely affected even though it was represented in much smaller amounts. It has been suggested that catechin which survives into finished beer may only have value as a protectant against new oxidation and not against staling which has already occurred during the brewing process (Walters et al., 1997). Another problem with the use of PVPP to remove polyphenols from beer is that polyphenols also contribute to the flavour of beer, i.e. mouth feel, astringency and after-bitterness (Langstaff and Lewis, 1993).

All of these treatments come at a cost to the breweries. By concentrating on the source of the HA components in the raw materials (barley) a potentially cheaper and more wholesome solution may be obtained.

1.7.4 Proanthocyanidin free lines

An alternative to the use of these stabilisation techniques is the removal of HA material from barley, and there has been the development of proanthocyanidin free (ant-free) barley varieties which significantly improve haze stability (Fukuda *et al.*, 1999; von Wettstein *et al.*, 1977 and 1980; Jende-Strid, 1997). Ant-free lines were first developed by the Carlsberg brewery group in 1976 by mutagenesis (Jende-Strid, 1976). With ant-free lines the biosynthetic pathway of proanthocyanidins is genetically blocked. As a consequence these lines contain very low levels of polyphenol monomers such as catechin (von Wettstein *et al.*, 1977). Although these mutant lines produced beer with excellent colloidal stability there were problems with their agronomic and malting qualities. Fukuda and colleagues have developed an ant-free barley (Mokkei 92-130) that has improved agronomic and malting quality (Fukuda *et al.*, 1999). Mokkei 92-130 was a line developed from the Carlsberg Ant13-347 line and the Japanese malting variety Haruna Nijo (Fukuda *et al.*, 1999). Although displaying excellent colloidal stability overall, beer flavour and stability were affected, with Mokkei 92-130 showing more rapid ageing then its control counterparts. It is also probably undesirable to remove polyphenols such as catechin, which can act as both an oxidant and an antioxidant throughout the brewing process. These antioxidant properties throughout production and during the storage of beer may assist in countering beer staling. More recently the ant-free variety Caminant (Ant 28), has shown that ant-free barley can be successfully malted and brewed. The haze stability of non-stabilised Caminant beer is

36

excellent and it is possible to mix ant-free and traditional malt in the ratio of 1:1 and still obtain a beer with satisfactory haze stability without using traditional stabilising treatments (anon) (http://www.crc.dk/flab/proantho.htm).

1.8 Conclusions

Any holistic approach or strategy for dealing with the problem of protein-polyphenol hazes must involve the removal of HA proteins or HA polyphenols or a combination of both, either before or during the brewing process. The removal of both HA proteins and HA polyphenols by fining and stabilisation agents during the brewing process is a costly exercise for the breweries. Combined with this are differences in haze stability that breweries note between brews of the same beer. The removal of polyphenols from the grain has so far not lead to the widespread adoption of these particular varieties, and in turn a reduction in the usage of colloidal stabilisation agents as was predicted. The minimisation of malt haze active proteins appears to be an attractive option to explore in order to improve the haze stability during the storage and transport of beer and in doing so reduce the need and or requirement for colloidal stabilisation treatments.

This research will therefore demonstrate how barley HA proteins can be managed to minimise the haze activity of these proteins and thus provide alternatives to the use of traditional colloidal stabilisation treatments in the brewery. Within this framework the project specifically aims to:

1. Utilise an antibody raised against proteins eluted from silica gel (SE), used for the colloidal stabilisation of beer, to identify by immunoblot, barley varieties which do or do not contain a low molecular weight MW ~12000 SE band.

2. Use pilot (10 L, 100 L, and 300 L) and small scale (600 mL – 800 mL) brewing trials to determine whether varieties that do not contain the MW ~12000 SE protein are less predisposed to haze formation.

3. Map the haze active proteins in (SE +ve) and (SE -ve) mapping populations to investigate the genetic basis of haze active proteins.

4. Isolate, sequence and characterise the SE protein as identified by the SE antibody and develop antibodies to the recombinant protein.

5. Examine beer filterability in relation to colloidal stability, as improved filterability may be directly related to improved colloidal stability in beer.

Chapter 2

Identification of haze active proteins in barley and malt

Abstract

SDS-PAGE immunoblot analysis using an antiserum that was raised against a silica eluent (SE) protein fraction (obtained from silica gel, used for the colloidal stabilisation of beer), detected a range of protein bands in barley, malt, beer and haze. A polymorphism was observed in which some barley varieties contained a molecular weight ~12000 band (SE +ve) while in other varieties this band was absent (SE -ve). A survey of 219 Australian and international barley varieties, including a comprehensive selection of current and past malting varieties, identified 181 varieties as SE +ve, and 38 varieties as SE -ve. The genetic basis for the presence or absence of the SE protein was determined by interval mapping analysis, which found that the gene encoding the MW ~12000 SE band mapped to the short arm of chromosome 3H.

2.1 Introduction

In bright beers, the formation of haze is a serious quality problem, which places limitations on the product's storage life. To the consumer, haze often represents a sign of ageing or contamination of the product. Beer contains a number of barley proteins that are modified chemically and proteolytically during the malting and brewing processes, which can influence final beer haze stability. Haze active proteins isolated from beer have been found to be derived primarily from barley (*Hordeum vulgare* L.) storage proteins (hordeins), composed of fragments of several different molecular weights, and are relatively rich in proline (Asano *et al.*, 1982).

The hordeins are a complex polymorphic mix, which when separated by electrophoresis, can be classified into four separate groups of polypeptides: B, C, D and γ according to their molecular size, sulphur content and amino acid compositions (Shewry, 1993). They have both monomeric and polymeric forms, with the monomeric (soluble fraction) able to be extracted by alcohol or detergent solutions, while the polymeric (insoluble) B/D disulfide linked aggregates are extracted in the presence of a reducing agent such as 2-mercapto-ethanol (Shewry, 1993; Shewry *et al.*, 1980). The hordein storage proteins consist of 20-30 proteins that account for around 50-60% of the total protein fraction of the barley endosperm (Shewry *et al.*, 1981). Genetic analysis of the hordeins has shown that they are encoded by families of genes at single, linked loci on chromosome 5 (1H) (Shewry, 1993). The N-terminal sequences of the hordeins contain repeats and motifs rich in glutamine and proline (Shewry, 1993).

During the colloidal stabilisation of beer, silica is used to remove proteins rich in proline that have the ability to interact with polyphenols to form haze in bright beer.

Analysis of the absorbed fraction of protein bound to silica gel following its use for the colloidal stabilisation of beer revealed that the mole percentage of proline ranged between 33.2% and 38.0%, and of glutamate/glutamine between 32.7% and 33.0% (Evans *et al.,* 2003, Sheehan *et al.,* 1999), consistent with the proline/glutamine rich composition of the hordeins. This silica eluent (SE) protein fraction was used to raise a polyclonal silica eluent (SE) antibody that when used in SDS-PAGE immunoblots appears to detect B and C hordeins and a protein of approximately MW ~12000 (Evans *et al.,* 2003).

The analysis of genotype marker segregation and phenotypic values of individuals enables the detection and location of loci that affect quantitative traits (Asíns, 2002). Quantitative trait loci (QTL) analysis is used extensively for marker-assisted selection (MAS) in breeding, and pre-breeding and QTL cloning (Asíns, 2002). A QTL defines the location of a gene that affects a trait which can be measured on a quantitative or linear scale. In eight germplasm combinations, 180 QTL have been reported in the literature for 29 barley and malt quality phenotypes (Hayes *et al.,* 2001). Regions associated with malting quality have been identified on all chromosomes in barley. In particular, 13 QTL including diastatic power, fermentability, fine-coarse difference, germination, germination speed, grain nitrogen, grain protein, hot water extract, malt β-glucan, milling energy, soluble/total protein, wort clarity and wort viscosity have been reported on chromosome 3H (Hayes *et al.,* 2001). A summary of this data may be found at http://barleyworld.org/northamericanbarley/qtllsum42401.htm.

The research described in this chapter investigates the genetic basis of the polymorphism associated with the presence or absence of a MW ~12000 protein.

2.2 Materials and Methods

2.2.1 Plant Material

Barley (*Hordeum vulgare* L. cv.) seed was obtained from the Australian Winter Cereals Collection, (Tamworth, Australia); the University of Adelaide (South Australian Barley Improvement Program) 2000 growing season's breeding experiments conducted at the Charlick experimental station (near Strathalbyn, South Australia), Port Wakefield, and Tuckey (both sites in South Australia); the 1998 season experiments at Yeelanna (South Australia) and the 1997 season experiments at Brinkworth in South Australia. Samples were also obtained from Dr Silja Home (VTT Biotechnology, Espoo, Finland), Christy Grime (The University of Western Australia, Western Australia), Dr Berne Jones (U.S. Department of Agriculture, Agricultural Research Service, Cereal Crops Research Unit, Madison, WI, USA) and Mr David Moody (Victorian Institute of Dryland Agriculture, Horsham, Australia). Commercial malt samples were obtained from Joe White Maltings (Adelaide, South Australia; Perth, Western Australia and Tamworth, NSW), Kirin Australia (Perth, Western Australia), Barrett Burston Malting Company (Melbourne, Victoria), International Malting Company (Corio, Victoria), Polttimo Companies (Lahti, Finland), Raisio Malt (Raisio, Finland), Coors Brewing Company (Golden, CO, USA), Carlsberg Breweries (Copenhagen, Denmark), and SABMiller (Miller Brewing Company, Milwaukee, WI, USA). Fifty-one of the genotypes selected were screened at least twice using samples obtained from different sites/sources.

F_2 seed was provided by Sue Broughton (Department of Agriculture, Western Australia). Stage 3 and 4 material was obtained from the 2003 University of Adelaide (South Australian Barley Improvement Program) trials grown at Tuckey, South Australia. Lines in stage 3 trials represent a preliminary malting quality evaluation and

seed multiplication phase for the most promising late generation breeding material from the program. Stage 4 trials involve a small number of entries that represent the most advanced testing stage for potential new malting quality varieties before commercial evaluation in South Australia. Hordein lines were grown at Charlick in 2000. The Chebec/Harrington mapping population was grown at the Charlick site in 1998.

Samples for the environmental trial were obtained from the 2001 and 2002 European Brewery Convention (EBC) trials grown in Finland, the Czech Republic, France, Sweden, Germany, the United Kingdom, and Denmark. Samples from Finland were obtained from three different sites; BOR (Jokioinen), LOU (Mietoinen), and LH (Hauho).

The major objective of the EBC barley trials is to provide reliable reference data for the performance of barley varieties grown under different conditions (Haeck and van den Berg, 1999). Due to variation in climatic conditions the trials are separated into four regions: North; Finland, Sweden, Denmark, and Estonia, West; the United Kingdom, France, the Netherlands, and Belgium, Central; Germany, Hungary, Slovakia, Slovenia, Austria, and the Czech Republic, and South; Portugal, Spain, Italy, and Bulgaria (Home, 1999). Differences between these regions include latitude (from $<40°N$ to $>60°N$), light hours, growing times and vegetative period.

2.2.2 Protein extraction, SDS-PAGE and immunoblotting

The polyclonal antibody used in this analysis was developed as described in Evans *et al.,* 2003. Briefly, silica (Lucilite; Crossfield, Melbourne, Australia) was added to a typical Australian lager beer. "Green" beer which had been conditioned at 0°C for at

least 1 week but had not been previously stabilised was centrifuged (6,000 x g for 15 min at 4°C) then dosed with silica at 200 mg/L to promote colloidal stabilisation and left overnight at room temperature. The silica was collected by centrifugation (10,000 x g at room temperature), washed twice with 5% (v/v) ethanol, centrifuged (10,000 x g at room temperature), then eluted with 2% (w/v) NH$_3$ followed by centrifugation (10,000 x g at room temperature) to remove the silica, before dialysis into water (Evans *et al.,* 2003). An antibody to this crude protein preparation was developed by standard methods in rabbits to produce a polyclonal antibody (Harlow and Lane, 1988). This SE antiserum was used to detect haze active proteins in barley, malt, beer and haze.

Ground barley and malt samples (20 mg) along with wort (300 µL) and beer (500 µL) samples were extracted or diluted into sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Appendix D) containing 1% (v/v) 2-mercapto-ethanol. For haze samples, hazy beer was centrifuged (4,000 x g for 10 min), the supernatant was discarded and the pellet resuspended in SDS-PAGE sample buffer. Samples were extracted at room temperature for 45 min on a rotary suspension mixer (Ratek Instruments, Melbourne, Australia), then centrifuged at 10,000 x g for 3 min. The supernatant (500 µL) was transferred to a new 2 mL Eppendorf tube containing 5 µL of 1% (w/v) bromophenol blue. Samples were frozen prior to analysis. Barley and malt samples were ground finely using a small coffee grinder. For each variety tested at least 100 seeds were ground for protein analysis.

One-dimensional SDS-PAGE was performed using homogeneous polyacrylamide gels (15% T, 2.7% C) according to the method of Laemmli (1970) (Appendix D). Samples were boiled for 5 min then centrifuged at maximum speed (10,000 x g) for 4 min. Per

well, 7.5 µL of each sample was loaded. A broad range pre-stained standard (5 µL) was also run along side the protein samples (See-Blue, (Novex), San Diego, CA, USA or Bio-Rad, Richmond, CA, USA). The chamber was filled with electrophoresis buffer (Appendix D) and the gels run at a constant voltage of 115V, for approximately 2 h. The extracted protein was separated using either Mini-Protean II or III electrophoresis units (Bio-Rad). The separated proteins were transferred to nitrocellulose (0.2 or 0.45 µm pore size; Schleicher and Schuell, Keene, NH, USA or Bio-Rad) by electroblotting (Bio-Rad – manufacture's instructions http://www.biorad.com/LifeScience/pdf/ Bulletin_2895.pdf). The buffer chamber was filled with transfer buffer (Appendix D) and the immunoblot run for 1 h at a constant voltage of 100V with cooling.

After transfer, the membrane was blocked with 5% (w/v) milk powder (non-fat) in 1X phosphate buffered saline (PBS) for 30-60 min with gentle agitation. Membranes were washed twice (5 min washes) with 1X PBS before overnight incubation with the primary polyclonal antibody (anti-SE) in 1% (w/v) bovine serum albumin (BSA)/PBS (antibody dilution 1/1000), at room temperature with gentle agitation. The membranes were washed three times with 1X Tween PBS (TPBS) (Tween 20 0.05% v/v), then twice with 1X PBS. The blots were incubated for between 1 and 3 h with a GAR-HRP antibody (Goat Anti-Rabbit IgG (H+L) - Horseradish Peroxidase conjugate, (Biorad) used at a 1/5000 dilution in 1% (w/v) BSA/PBS at room temperature (with agitation). The blots were washed three times with 1X TPBS, then twice with 1X PBS before a final wash with 1X Tris Buffered Saline (TBS). All washes were for 5 min with gentle agitation. The immunoblots were developed at room temperature using 4-chloro-1-naphthol as the substrate (Appendix D). The reaction was stopped by placing the membrane into a 0.3% (w/v) oxalic acid solution.

2.2.3 Genetic analysis of the SE trait

The SE polymorphism was screened by immunoblot in 92 lines from the Chebec (SE +ve)/Harrington (SE -ve) mapping population (Barr *et al.*, 2003b; Kretschmer *et al.*, 1997). Results were scored using a + or − scale, + being positive for the presence of the MW ~12000 SE protein, - being negative for its occurrence. The Chebec/Harrington mapping population was derived from 120 F_1 double haploid (DH) lines and a linkage map for this population was constructed using 259 restriction fragment length polymorphisms (RFLPs), 47 amplified fragment length polymorphisms (AFLPs), and 34 simple sequence repeat (SSR) markers (Barr *et al.*, 2003b). Chebec (Orge Martin/2/Clipper(86)//Schooner) is an Australian feed variety, Harrington (Klages/((Gazelle/Betzes)/Centenial) a Canadian malting variety. The SE trait data obtained from the Chebec/Harrington mapping population was applied to the linkage map using the software package MapManager QTX (Manly *et al.*, 2001), using the Kosambi map unit function (Kosambi, 1944).

To examine the segregation of the SE trait, single grains from a F_2 population of Unicorn (SE –ve)/Gairdner (SE +ve) were screened by immunobloting for the presence or absence of the SE protein. The segregation ratio was tested for conformity with Mendelian expectations for a single locus using the chi-square test.

2.3 Results

2.3.1.1 Immunodetection of SE polymorphism

One-dimensional SDS-PAGE immunoblot analysis using the SE antiserum detected a range of protein bands in barley, malt, beer and haze samples. A polymorphism was observed in which some barley varieties contained a molecular weight (MW) ~12000 band (SE +ve), while in other varieties this band was absent (SE -ve) (Figure 1). Incubation of the blots with the SE antiserum resulted in the detection of an intensively staining protein band with an apparent MW of ~12000, with additional faint bands located between 32000 and 98000 (Figure 1). These higher MW bands typical of patterns expected for B, C and D hordeins (Shewry, 1993) were observed in both SE +ve and SE –ve varieties (Figure 1).

The SE MW ~12000 protein detected in barley could also be detected in malt as well as in beer and haze (Figure 1). The quantity of the immunodetected protein band of MW ~12000 in the SE +ve varieties increased in malted samples along with the appearance of a less intense secondary band with an apparent molecular mass of ~8000, suggesting that a limited amount of proteolysis occurred during malting. About 30% of hordein is known to be degraded during malting (Smith, 1990). Two hundred and nineteen Australian and international barley varieties, including a comprehensive selection of current and past malting varieties were screened. Overall, one hundred and eighty one varieties were identified as SE +ve, while thirty-eight were identified as SE –ve varieties (Table 1). An additional ten lines varying in their level or composition of B/C hordein were screened for the SE trait. All hordein lines screened were identified as SE +ve (data not shown).
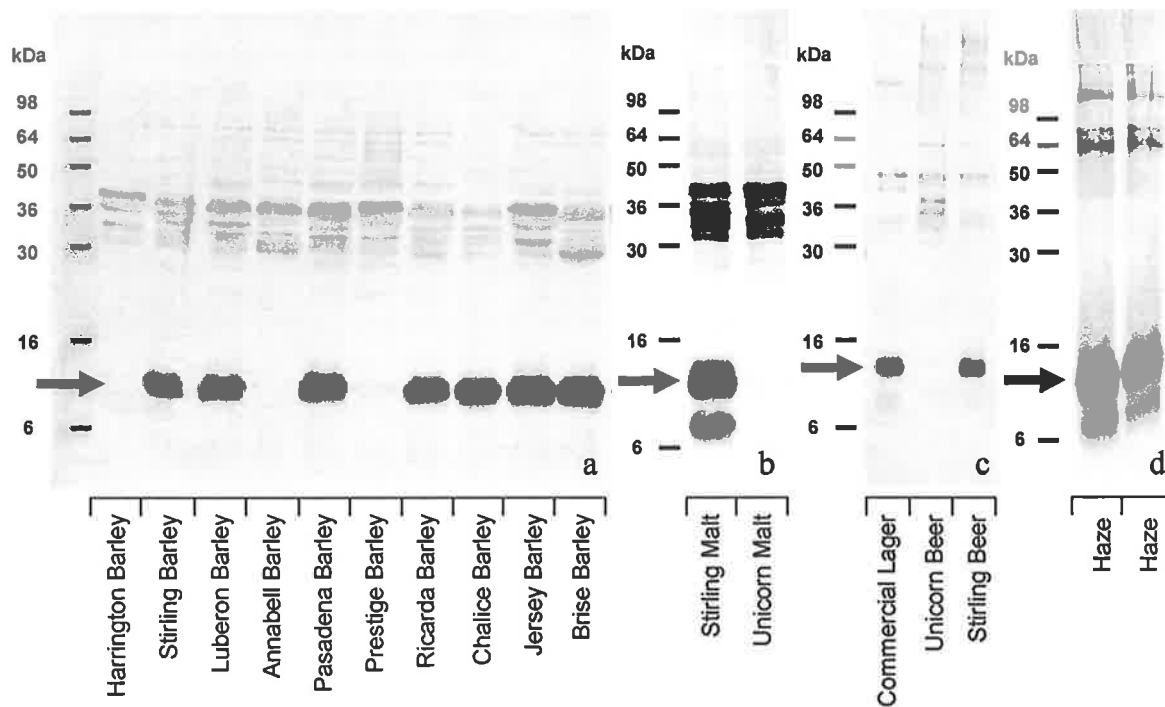
Figure 1: SDS-PAGE immunoblot analysis of total protein extracts from barley (a), malt (b), beer (c), and haze (d) using the anti-SE antibody. The arrow indicates the MW ~12000 SE protein.

Table 1: Overall classification of varieties

Seed source description (listed first in the table)

A	Australian Winter Cereals Collection (Tamworth, Australia)
C	Charlick experimental station (near Strathalbyn, South Australia)
S	Dr Silja Home (VTT Biotechnology, Espoo, Finland)
Y	Yeelanna (South Australia)
B	Brinkworth (South Australia)
PTW	Port Wakefield (South Australia)
T	Tuckey (South Australia)
W	Waite germplasm
M	SABMiller (Miller Brewing Company, Milwaukee, WI, USA)
P	Polttimo Companies (Lahti, Finland)
R	Raisio Malt, (Raisio, Finland)
Coors	Coors Brewing Company (Golden, CO, USA)
CB	Carlsberg Breweries (Copenhagen, Denmark)
EBC	European Brewing Convention (EBC) barley trials
IMC	International Malting Company, (Corio, Victoria)
JW-T	Joe White Maltings (Tamworth, NSW)
JW-SA	Joe White Maltings (Adelaide, South Australia)
JW-WA	Joe White Maltings (Perth, Western Australia)
BBM	Barrett Burston Malting Company (Melbourne, Victoria)
K	Kirin Australia (Perth, Western Australia)
CG	Christy Grime (The University of Western Australia, Western Australia)
BJ	Dr Berne Jones (U.S. Department of Agriculture, Agricultural Research Service, Cereal Crops Research Unit, Madison, WI, USA)
DM	David Moody (Victorian Institute of Dryland Agriculture, Horsham, Australia)

Seed origin description (listed second in the table)

AU	Australia
AT	Austria
CA	Canada
CL	Chile
CZ	Czech Republic
CSK	Former Czechoslovakia
DK	Denmark
DZ	Algeria
EG	Egypt
EE	Estonia
ET	Ethiopia
FI	Finland
FR	France
DE	Germany
JP	Japan
MX	Mexico
SE	Sweden
GB	United Kingdom
US	United States
NL	Netherlands
NO	Norway
UNK	Unknown

Varieties Underlined: Malt sample analysed; Varieties Double Underlined: Barley and Malt sample analysed; TG-Harr 6 and 58 = Tallon Grimmett-Harrington 6 and Tallon Grimmett-Harrington 58.

SE +ve Varieties				SE −ve Varieties
Abyssinia (Seln)[A/ET]	Diva[C/NL]	Loke Abed[C/DK]	Resibee[C/AU]	Annabell[S/DE]
Acclaim[C/DE]	Doublet[A/UK]	Luberon[EBC & S/FR]	Ricarda[S/UK]	Arra[A/FI]
Akka[A/SE]	Ellice[C/CA]	Lumar[C/CZ]	Rifle[C/UK]	Barke[EBC & P/AT]
Alexis[A & C/DE]	Elo[A & C/EE]	Magda[C/AT]	Rubin[C/CZ]	Beka[A/UNK]
Alliot[S/DK]	Esterel[M/FR]	Maltine[C/NL]	Sahara[C/DZ]	Bowman[A & Y/US]
Alondra[C/AT]	Etu[C/FI]	Manley[A & C/CA]	Scarlett[A, C, EBC & P/AT]	Brenda[A/AT]
Amagi Nijo[A & C/JP]	Feebar[A/US]	Maresi[C/DE]	Schooner[A & BBM/AU]	Conquest[A/CA]
Antto[S/SE]	Felicie[C/FR]	Maris Mink[A/UK]	Semal[C/DK]	Copeland[M/CA]
Arapiles[A & C/AU]	Fergie[C/UK]	Maris Puma[C/UK]	Shannon[A & C/AU]	Defra[A/DE]
Astoria[S/FR]	Forrest[C/AU]	Maris Trojan[C/UK]	Sherpa[A/UK]	Dicktoo[A/US]
Aura[A/DE]	Franklin[A & IMC/AU]	Mars[A/UNK]	Shonkin[C/US]	Drummond[M/UNK]
Ayr[C/UNK]	Gairdner[C & JW-SA/AU]	Maud[C/SE]	Skiff[C/AU]	Excel[M/US]
Baronesse[A & C/UNK]	Galan[C/CSK]	Meltan[C/SE]	Sloop[A & C/AU]	Fatran[A/CZ]
Barque[A & C/AU]	Galaxy[C/AU]	Melusine[C/FR]	Steptoe[A & C/US]	Guardian[A/CA]
Baudin[W & CG/AU]	Galleon[A & C/AU]	Metcalfe[C & CG/CA]	Stirling[JW-WA/AU]	Harrington[A, C & K/CA]
Berolina[A & C/UNK]	Gleam[A & C/UK]	Monarch[C/UNK]	Sultan[C/NL]	Haruna Nijo[A/JP]
Blenheim[A & C/UK]	Gobernadora[C/MX]	Montcalm[A/CA]	Tadmore[C/UNK]	Kustaa[EBC, R & C/FI]
Bonanza[A & Y/CA]	Golden Promise[A & C/UK]	Moravian 37[M/US]	Tallon[A & C/AU]	Landlord[A/UK]
Brindabella[A/AU]	Golf[A/UK]	Mundah[A/AU]	Terno[C/UNK]	Legacy[M/CA]
Brise[S/UK]	Grimmett[A & JW-T/AU]	Narin[C/UNK]	TG-Harr 6 & 58[C/AU]	Moravian III[Coors/US]
Budvar[M/UNK]	Grit[C/DE]	Natasha[A/FR]	Thuringia[A & C/AT]	Morex[A & BJ/US]
Calicuchima[A/MX]	Halcyon[A & C/UK]	Neruda[S/UK]	Tilga[C/AU]	Olli[A/FI]
California Mariout[A & C/EG]	Hamelin[W & CG/AU]	Novum[C/UNK]	Tore[C/NO]	Onslow[A/AU]
Caminant[A & CB/DK]	Heriot[A & C/UK]	O'Connor[C/AU]	Torkel[C/SE]	Osiris[A/FR]
Carlsberg II[A/DK]	Herta[A/SE]	Optic[C/UK]	Torrent[C/UK]	Pasadena[S/DE]
Carmaque[C/DE]	Hiproly[A/UNK]	Orbit[C/CSK]	Tremois[C/FR]	Pirkka[B/FI]
Caruso[C/DK]	Jarek[C/CSK]	Ortolan[C/DE]	Trine[C/NO]	Polygena[A/AT]
Cask[C/UNK]	Jersey[S/NL]	Oxbow[A & C/CA]	Trinity[A & C/UK]	Pomo[C/FI]
Chalice[S/UK]	Jubilant[C/UNK]	Parwan[C/AU]	Triumph[A/UNK]	Prefect[S/SE]
Chariot[C/UK]	Kaputar[A & C/AU]	Patty[A & C/FR]	Tweed[C/UK]	RISO 1508[A & C/DK]
Chebec[A & C/AU]	Keel[A & C/AU]	Picola[C/AU]	Tyne[A/UK]	Robust[M/US]
Chinook[C/US]	Kendall[M/CA]	Pipkin[C/UK]	Ulandra[C/AU]	Saana[P/FI]
Cicero[S/DK]	Kilta[C/FI]	Pitcher[A & C/UK]	Vada[C/UNK]	TR 306[A/CA]
Clarity[C/UK]	Klaxon[A/UK]	Plaisant[A/FR]	Vantage[A/CA]	Trebon[A/UNK]
Clipper[A, C & PTW/AU]	Koral[C/CZ]	Pokko[C/FI]	Varunda[C/NL]	Unicorn[K/JP]
Conlon[M/UNK]	Korinna[C/DE]	Pompadour[C/FR]	Venture[C/UNK]	Vantmore[A/CA]
Cooper[C/UK]	Koru[C/UK]	Prestige[S/UK]	Viking[A/UNK]	Vigdis[A/NO]
Cork[C/FR]	Kredit[C/UNK]	Prisma[A/UK]	Viktor[C/UNK]	Viskosa[S/DE]
Corniche[A & C/DE]	Krona[C/DE]	Proctor[A & C/UK]	Volga[C/FR]	
Daphne[C/UK]	Kympii[C/FI]	Profit[C/UNK]	Waranga[C/AU]	
Dash[C & DM/UNK]	Ladik[C/UNK]	Puffin[A & C/UK]	Waveney[C/UK]	
Derkada[C/UNK]	Lara[C/AU]	Reform[S/DK]	Weeah[C/AU]	
Derkado[AWCC & C/AT]	Libra[C/CL]	Regatta[A/UK]	Wikingett[EBC & S/SE]	
Dhow[T/AU]	Lindwall[JW-T/AU]	Reggae[C/NL]	Yagan[C/AU]	
Digger[AWCC/UK]	Lofty Nijo[W/JP]	Research[C/AU]	Zephyr[A/UNK]	

2.3.1.2 Effect of environment on expression of the SE trait

The effect of environment on the presence and or absence of the SE protein was analysed by screening four varieties (3 SE +ve; 1 SE –ve) grown over two years (2001 and 2002) at nine different sites throughout Europe (Table 2). The SE +ve varieties were Luberon, Scarlett and Wikingett, the SE –ve variety was Barke. In this trial the effect of the environment did not appear to have an influence on the SE protein. Varieties identified as SE +ve, remained SE +ve across all sites tested. With three samples of Barke (SE –ve), two from the Czech Republic (2001 and 2002), and one from Germany (2001), there was a faint MW ~12000 SE band immunodetected (+/-) (Table 2). Given that the banding was quite faint this is most likely as a result of some cross-contamination of the individual samples, rather then a change to the presence of the SE protein in Barke. Control samples of Luberon, Scarlett, Wikingett (SE +ve) and Barke (SE –ve) were also obtained from a separate source to the samples analysed in this trial. These samples were electrophoresed on the same SDS-PAGE screening gels as the samples from different growth locations.

Table 2: The environmental effect on the presence and or absence of the SE protein. Samples were grown at 9 different sites over two years in 7 countries. Barley was grown at three separate sites across Finland. [1]BOR is Jokioinen, LOU is Mietoinen, LH is Hauho, and they are the names of the sites/villages in Finland.

Country	Barke (SE −ve)		Luberon (SE +ve)		Wikingett (SE +ve)		Scarlett (SE +ve)	
Year	2001	2002	2001	2002	2001	2002	2001	2002
Finland								
-BOR[1]		-		+		+		+
-LOU[1]		-		+		+		+
-LH[1]	-	-	+	+	+	+	+	+
Sweden	-	-				+	+	+
France	-	-	+	+		+	+	+
Germany	+/-	-			+	+	+	+
Czech Republic	+/-	+/-	+	+		+	+	+
UK	-	-			+	+	+	+
Denmark	-	-	+	+		+	+	+

2.3.1.3 Sensitivity of SE immunoblot test to contamination

The sensitivity of the SE antibody for the level of contamination within a sample was tested by mixing pure seed of Barke (SE −ve) with either 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 40%, 50% or 60% of Scarlett (SE +ve), with a minium of 100 seeds tested. The results obtained indicate the antibody is sensitive to small level of contamination, with as little as 4% contamination detectable (i.e. 4/100 seeds) (Figure 2).
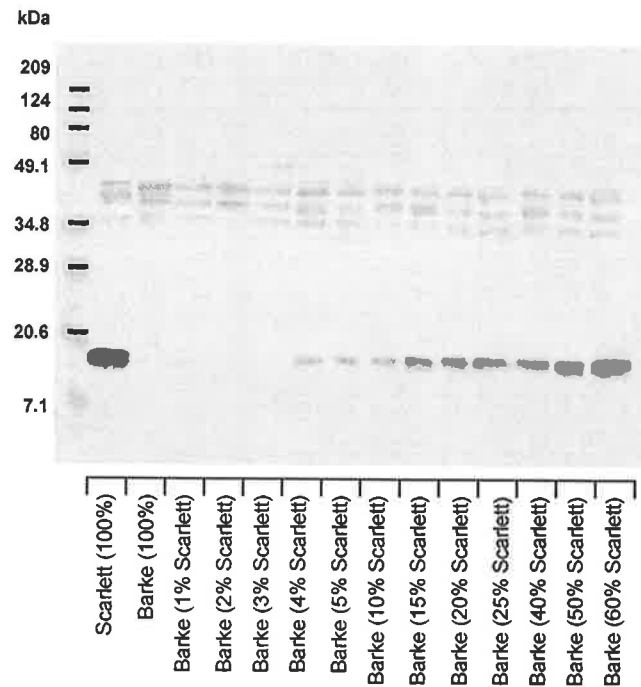
Figure 2: SDS-PAGE immunoblot analysis using the anti-SE antibody (1/1000) dilution of total protein extracts from barley for the level of contamination detectable with this antibody.

2.3.1.4 Examining the SE trait in a breeding program

One hundred and thirteen lines from the stage 3 and twenty-three lines from the stage 4 South Australian Barley Improvement Program trials grown in 2003 were screened for the SE protein. All stage 4 material tested was identified as SE +ve (Table 3), however, four lines from the stage 3 trials were identified as SE –ve (Table 4). Two of the four stage 3 lines identified were derived from the same cross. The lines WI3968 and WI3969 (SE –ve) were derived from a cross between Dhow/Cork//TG-Harrington-58, however WI3967 was also derived from the same cross and was found to be SE +ve. Similarly, WI3954 (SE –ve) was derived from a cross between Baronesse/Keel//Dhow, however WI3941 and WI3953 were also derived from this same cross and they were

found to be SE +ve. The parental varieties in the crosses producing the SE –ve lines (WI3968, WI3969 and WI3954) have all been identified as positive for the SE protein (Table 1). The WI3978 line identified as SE –ve, is a cross between MCAL21/Chebec-Harrington-113//TG-Harrington, and although MCAL21 and this specific TG-Harrington line have not been tested, there is both SE -ve (Chebec-Harrington-113 and Harrington) and SE +ve (Chebec, Tallon and Grimmett) material in this cross. This breeding program data obtained on the SE protein indicates that as only four out of the 136 varieties screened were found to be SE –ve, that the SE +ve trait is dominant in current and developing Australian germplasm.

Table 3: SE screening of the South Australian Barley Improvement Programs stage 4 lines grown at Tuckey, South Australia in 2003.

Name	Synonym	Pedigree	Quality	SE +/-
SloopSA	WI3167	CCN 6-3*WI2875/22/3D/20	Malt	+
WI3668	WI2976*WI2875-22*MONARCH//40	WI2976*WI2875-22*MONARCH//	Malt	+
WI3786	BX97S;033/40	CHIEFTAN/VB 9623//MANLEY/VB 9104	Malt	+
WI3408	BX 97S;30D/508	Chieftain/Barque//Manley/VB9104	Malt	+
WI3416	WI2976*Sloop**Galaxy//1043	WI2976*Sloop**Galaxy//	Malt	+
WI3837	BX98A;080/280	WI 3102//KEEL/FITZGERALD	Malt	+
Hamelin	WABAR2104	Stirling/Harrington	Malt	+
VB9935		Chebec/9104//WI2875*22	Malt	+
WI3851	BX98A;080/398	WI 3102//KEEL/FITZGERALD	Malt	+
VB0024		9018/Alexis//9104	Malt	+
SBWI-3	SA95319-2		Malt	+
WI3586res1747		Keel/Gairdner//Gairdner	Malt	+
WI3823	BX98A;062/67	WI2976/Gairdner//WI3102	Malt	+
VB0105		Franklin/9104//(9104*1)	Malt	+
Baudin	WABAR2080	Stirling/Franklin	Malt	+
VB0021		9018/Alexis//9104	Malt	+
WI3835	BX98A;080/266	WI 3102//KEEL/FITZGERALD	Malt	+
Dhow	WI3102	(WI-2808*(Skiff*Haruna nijo)/9)	Malt	+
WI3842	BX98A;080/323	WI 3102//KEEL/FITZGERALD	Malt	+
WI3838	BX98A;080/282	WI 3102//KEEL/FITZGERALD	Malt	+
WI3580	BX98A;019/42 (+5H)	Chebec/Harrington-113//VB9525/3/VB9524/4/VB9524	Malt	+
SBWI-4	SA99043		Malt	+
SloopVic	VB9935	VB9743/SLOOP//SLOOP/3/SLOOP	Malt	+

Table 4: SE screening of the South Australian Barley Improvement Programs stage 3 lines grown at Tuckey, South Australia in 2003. SE −ve varieties are in **bold** and are underlined; SE +ve varieties derived from the same cross as these SE −ve varieties are in *italics* and are underlined. WI3102 = Dhow, WI2976 = Keel.

Name	Synonym	Pedigree	Quality	SE +/-
WI3953	*BX98S;136-52*	*Baronesse/WI2976//WI3102*	*Malt*	+
WABAR2213			Malt	+
WI3988	BX98S;273-10	Pitcher/WI2976//WI3102	Malt	+
WI3600res45/5		Keel/Gairdner//Gairdner	Malt	+
SA99036	G97071-2		Malt	+
WI3866	FITZGERALD*KEEL/10	FITZGERALD/KEEL	Malt	+
WI3821	BX98A;062/113	WI2976/Gairdner//WI3102	Malt	+
VB0112		9307/(9104*1)	Malt	+
94S931W-42-29		Onslow/Franklin sib//Mundah/Blenheim	Malt	+
VB0229	97-121D*028	9524/9507*16//ND11231-12	Malt	+
WI3966	BX98S;326-84	WI2976///Sahara/2723-30//Sloop/4/BX98A;070	Malt	+
WI3856	BX98S;120D/12	19IBON97/WI2976//WI3102/Optic	Malt	+
WI-3416-1576		WI2976*Sloop**Galaxy//	Malt	+
WI3935	BX98S;346-3004	WI3102/BX98A;079	Malt	+
WI3958	BX98S;302-53	TR232/WI2976//WI3140	Malt	+
WI3944	BX98S;157-1007	BX98A;032/WI3102	Malt	+
WI3937	BX98S;185-1001	Cooper/WI2976//WI3102	Malt	+
WI3941	*BX98S;136-1016*	*Baronesse/WI2976//WI3102*	*Malt*	+
WI3661	Monarch**Sloop*WI2976//1006	Monarch**Sloop*WI2976//	Malt	+
WI-3416-1572		WI2976*Sloop**Galaxy//	Malt	+
WI3864	BX98S;315D/78	VB9524/Fanfare//Gairdner/WI2976	Malt	+
WI3991	BX98S;278-37	Pompadour/WI2976//WI3102	Malt	+
Loftynijo	SBWI-1	SA93013	Malt	+
WI3979	BX98S;360-10	WI3145/Scarlet//WI3102	Malt	+
WI3938	BX98S;276-1017	Pompadour/Barque//WI3102	Malt	+
VB9926		WI2808/Alexis	Malt	+
VB0111		(9104*1)/PI366444	Malt	+
WI-3416-1570		WI2976*Sloop**Galaxy//	Malt	+
WI3940	BX98S;353-1009	WI3102/Scarlett//WI3102	Malt	+
WI3974	BX985;221-82	Gairdner/WI2976//WI3102	Malt	+
WI3863	BX98S;315D/7	VB9524/Fanfare//Gairdner/WI2976	Malt	+
WI3850	BX98A;080/395	WI 3102//KEEL/FITZGERALD	Malt	+
WI3827	BX98A;080/18	WI 3102//KEEL/FITZGERALD	Malt	+
WI3981	BX98S;362-25	WI3148/Barke//WI3102	Malt	+
VB0039		Chebec/9104//WI2875*22	Malt	+
WI3820	BX98A;061/129	WI2976/Gairdner//Gairdner	Malt	+
WI3789	BX97S;042/27	CHIEFTAN/WI 2976//FITZGERALD	Malt	+
WI3967	*BX98S;348-2*	*WI3102/Cork//TG-Harrington-58*	*Malt*	+
WI3945	BX98S;270-1008	Pitcher/Chebec-Harrington-113//WI3102	Malt	+
VB0135		9524/VB9622*1	Malt	+
WI3983	BX98S;364-7	WI3148/Optic//WI3102	Malt	+
WI3960	BX98S;302-94	TR232/WI2976//WI3140	Malt	+
WI3869	FITZGERALD*KEEL/9	FITZGERALD/KEEL	Malt	+

Name	Synonym	Pedigree	Quality	SE +/-
SA99026	G97058		Malt	+
WI3831	BX98A;080/256	WI 3102//KEEL/FITZGERALD	Malt	+
WI3971	BX98S;267-16	Oxbow/WI2976//WI3140	Malt	+
WI3936	BX98S;346-3049	WI3102/BX98A;079	Malt	+
WI3973	BX985;221-67	Gairdner/WI2976//WI3102	Malt	+
SA99013	G97046		Malt	+
WI3839	BX98A;080/292	WI 3102//KEEL/FITZGERALD	Malt	+
WI3987	BX98S;270-52	Pitcher/Chebec-Harrington-113//WI3102	Malt	+
WI3663	BX96-95D/29	Chieftain//WI2976/WI2875-22	Malt	+
WI3849	BX98A;080/389	WI 3102//KEEL/FITZGERALD	Malt	+
WI3841	BX98A;080/318	WI 3102//KEEL/FITZGERALD	Malt	+
WI3934	BX98S;291-2078	TG-Harrington-6/4/WI2976///Sahara/2723-30/	Malt	+
WI3975	BX98S;230-17	Landlord/BX98A;068	Malt	+
WI3957	BX98S;257-43	ND1123-12/VB9623//WI3102	Malt	+
WI3670	Monarch**Sloop*WI2976//1008	Monarch**Sloop*WI2976//	Malt	+
VB0128		WI2875/(9316*104)	Malt	+
WI3587-5	WI3587-b	Keel/Gairdner//Gairdner	Malt	+
WI3993	BX98S;286-71	SD3-Bamy/Gairdner//Gairdner/WI2976	Malt	+
VB0227	97-116D*117	9524/9507*16//Alexis	Malt	+
WI3826	BX98A;080/115	WI 3102//KEEL/FITZGERALD	Malt	+
WI3990	BX98S;278-23	Pompadour/WI2976//WI3102	Malt	+
WI3965	BX98S;326-71	WI2976///Sahara/2723-30//Sloop/4/BX98A;070	Malt	+
WI3994	BX98S;292-4	TG-Harrington-58/4/WI2976///Sahara/2723-30	Malt	+
WI3995	BX98S;118D/9	19IBON97/WI2976//WI3102	Malt	+
WI3397	(Waveney*Chebec)*Vic-9104//16	(Waveney*Chebec)*Vic-9104/	Malt	+
SA99045	G97077-2		Malt	+
WI3978	**BX98S;234-3**	**MCAL21/Chebec-Harrington-113//TG-Harrington**	**Malt**	-
WI3986	BX98S;264-131	Oxbow/WI2976//WI3102	Malt	+
WI3968	**BX98S;348-17**	**WI3102/Cork//TG-Harrington-58**	**Malt**	-
WI3951	BX98S;116-39	19IBON97/WI2976//WI3099/Barke	Malt	+
WI3586res1739		Keel/Gairdner//Gairdner	Malt	+
WI3959	BX98S;302-59	TR232/WI2976//WI3140	Malt	+
WI3587-14	WI3587-f	Keel/Gairdner//Gairdner	Malt	+
WI3632	SH93029*WI2976*CHEIFTAN//100	SH93029*WI2976*CHEIFTAN//	Malt	+
WI3396	(Alexis*Chebec)*WA-87s671//13	(Alexis*Chebec)*WA-87s671//	Malt	+
WI3996	BX98S;118D/49	19IBON97/WI2976//WI3102	Malt	+
WI3982	BX98S;362-41	WI3148/Barke//WI3102	Malt	+
WI3969	**BX98S;348-45**	**WI3102/Cork//TG-Harrington-58**	**Malt**	-
WI3855	BX98A;080/90	WI 3102//KEEL/FITZGERALD	Malt	+
WI3962	BX98S;317-21	VB9524/Fanfare//VB9728	Malt	+
VB0114		9307/(9104*1)	Malt	+
WI3992	BX98S;278-40	Pompadour/WI2976//WI3102	Malt	+
WI-3416-1573		WI2976*Sloop**Galaxy//	Malt	+
WI3662	WI2976*WI2875-22**GALAXY//14	WI2976*WI2875-22**GALAXY//	Malt	+
WI3970	BX98S;114-78	19IBON97/WI2976//TR232/Chebec-Harrington-1	Malt	+

Name	Synonym	Pedigree	Quality	SE +/-
WI3423	(DH115*WI-2875/1)*(Amaji nijo*A	(DH115*WI-2875/1)*(Amaji nijo *Alexis)/	Malt	+
WI3954	**BX98S;136-82**	**Baronness/WI2976//WI3102**	**Malt**	-
WI3825	BX98A;080/103	WI 3102//KEEL/FITZGERALD	Malt	+
WI3984	BX98S;366-19	WI3148/Scarlett//WI3102	Malt	+
VB0209	95-019B*9-7-011	Harrington/9104*1//WI2875*22	Malt	+
SA95302s/1			Malt	+
WI3846	BX98A;080/363	WI 3102//KEEL/FITZGERALD	Malt	+
WI3946	BX98S;224D-133	Gleam/VB9524//Gairdner/WI2976	Malt	+
WI3854	BX98A;080/61	WI 3102//KEEL/FITZGERALD	Malt	+
WI-3416-1574		WI2976*Sloop**Galaxy//	Malt	+
WI3587	BX98A;061/19	WI2976/Gairdner//Gairdner	Malt	+
WI3963	BX98S;326-16	WI2976///Sahara/2723-30//Sloop/4/BX98A;070	Malt	+
WI3587-9	WI3587-e	Keel/Gairdner//Gairdner	Malt	+
WI3961	BX98S;302-97	TR232/WI2976//WI3140	Malt	+
WI3980	BX98S;362-16	WI3148/Barke//WI3102	Malt	+
WI3972	BX98S;267-28	Oxbow/WI2976//WI3140	Malt	+
WI3947	BX98S;120D-212	19IBON97/WI2976//WI3102/Optic	Malt	+
WI3586	BX98A;061/124	WI2976/Gairdner//Gairdner	Malt	+
WI3985	BX98S;264-73	Oxbow/WI2976//WI3102	Malt	+
WI3964	BX98S;326-55	WI2976///Sahara/2723-30//Sloop/4/BX98A;070	Malt	+
WI3989	BX98S;273-103	Pitcher/WI2976//WI3102	Malt	+
WI-3416-1569		WI2976*Sloop**Galaxy//	Malt	+
WI3939	BX98S;349-1003	WI3102/Cork//WI3102	Malt	+
WI3788	BX97S;041/208	CHIEFTAN/VB 9624//WI 2976/(SAH/WI 2723//CHEBEC)	Malt	+
94S931W-6-18		Onslow/Franklin sib//Mundah/Blenheim	Malt	+

2.3.2.1 Inheritance of the SE trait

The genetic basis of the SE polymorphism was identified in an F_2 population of Unicorn (SE –ve)/Gairdner (SE +ve). The ratio of present SE +ve to absent SE -ve was found to be segregating 3:1 (83 SE +ve: 29 SE –ve; 3:1 ratio $\chi^2 = 0.0476$, P > 0.01). This ratio demonstrates that the SE trait is controlled by a single locus with a dominant (SE +ve) and a recessive (SE –ve) allele.

2.3.2.2 Mapping of the SE trait

Thirty three varieties selected from 19 different mapping populations were screened by immunoblot for the SE trait (Table 1), to determine which populations would be suitable for interval mapping of the SE trait. The polymorphism identified with the SE

antiserum was screened by immunoblot in 92 lines from the Chebec (SE +ve)/Harrington (SE −ve) mapping population (Barr *et al.,* 2003b; Kretschmer *et al.,* 1997). The variation in the presence or absence of the SE protein was mapped to the short arm of chromosome 3H in this double haploid population (Figure 3). The SE trait mapped between the markers PSR1196/ABC171/PSR1316 and BCD089. The SE trait was found to be flanked by PSR1196/ABC171/PSR1316 (21.8 cM) and by BCD089 (22.5 cM) (Figure 3). Unfortunately, due to the large distance between these flanking markers (between 10 and 55 cM) a closer linkage of the SE trait to one of these markers was not possible. Linkage of the markers (PSR1196/ABC171/PSR1316 and BCD089) on the recently published barley consensus map (Karakousis *et al.,* 2003b) as well as in two other mapping populations (Galleon/Haruna Nijo (Karakousis *et al.,* 2003a) and Alexis/Sloop (Barr *et al.,* 2003a)) confirms that the SE locus is located within this region on chromosome 3H.
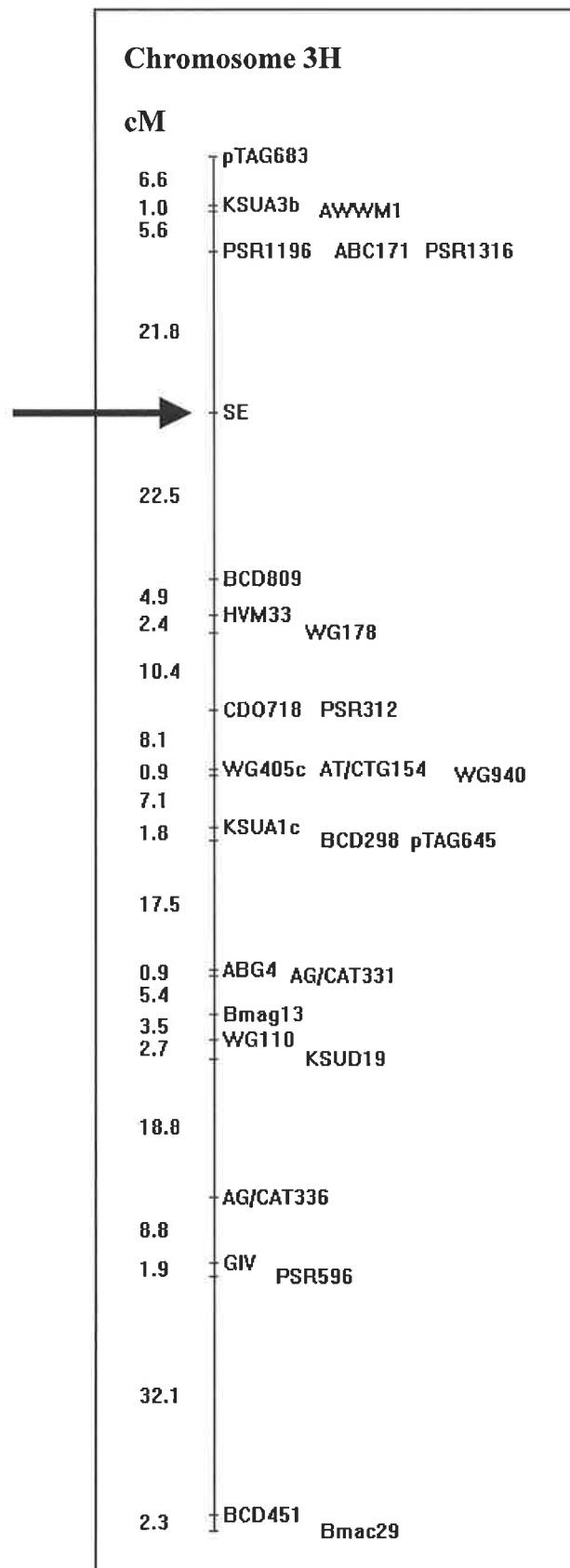
Figure 3: The map location of the SE trait on chromosome 3H (P = 0.0001), in the Chebec (SE +ve)/Harrington (SE –ve) population. The arrow marks the SE trait.

2.4 Discussion

The hordein polypeptides that are present in both barley and malt have been well characterised (Shewry, 1993), but to date their precise identities and roles in beer have not been well characterised. This study investigated a polymorphism that was observed in which some barley varieties contained a MW ~12000 band (SE +ve), while in other varieties this band was absent (SE -ve). This SE MW ~12000 protein detected in barley could also be detected in malt as well as in beer and in preparations made from beer haze indicating that this protein may influence final beer haze stability.

To examine the diversity of the SE protein, 219 Australian and international barley varieties, including a comprehensive selection of current and past malting varieties were surveyed (Table 1). Of these barley varieties tested, 83% were found to be SE +ve as detected using the SE antiserum, making this protein very common in both Australian and international germplasm. Of all the germplasm screened, the North American (e.g. Morex and Harrington) and the Scandinavian (e.g. Pirkka and Kustaa) had the highest incidence of SE –ve varieties. With regard to the Australian germplasm, given that in many instances the varieties used in the breeding programs have originated from such SE +ve varieties as Proctor, Prior, Research and Resibee (Fitzsimmons and Wrigley, 1984; Sparrow *et al.,* 2000, see Appendix A for this reference) it is not surprising that the Australian germplasm was found to be segregating as SE +ve. The pedigrees of the Finnish germplasm identified as SE –ve (Kustaa, Pirkka, Olli, Arra, Pomo, and Saana) contain both SE -ve varieties such as Olli and Kustaa and SE +ve varieties in Bonus (*pers. comm.* Dr Silja Home and Mr Reino Aikasalo) along with other varieties not tested in this study for the SE trait. SE –ve varieties such as Morex and Harrington have been widely used in the development of new varieties in the North American

breeding programs (anon), making the SE –ve allele very common in North American germplasm.

Western immunoblotting of protein samples from barleys, malts and beers using the SE antibody revealed complex patterns of polymorphism. A polymorphism at MW ~12000, along with a number of higher MW bands between MW 32000 and 98000 in both SE +ve and SE –ve varieties, indicative of hordein origin, were detected. The *lys 3a* Risø 1508 mutant (a variety known to be deficient in B/C hordeins) (Sørensen *et al.,* 1989) was tested for the SE trait. Risø 1508 was identified as SE –ve, however, the presence also of higher MW bands made the relationship of these bands with hordeins ambiguous. The B and C hordeins are highly polymorphic, both within and between genotypes (Shewry, 1993). Lines varying in their level or composition of B/C hordein were screened by immunoblot for the SE trait. All hordein lines screened were found to be SE +ve, and similar to the Risø 1508 mutant, the higher MW banding patterns between (32000-48000) were typical of those observed in both SE +ve and SE –ve varieties.

Characters that affect malting and or brewing quality (i.e. malt extract content, α- and β-amylase activity, diastatic power, malt β-glucan content, malt β-glucanase activity, grain protein content, kernel plumpness and dormancy) are quantitatively inherited and are variously influenced by the environment (Zale *et al.,* 2000). Studies evaluating genotype by environmental interactions have examined a range of malt quality traits and interactions between genotypes and sites. Studies have investigated β-glucanase, β-glucan and hordein fractions (Molina-Cano *et al.,* 1995; Molina-Cano *et al.,* 2000a,b; Swanston *et al.,* 1995). Australian studies have reported genotype and environmental

effects on malt quality including the key malt enzymes involved in diastatic power (Arends *et al.,* 1995; Gibson *et al.,* 1995), the barley α-amylase/subtilisin inhibitor (BASI) (Jarrett *et al.,* 1997) and Protein Z and lipid transfer protein (Evans *et al.,* 1999). Interestingly, in the study by Jarrett *et al.,* (1997), it was shown that the BASI inhibitor in Australian barleys was influenced by genotype but not by environmental factors. However, the genotype effect with BASI is most likely the effect of the quantitative variation that is described in this study compared to the qualitative variation, which is described in the other studies examining genetic x environment variation.

To examine environmental differences between malting barley cultivars in relation to the SE protein, samples grown in Finland, the Czech Republic, France, Sweden, Germany, the United Kingdom, and Denmark were analysed for the presence or absence of the SE protein (Table 2). In addition, of the 219 varieties screened for the SE trait, 51 were screened at least twice with samples obtained from different seed sources/growth locations (Table 1). Varieties identified as either SE +ve or SE –ve, were identified as SE +ve or SE -ve across all the different growth sites or sources of the seed tested. The results obtained from these trials indicate that it is likely that the presence of the SE protein is influenced only by genotype rather than by environmental factors. This conclusion can be extended further as the SE antibody is sensitive to a very small level of contamination, with as little as 4% contamination detectable (Figure 2), indicating that those varieties identified as either SE +ve or SE –ve are definitely SE +ve or SE –ve varieties.

As discussed previously, traditionally SE +ve varieties such as Proctor, Prior, Research and Resibee have been used as the basis of Australian barley breeding programs along

with the incorporation of a number of wild types and landraces. More recently, SE –ve varieties such as Harrington and Haruna Nijo have been incorporated into the breeding programs. It is of interest from a plant breeding perspective to examine the diversity of the SE protein within a breeding program and to examine this potential SE –ve material that has entered into the breeding programs. Stage 3 and 4 material from the South Australian Barley Improvement Program was screened for the SE trait (Tables 3 and 4). Four lines from the stage 3 trials were identified as SE –ve, however 2 of these lines were derived from the same crosses. The varieties used in the crosses for 2 of these SE –ve stage 3 lines have been identified as SE +ve (Table 1).

With regard to the pedigree of the SE –ve line WI3978 (MCAL21/Chebec-Harrington-113//TG-Harrington), the Chebec-Harrington-113 line is a fixed double haploid line from the Chebec/Harrington mapping population. When this line was tested it was found to be SE –ve, indicating that the source of the SE –ve material in WI3978 is the Chebec/Harrington-113 line. Given the pedigrees of the SE –ve lines WI3968 and WI3969 (Dhow/Cork//TG-Harrington-58) (Table 4) the most likely candidate for the SE –ve source material is TG-Harrington-58. TG-Harrington 58 when tested was identified as SE +ve (Table 1), however, TG-Harrington-58 is not a fixed line. F_2 seed was multiplied to the F_3 generation, then individual lines from this F_3 population were numbered as TG-Harrington lines. This indicates that there was still a 12% chance of an individual TG-Harrington plant being heterozygous for the Harrington allele (SE –ve). This segregation is the most likely explanation for the SE –ve status of WI3968 and WI3969.

The Baronesse/Keel//Dhow derived line (WI3954), was identified as SE −ve (Table 4), however all three of the parental lines are SE +ve (Table 1). This suggests an error was made in the actual parents used in the cross. Substitution of WI3954 with an alternative line during the breeding process appears unlikely given the very low frequency of SE −ve genotypes within the South Australian barley program (Tables 3 and 4).

Examination of stage 3 and 4 material from the South Australian Barley Improvement Program for the SE trait was undertaken to understand the diversity of the SE trait, and to examine those varieties whose pedigrees consisted of both SE +ve and SE −ve varieties. These varieties included Hamelin (Stirling/Harrington), Dhow (WI-2808*(Skiff*Haruna Nijo)/9), VB0209 (Harrington/9104*1//WI2875*22), and WI3980 (WI3148/Barke//WI3102). As only four out of the 136 stage 3 and stage 4 varieties screened were found to be SE −ve, this demonstrates that the SE +ve trait is dominant within both past, current and developing Australian germplasm.

Haze active proteins isolated from beer have been found to be derived primarily from barley (*Hordeum vulgare* L.) storage proteins (hordeins) (Asano *et al.*, 1982). The B and C hordeins have been found to be encoded by linked loci, designated *Hor1* and *Hor2* on the short arm of chromosome 5 (1H) and D hordein by a loosely linked locus (*Hor3*) in the proximal region of the long arm (Shewry, 1993). The γ hordeins have not yet been conclusively identified but are likely to be encoded by the *HrdF* (*Hor5*) locus, which is located distally to *Hor2* (Netsvetaev and Sozinov, 1982; Shewry and Parmar, 1987). The SE protein has been identified on the short arm of chromosome 3H in the double haploid population, Chebec (SE +ve)/Harrington (SE −ve) (Figure 3), thus making it unlikely that the SE protein is of hordein origin. However, it may be possible

that this 3H locus may encode a protease that cleaves hordein in a specific way, thus resulting in the presence or absence of a specific hordein fragment that has specific immunogenicity, i.e. the presence or absence of the SE protein as detected by the SE antiserum. To conclude that the SE protein is definitely not of hordein origin, sequence data of the protein is required. The identification of the SE protein will be discussed in Chapter 5.

Harrington is a high quality malting line, thus the Chebec (SE +ve)/Harrington (SE –ve) population was selected to try and identify the map location of the SE malting/brewing quality trait. The chromosomal location of the SE trait was identified on the short arm of chromosome 3H in the Chebec/Harrington mapping population. From a breeding perspective, the location of the SE trait between markers PSR1196/ABC171/PSR1316 and BCD089 could potentially be used to facilitate marker-assisted selection (MAS) of the SE trait. However, it would be useful if molecular markers more closely linked to the SE trait could be developed, as they could be valuable in assessing large numbers of cross-progeny in diverse crosses for the SE trait.

In the Chebec/Harrington mapping population no QTL's associated with malt and or beer quality have so far been identified on chromosome 3H (Barr *et al.*, 2003b). Of the 180 QTL's reported in the literature for 29 barley and malt quality phenotypes, only 19 have been located on chromosome 3H (Hayes *et al.*, 2001). Of these 19 phenotypes only 5 have been associated with the same chromosome region as the SE trait identified in this study. These include fine-coarse difference in the Harrington/TR306 population (Mather *et al.*, 1997), extract (wort) viscosity in Steptoe/Morex population (Larson *et al.*, 1997), β-glucan (malt), in both the Harrington/TR306 and Steptoe/Morex

populations (Han *et al.*, 1995; Mather *et al.*, 1997) and grain N or protein in the Steptoe/Morex population (Larson *et al.*, 1997). Many malting quality traits have been examined in mapping populations. In this current study, the SE trait has been shown to be one of the few specific malting/brewing quality traits that has been traced back to genetic variation in barley and the genetic basis of the SE trait has been characterised.

2.5 Conclusions

In this study, a polymorphism for haze active proteins was identified and surveyed by immunoblot throughout the brewing process, in barley, malt, beer and in haze. The identification and minimisation of malt haze active proteins in beer could be a useful option to improve beer haze stability during storage. This would reduce the need or requirement for traditional colloidal stabilisation treatments, reducing brewery costs and environmental wastes. Using interval mapping analysis the presence or absence of the SE protein was determined to a single locus on chromosome 3H with a recessive (SE − ve) and dominant (SE +ve) allele. The information generated in this study on the SE trait could be used by breeding programs to transfer, by conventional crossing methods in combination with MAS, these variants into the Australian germplasm and improve the quality of Australian malting barley.

Chapter 3

The influence of the SE –ve and SE +ve phenotypes on beer haze stability

Abstract

The formation of permanent haze in bright beers is a serious quality problem, which places limitations on the storage life of the product. Pilot brewing trials found that beer brewed from SE –ve malt varieties formed less haze in haze force testing trials (5 days at 55°C, 1 day at 0°C) and in natural ageing testing trials, than beer produced from SE +ve malt varieties. These results demonstrate that the selection of SE -ve malt varieties for brewing provides an opportunity to improve the haze stability of beer.

3.1 Introduction

There are a number of factors that can impact upon haze formation in beer. Most commonly the mechanism attributed to haze formation is the formation of protein-polyphenol complexes (Bamforth, 1999a; Siebert, 1999). Beer contains a number of barley proteins, modified chemically and proteolytically during the malting and brewing processes, which can influence haze stability. Proteins with high levels of proline, and polyphenols with higher degrees of polymerisation, are most likely to form haze. During beer storage, polyphenol monomers (the proanthocyanidins catechin, epicatechin and gallocatechin) are slowly oxidised to form proanthocyanidin dimers and higher polymers. These include procyanidin B3 and prodelphinidin B3, which can bind more efficiently to the proline residues to form large cross-linked protein-polyphenol networks, that produce large colloidal particles causing maximum light scattering (Siebert, 1999).

When a low molecular weight polyphenol cross-links with a protein through weak interactions such as hydrogen bonding, a chill haze is formed (Bamforth, 1999a). Chill haze forms when beer is cooled to 0°C but redissolves when the beer is warmed again to 20°C or more. Any haze remaining in beer at 20°C or higher is defined as permanent haze.

Haze active proteins isolated from beer, have been found to be derived primarily from fragments of the main barley storage protein group, the hordeins. These protein fragments consist of several different molecular weights, and are relatively rich in proline (Asano *et al.,* 1982). This description matches the characteristics of the N-terminal sequence repeats of hordeins that are rich in glutamine and proline (Shewry, 1993), and are produced by proteolytic modification during malting and mashing. The distribution and mole percentage of proline within haze active proteins (hordeins) has also been shown to be directly related to haze forming potential (Outtrup *et al.,* 1987; Outtrup, 1989).

There are a number of critical time points and components of the brewing process, from the raw materials through to packaging and storage that can have an influence on beer haze stability. These have been shown to include the duration, temperature, concentration and pH of the mash and the dissolved oxygen throughout the mashing process (Moll, 1987), the kettle boil, cold conditioning, temperature during filtration (Hardwick, 1978), oxygen content after day 1 of fermentation and once the beer has been packaged (Bamforth, 1988), beer agitation and elevated temperatures during storage and transport (Glenister, 1975; Walters *et al.,* 1996; Wenn *et al.,* 1989), and the pH and alcohol content of beer (Siebert and Lynn, 2003; Siebert *et al.,* 1996a).

Conversely, during the brewing process, haze active proteins and polyphenols are removed from beer during wort boiling, fermentation and maturation and filtration. Overall levels of haze active (HA) proteins and polyphenols may therefore reflect conditions favoring both their formation and loss from the final product. Haze formation in beer has been shown to occur at a rate which is a function of the concentration of the HA proteins and HA polyphenols at the time of packaging (McMurrough *et al.*, 1992).

To improve the colloidal stability of beer, residual HA protein, HA polyphenol or a portion of both need to be removed. In brewing, this is typically achieved by using stabilisation treatments such as silica hydrogel for the removal of HA proteins or polyvinylpolypyrrolidone (PVPP) for the removal of HA polyphenols (Siebert and Lynn, 1997a).

In this chapter, pilot brewing trials using SE +ve or SE −ve malt varieties as defined in Chapter 2, section 2.3.1.1 were used to test the hypothesis that the absence of the MW~12000 silica eluent (SE) protein in malt resulted in improved beer haze stability.

3.2 Materials and Methods

3.2.1 Malt Samples

Experiment 1 - Commercial malt samples of Unicorn (SE −ve) and Harrington (SE −ve), (Kirin Australia, Perth, Western Australia); Stirling (SE +ve), (Joe White Maltings, Perth, Western Australia); Schooner (SE +ve), (Barrett Burston Malting Company, Melbourne, Australia); Franklin (SE +ve), (International Malting Company, Corio, Victoria); Grimmett (SE +ve), (Joe White Maltings, Tamworth, NSW); Lindwall (SE

+ve), (Adelaide Malting Company, Adelaide, South Australia); and Gairdner (SE +ve) (Joe White Maltings, Adelaide, South Australia), were obtained from barley grown in Australia during the 1999 season.

Experiment 2 - Commercial samples of Stirling (SE +ve), (Joe White Maltings, Perth, Western Australia); Harrington (SE -ve) and Unicorn (SE -ve) (Kirin Australia, Perth, Western Australia) malts were obtained from the 2000 Western Australian growing season.

Experiment 3 - Commercial malt samples Barke (SE -ve), Saana (SE -ve) and Scarlett (SE +ve) (Polttimo Companies, Lahti, Finland) and Kustaa (SE -ve) (Raisio Malt, Raisio, Finland), were obtained from barley grown in Finland during the 2002 season. The malt quality characteristics of these samples were typical of commercially used malts in Australia and Finland (Appendix B -Tables 1-3).

3.2.2 Protein extraction, SDS-PAGE and immunoblotting

Ground barley and malt samples (20 mg) along with wort (330 µL), beer and haze (500 µL) samples were extracted or diluted into 1 mL SDS-PAGE sample buffer (5M urea, 4% SDS, Tris buffer pH 8.0) and 1% (v/v) 2-mercapto-ethanol. SDS-PAGE and immunoblotting was performed essentially as described in Chapter 2, section 2.2.2.

3.2.3 Small Scale Brewing Procedure (600 – 800 mL) - Experiment 1

Small scale brewing (SSB) was conducted as previously described by Stewart *et al.* (1998). Brewing trials were conducted using a 25 place IEC mash bath (Industrial Equipment and Control Pty. Ltd., Melbourne, Australia). The malt was ground to 0.7

mm using a Bühler-Miag (Braunschweig, Germany) mill. For each brew, 142.5 g of malt was added to 443 mL pre-warmed (69°C) deionised water (grist:water ratio 1:3). Mash pH was checked with pH 4 – 7 indicator strips to ensure it was between 5.2 and 5.7, with the pH adjusted with $CaSO_4$ or $CaCO_3$ if required. To the mash there was also the addition of 0.22 g $CaSO_4$ along with 55 U/L of Bioglucanase and Biocellulase (Quest International, Melbourne, Australia). Bioglucanase (1.4 mL) and Biocellulase (0.4 mL) were added to 30 mL ddH_2O and 1 mL of this was added to the mash. Mashing in was at 64°C for 40 min followed by an increase in temperature of 1°C/min to 74°C over 10 min, then the mash was held at 74°C for 10 min. The mash was separated by filtration through Postlip paper (330 mm Hollingsworth and Vose Company Ltd., Winchcombe, England) into 1-litre Schott bottles (Schott Glas, Mainz, Germany). Sparging was completed at 78°C to a final wort volume of 700 mL. The wort was boiled in a water bath for 60 min, 15 min before the end of the boil kettle finings were added (90 mg/L Whirlfloc T P424, Quest International). The wort was cooled to 20°C immediately after boiling and then transferred to 4°C overnight.

The wort was centrifuged at 10, 000 x g for 10 min to remove both the hot and cold trub, under sanitary conditions. The clarified wort was diluted to 10°P with sterile water and transferred to sterile 1-litre Schott bottles. The bottles were shaken vigorously to aerate the wort. A commercial lager yeast strain (slurry obtained from the South Australian Brewing Company, Adelaide, Australia) was pitched at a rate of 0.5% w/v. The wort was fermented with a sanitised (ethanol 70% v/v) airlock fitted, at 20°C for 4 days until the specific gravity was between 1-2 °P. The fermentation was transferred to 4°C for 72 h before finings were added (35 mg/L Biofine P19, Quest International). The ferments were then stored for a further 7 days at 4°C. After cold conditioning iso-

hop extract was added (96 μL/L, 30% α-acid) (Hop Suppliers, Australia). The iso-hop was prepared by adding 95 μL of the 30% α-acid extract to 1 mL EtOH before adding 1 μL per 1 mL of beer. The beer was centrifuged at 10 000 x g for 10 min before being filtered through Miracloth (Calbiochem, La Jolla, CA, USA) to remove the bulk of the remaining yeast.

Samples for haze force testing were also filtered through 0.45 μm sterile filters (Schleicher and Schuell, Keene, USA). The filtered beer (10 mL) was placed into a round bottom sample tube (Kimax culture tube 16 x 100 mm, Kimble Glass Inc., Vineland, NJ, USA) and pasteurised in a water bath (63°C for 22 min, equivalent to ~20 pasteurisation units (PU)). The vials were purged with CO_2 (5 sec) prior to adding the beer, and also after they were filled (head space). Each malt variety was brewed in triplicate.

3.2.3.1 Wort and Beer Analysis

The density and viscosity of wort and beer samples was determined using an AMV-200 Anton PAAR KG densitometer/microviscometer (Graz, Austria), using the 'rolling ball principle' (Appendix B, Table 4). Beer alcohol and bitterness were measured at the South Australian Brewing Company, Adelaide using a SCABA® automated analyser (Tecator, AB, Höganäs, Sweden) or the European Brewing Convention (EBC) methods (Analytica - EBC, 1998) method (9.8), respectively. Beer total polyphenols were determined using a modified EBC method (9.11) with 400 μL of beer used instead of the 8 mL as suggested in the assay. Beta-glucan in beer was determined using the EBC method (8.13.1). Total protein in beer was measured using the Bradford (1976), Coomassie blue dye binding assay using bovine serum albumin (BSA) as the standard.

Briefly, 200 μL of the Coomassie protein reagent (Bio-Rad, Richmond, CA, USA) was added to 50 μL of beer diluted in 750 μL of ddH$_2$O, and mixed by inversion. After 10 min the samples were read on a spectrophotometer (Shimadzu UV-160A) at 595 nm. Protein concentration was determined by referring to a standard curve prepared using BSA. Wort and beer analysis is shown in Appendix B, Table 4.

3.2.3.2 Haze Measurement - SSB

The haze stability of beer produced in the SSB trials was analysed in triplicate in the sample tubes previously described in section 3.2.3. Haze force testing was conducted by heating the vials at 55°C for 5 days in a water bath and then cooling them to 0°C in a cold room for 1 day before measurement. Three treatment regimes for each brew were used: Treatment 1; samples were filtered (0.45 μm), pasteurised and then haze force tested. Treatment 2; samples were pasteurised and then force tested and treatment 3; samples were filtered (0.45 μm), pasteurised then stored in the dark for 7 days at 20°C before measurement. Chill haze was measured at 0°C, permanent haze at 20°C. The vials were kept on ice while measuring the chill haze (0°C) and then were returned to 20°C for permanent haze measurements. Haze measurements were recorded in EBC formazin units (EBC FU) using a Hach 2100N Laboratory Turbidimeter (90°) (Hach, Loveland, CO, USA). Each vial was inverted gently (1-2 times) before reading to ensure no sediment remained on the bottom. SSB haze analysis data is contained in Appendix B, Tables 5a, b and c.

3.2.4 Pilot Brewing Trials (300 L) – Experiment 2

Three brewing trials were conducted at the University of Ballarat (Mt Helen Campus, Ballarat, Australia) using a DME brewing system (DME Brewing Services,

Charlottetown, PE, Canada), with a 300 L brew length. Trials were conducted with the assistance of Dr Peter Aldred (University of Ballarat). Trials 1 and 2 used Unicorn (SE –ve), Harrington (SE –ve) and Stirling (SE +ve) malts, trial 3 Harrington (SE –ve) and Stirling (SE +ve) malts. The timing of these three trials was separated with trial 1 completed in August/September 2001, trial 2 November 2001 and trial 3 August 2002. The malts were milled using a 2-roller mill (Wes Smith and Associates, NSW, Australia), with a gap setting of 0.8 mm. For each brew, 60 kg of malt was added to 180 L water (grist:water ratio 1:3). The mash conditions were those of a typical one-temperature infusion mash held at 65°C for 1 h. In addition, 20 mL Bioglucanase (Quest International), 6 mL Biocellulase (Quest International) and 100 g $CaCl_2$ were added at the start of mashing.

The wort was separated using a lauter tun with sparging completed at 78°C to a final kettle target volume of 330 L. The wort was boiled for 75 min and after 15 min at the boil bittering hops were added (125 g NZ super alpha pellets) (DME, Sydney, Australia). Fifteen minutes before the end of the boil aroma hops (125 g Willamette pellets) (DME) were added along with kettle finings (30 g Koppakleer G) (Progressive group, Melbourne, Australia) and 100 mL (0.1 w/v) $ZnSO_4$. The hot break was removed in a whirlpool and the wort pumped through a heat exchanger and cooled to pitching temperature (20°C). An ale yeast strain was used in all fermentations and the temperature was held at 18°C. The slurry was pitched at a rate of 0.5% w/v. Fermentations were for 12 to 16 days. The beer was conditioned for 2 weeks at 0°C before filtration using a plate and frame filter (TMCI Padovan, Conegliano (Treviso), Italy) containing 20, 40 x 40 cm Cuno Zeta Plus 10SL filter sheets (Cuno, Blacktown, NSW, Australia) and packaged into 20 L kegs (Stainless steel lined, poly-urethane

coated, Schäfer "Junior plus" kegs, Toronto, ON, Canada). A keg chosen at random was selected for bottling.

The bright beer was bottled at Lion Nathan (Sydney, Australia) using a counter pressure filler (Skerra Pty. Ltd., Sydney, Australia) with bottles filled at 200 kPa and sparged for 10 sec with CO_2 prior to filling. Final CO_2 levels were adjusted in trial 1 to 5.20 g/L (Unicorn), 5.85 g/L (Harrington) and 5.90 g/L (Stirling), in trial 2 to 5.57 g/L (Unicorn), 6.10 g/L (Stirling) and 5.30 g/L (Harrington) and in trial 3 to 5.60 g/L (Harrington), and 5.80 g/L and 5.65 g/L for the two Stirling brews. The beer was pasteurised to (~20 PU). The pasteurisation program was for 1 h, starting at 20°C before ramping to 40°C, then to 65°C before returning to 5°C. Haze force testing was conducted at the South Australian Brewing Company (Adelaide, Australia) with haze force test data obtained using a five day haze challenge protocol (5 days at 55°C in a water bath, 1 day at 0°C in a water bath). Haze was measured in EBC FU using a Hach 2100N Laboratory Turbidimeter (90°).

3.2.4.1 Wort and Beer Analysis

Wort specific gravities were determined using an Anton-Parr DMA 35 density meter (Graz, Austria) with the pH determined as measured at 20°C using a TPS LC80A pH meter (TPS, Springwood, Queensland, Australia) (Appendix B, Table 6a). Analysis of beers was completed by the Technical Department – Central Laboratory, Tooheys Brewery (Lion Nathan, Sydney, Australia) (Appendix B, Table 6a and 6b). The analysis included SCABA – Alcohol (%v/v), original, apparent and real extract (°P), and apparent fermentability (%). SCALA – Bitterness (BU), pH and Colour (EBC). Volatile flavour components – Acetaldehyde (mg/L), dimethyl sulphide (μg/L) ethyl

acetate (mg/L), n-propanol (mg/L), iso-butanol (mg/L), iso-amyl acetate (mg/L) iso-amyl alcohol (mg/L), ethyl hexanoate (mg/L) ethyl octanoate (mg/L) total fused alcohol (mg/L), ester index, ester ratio, diacetyl (mg/L), pentanedione (mg/L) and total VDK (mg/L) were measured in trials 2 and 3 (Appendix B, Table 6b).

3.2.5 Pilot Brewing Trials (100 L) – Experiment 3

Pilot brewing trials were conducted in a 100 L capacity pilot brewery at VTT Biotechnology (Espoo, Finland) (Ahvenainen, 1983). Trials were conducted in Finland under the guidance and with the assistance of Mr Arvi Vilpola, Mr Eero Mattila and Dr Anu Kaukovirta-Norja. The malts (Barke, Kustaa, Scarlett, Saana) (26 kg) were hammer milled (2.25 mm sieve) and added to 78 L of water (grist:water ratio 1:3). Mashing salts $CaCl_2.2H_2O$ (30 g) and $CaSO_4.2H_2O$ (10 g) were added and the pH adjusted to give mash pHs between 5.28-5.38 (as measured at 20°C). Mashing in was at 48°C for 15 min (protein rest) followed by an increase in temperature of 1°C/min to 63°C for 30 min, then 72°C over 30 min, finally the mash was held at 80°C for 10 min. Wort separation was achieved using a Meura 2001 mash filter (3 frames, 500 mm x 500 mm, Meura, Tournai, Belgium). Boiling was for 60 min, bitter hop extract, Hallertau (α = 60%, dosing 21 g) was added at the beginning of the boil.

The hot break was removed in a whirlpool and the wort pumped through a heat exchanger where it was cooled to pitching temperature and adjusted to 10°P. A lager yeast strain was used in all fermentations. Primary fermentation was for 8 days at 10°C, secondary fermentation was for 15 days at 15°C in keg, stabilisation was for 5 days at 0°C in keg. Following conditioning, the beers were filtered using a plate and frame filter (SeitzSchenk Filtersystems GmbH, Bad Kreuznach, Germany) using 7, 20 x 20 cm

Seitz-EK filter sheets (Seitz-Werke GmbH, Type 20z, Bad Kreuznach, Germany). Filtration was conducted in a temperature-controlled room at 5°C. In this trial, three beers were filtered in succession using the same set of filter sheets. The bright beer was bottled using a counter pressure filler with bottles filled at 2.5 bar (initial pressure), 2.0 bar (counter pressure), gas flow rate equal to 5L/min, and sparged with CO_2 prior to filling.

3.2.5.1 Wort and Beer Analysis

The pH of wort and beer samples was determined using a PHM 83 pH meter (Radiometer, Copenhagen, Denmark) as measured at 20°C. Wort and beer analysis was carried out according to the following EBC methods (Analytica - EBC, 1998). Wort analysis included extract (°P) (Method 8.3), measured using an Anton-Parr DMA 58 density meter (Graz, Austria), colour (25 mm cuvette) (Method 4.7.2), F.A.N (mg/L) (Method 8.10) and soluble nitrogen (mg/L) (Method 4.9.1), measured using a Kjeltec Auto 1030 Analyser (Tecator). Fatty acids (mg/g) in wort were measured using a method described by Anness (1984) (Appendix B, Table 7).

Beer analysis included specific gravity (Method 8.3), measured using an Anton-Parr DMA 58 density meter (Graz, Austria), alcohol (%v/v) (Method 9.2.1), colour (25 mm cuvette) (Method 4.7.2) and polyphenols (mg/L) (Method 9.11). Total protein (mg/L) was measured using the Bradford (1976) Coomassie blue dye binding test, using BSA as the standard (100, 250 and 500 mg/L). Briefly, 50 µL of beer was added to 1.5 mL of the Coomassie protein reagent (Pierce, Oud-Beijerland, The Netherlands), and mixed by inversion. After 30 min the samples absorbance was read at 595 nm using a Shimadzu, UV-1601, spectrophotometer. Protein concentration was determined by

reference to a standard curve prepared using BSA. A PT standard analyser (2000PT) (Schneider Brew Service/Opto-ems, Wiesloch/Schriesheim, Germany) was used to measure the tannin and protein content of the beer as a predictor of beer haze stability. Haze force testing was conducted by heating the bottled beer at 55°C for 5 days in a temperature controlled oven and then cooling the beer to 0°C in a water bath for 1 day before measuring. Beer analysis data is shown in Appendix B, Table 8.

3.2.6 Haze stability analysis

The colloidal stability of beer produced from the pilot (100 L) – Experiment 3 and (300 L) – Experiment 2 trials was analysed in bottle (100 L trial) or transferred to a 1 inch round glass, 30 mL volume, sample cell (300 L trial), in triplicate using a (5 day 55°C, 1 day 0°C) haze force test procedure. Haze measurements were recorded (EBC FU) using a HZ-013 Lg – automatic ApS (Frederiksværk, Denmark) (100 L trials) or a Hach 2100N Laboratory Turbidimeter (90°) (300 L trials) calibrated in nephelos turbidity units (NTU) using formazin or StablCal® stabilised formazin turbidity standards (20, 200, 1000, and 4000 NTU), according to the supplier's instructions (Hach). Each bottle was inverted gently (1-2 times) before reading or decanting into the sample cell to ensure no sediment remained on the bottom. Chill haze was measured at 0°C, permanent or room temperature haze at 20°C.

In the HZ-013 Lg – automatic ApS system the chamber was filled with sterile ddH_2O to prevent the formation of condensation on the outside of the bottle when measuring the chilled (0°C) samples. The bottles were rotated in the chamber at least three times with readings taken at each rotation to ensure that imperfections in the glass did not bias the results. With samples measured in the Hach 2100N Laboratory Turbidimeter, the

optical compartment was purged with instrument grade air to minimise condensation on the outside of the sample cell when measuring the chilled (0°C) samples.

3.3 Results

3.3.1 Brewing Trials - Brewing with SE +ve and SE-ve malt varieties.

3.3.1.1 Experiment 1 - Small Scale Brewing (SSB) Trials

In a preliminary 50 L pilot-brewing trial conducted at Lion Nathan (Sydney, Australia), by Dr Evan Evans and Dr Marian Sheehan, eight barley varieties including Unicorn (SE –ve), Harrington (SE –ve), Schooner (SE +ve), Franklin (SE +ve), Gairdner (SE +ve), Stirling (SE +ve), Grimmett (SE +ve) and Lindwall (SE +ve) were used. In this single repetition trial it was found that beer brewed from varieties which did not contain the MW ~12000 SE protein were more resistant to haze force testing then beer which was brewed from varieties that did contain the MW ~12000 SE protein (Evans *et al.,* 2003) (Figure 1).
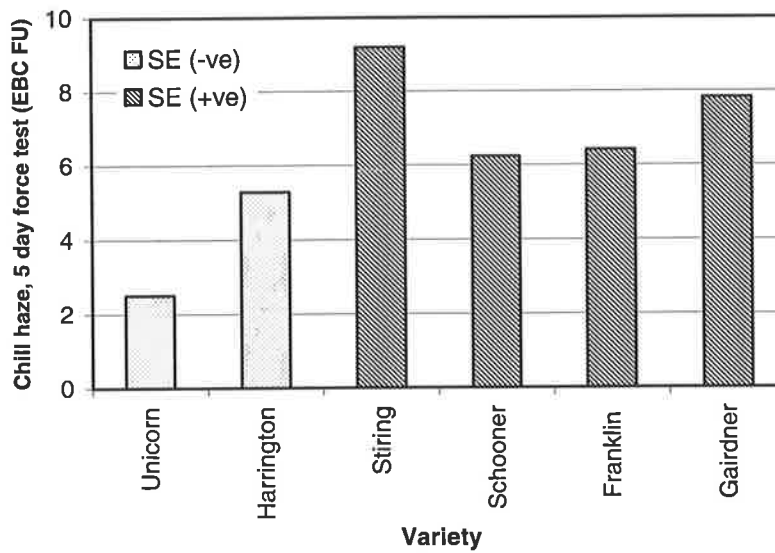
Figure 1: The colloidal stability of beer, produced from six pilot-scale brews, to the five-day chill haze force test. SE = silica eluent. (Reproduced from Evans *et al.,* 2003).

The previously described eight commercially malted samples were used to assess the haze stability of these malts using a SSB procedure in which each malt was used to produce experimental beers in triplicated brewing trials (Experiment 1). Levels of total protein, polyphenol and β-glucan, beer constituents potentially relevant to haze formation, were not significantly different in beers brewed from the different malts ($P < 0.05$). Moreover, no significant difference was observed between beers brewed from the 8 malts for haze stability ($P > 0.05$). There was substantial variability/error observed between replicate brews of the same malt (Figure 2 and Appendix B, Table 5a, b and c).
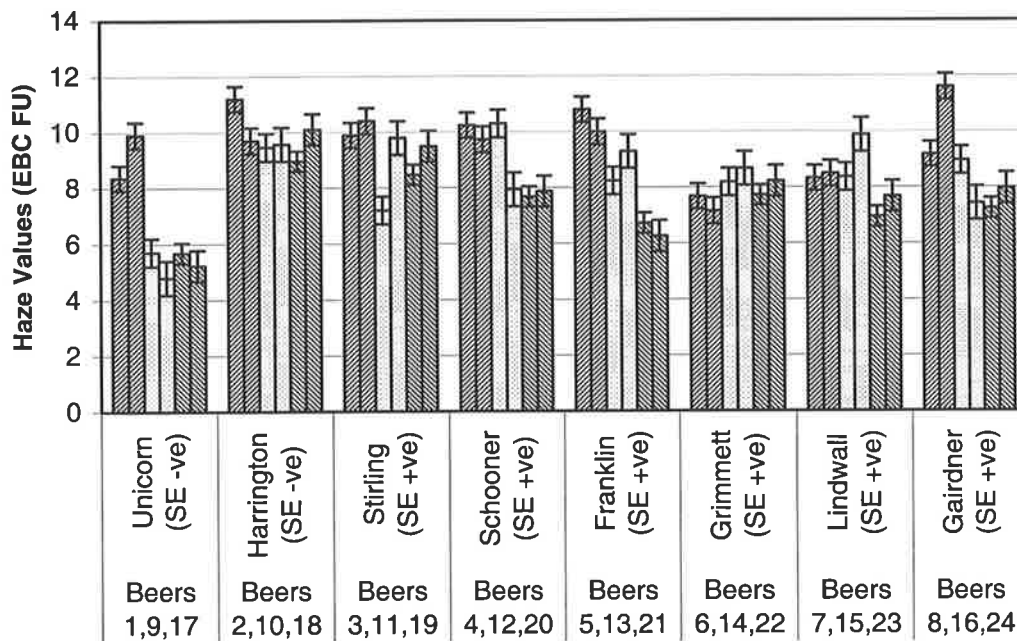
Figure 2: The colloidal stability of beer produced from SSB trials (Experiment 1) using a 5 day 55°C, 1 day at 0°C chill haze force protocol. Samples were filtered (0.45 µm) and pasteurised prior to force testing (treatment 1, section 3.2.3.2) ▨ Beers 1-8 (refer to Appendix B, Table 4), ⊡ Beers 9-16 (refer to Appendix B, Table 4), ▧ Beers 17-24 (refer to Appendix B, Table 4). Bars = Standard error of the mean; chill haze measurement. Duplicate samples of each brew were analysed.

Three haze force testing treatment regimes were used in this SSB trial as described in section 3.2.3.2. No improved haze stability was observed with those samples which were filtered (0.45 µm) before pasteurisation (treatment 1) compared to those that were only pasteurised (treatment 2) (Figures 2 and 3). The initial chill hazes ranged between 1.12 and 2.14 EBC FU (treatment 1 beers) (Appendix B, Table 5a) and 1.56 and 2.45 EBC FU (treatment 2 beers) (Appendix B, Table 5b). Five day permanent hazes ranged between 1.70 and 7.83 EBC FU (treatment 1 beers) (Appendix B, Table 5a) and 2.44 and 7.73 EBC FU (treatment 2 beers) (Appendix B, Table 5b). The haze results

obtained in this SSB trial were inconclusive in determining whether SE –ve malts could

be differentiated as having improved haze stability as compared to SE +ve malts.
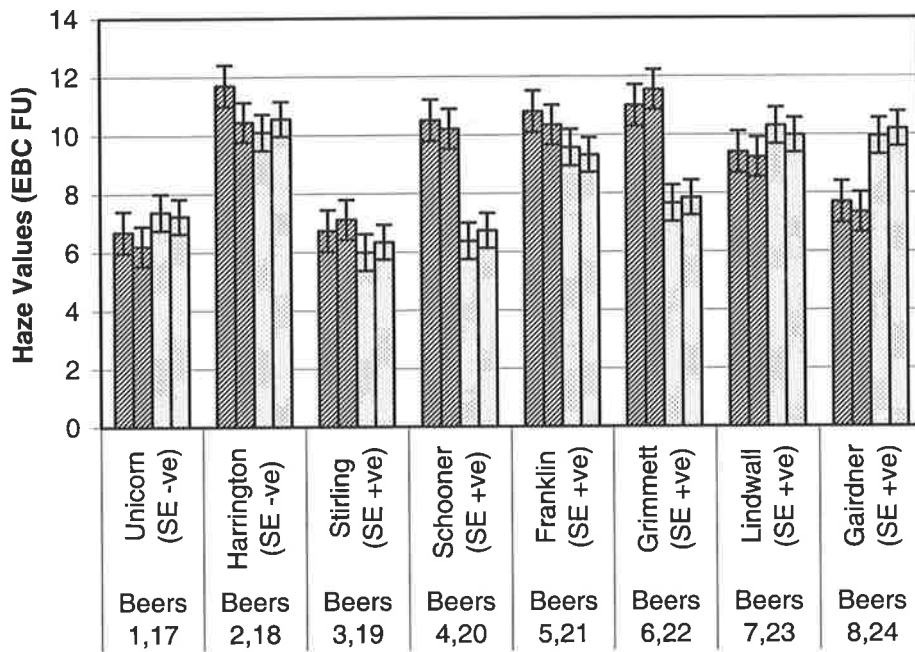
Figure 3: The colloidal stability of beer produced from SSB trials (Experiment 1) using

a 5 day 55°C, 1 day at 0°C chill haze force protocol. Samples were pasteurised prior to

force testing (treatment 2, section 3.2.3.2). ▨ Beers 1-8 (refer to Appendix B, Table 4)

▢ Beers 17-24 (refer to Appendix B, Table 4). Bars = Standard error of the mean, chill

haze measurement. Duplicate samples of each brew were analysed.

3.3.1.2 Experiment 2 - 300 L Trials (University of Ballarat)

Pilot brews (300 L), were completed at the University of Ballarat (Victoria, Australia),

with Australian grown and malted samples. Three malt varieties were selected for the

300 L pilot brewing trials; Unicorn (SE –ve), Harrington (SE -ve) and Stirling (SE +ve).

This selection was based on the five day forced haze results obtained in the preliminary

50 L pilot brewing trials conducted at Lion Nathan, with Unicorn the lowest at 2.50

EBC FU, Harrington at 5.20 EBC FU and Stirling the highest (SE +ve) variety at 9.20 EBC FU (Evans *et al.,* 2003) (Figure 1).

The 5-day force test chill hazes for the trial 1 beers in EBC FU were Unicorn 14.36, Harrington 7.57 and Stirling 8.25 (Figure 4). In trial 2 in EBC FU were Unicorn 15.69, Harrington 7.94 and Stirling 12.57 (Figure 4). In trial 3 in EBC FU were Harrington 15.19, Stirling 15.59 and 11.14 (Figure 4). The initial chill hazes for all nine beers were <1 EBC FU (Figure 4).

It was noted in trial 1 and trial 2 that mashing and lautering with the Unicorn malt was extremely difficult. During mashing, the surface of the grain bed was gelatinised and the bed pulled away from the side of the mash tun. At the end of mashing residual starch could still be detected by iodine testing. During recirculation, the wort took a long time to clarify and during lautering the wort required extra recirculation before it could be transferred to the kettle. These difficulties in brewing, and high haze results for the Unicorn malt, may in part have been due to a poor growing season (caused by drought conditions) in Western Australia and the relatively high total protein (12.4%) and a relatively low KI (40.6%) in the Unicorn malt sample compared to the other malt samples used in this trial (Appendix B, Table 2).
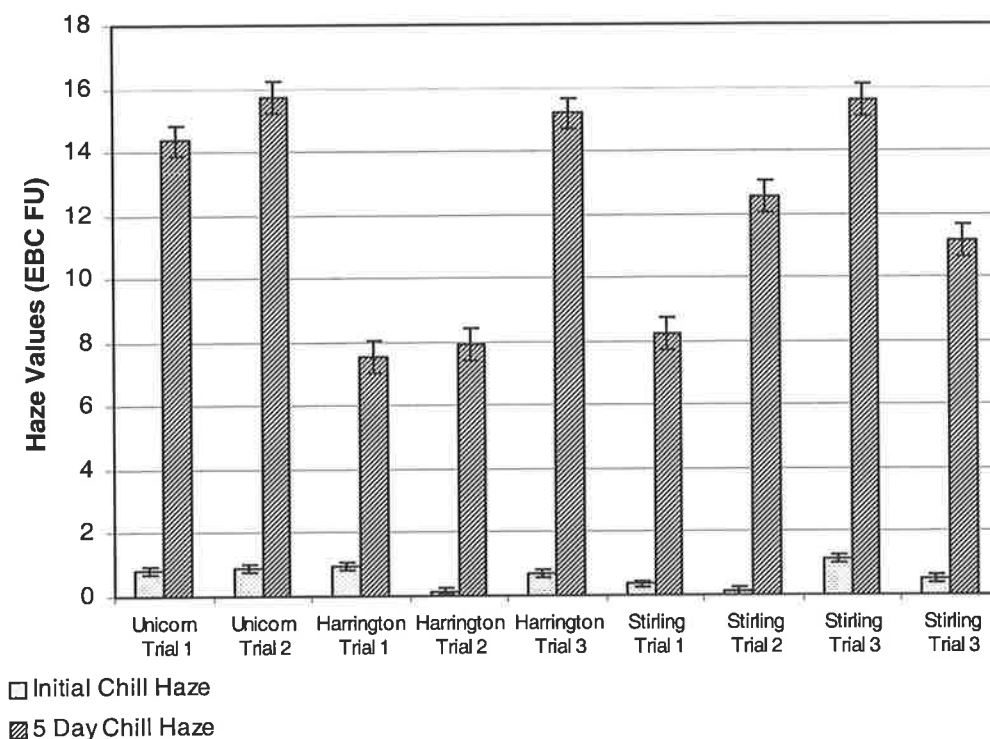
Figure 4: The colloidal stability of beer produced from 300 L brewing trials conducted at the University of Ballarat (Experiment 2) using a 5 day 55°C, 1 day at 0°C chill haze force protocol. Two brews were completed with Unicorn (SE –ve), three brews with Harrington (SE –ve) and four brews were completed with the Stirling malt (SE +ve). Bars = Standard error of the mean, chill haze measurement.

In this 300 L brewing trial the haze stability results were somewhat contradictory. Haze force testing (5 days at 55°C, 1 day at 0°C) of the brews found that two of the Harrington SE –ve brews (trials 1 and 2) showed relatively good colloidal stability (~7.0 EBC FU), however in trial 3 the colloidal stability was reduced (~15 EBC FU) (Figure 4). Three of the Stirling SE +ve brews (trials 2 and 3) showed reduced colloidal stability as expected, while the other Stirling SE +ve brew (trial 1) had comparable colloidal stability to the Harrington SE –ve brews (trials 1 and 2) (Figure 4). The 5-day

haze force test results for both the Unicorn brews (trials 1 and 2) were unexpectantly

high (Figure 4), given the result observed for Unicorn in the 50 L pilot brews conducted

at Lion Nathan (Evans *et al.,* 2003) (Figure 1).

SDS-PAGE immunoblot analysis with the SE antibody showed that the MW ~12000 SE

protein was present in the SE +ve malt and unfiltered beer, but was absent from the

filtered beer from both SE –ve and SE +ve varieties (Figure 5).
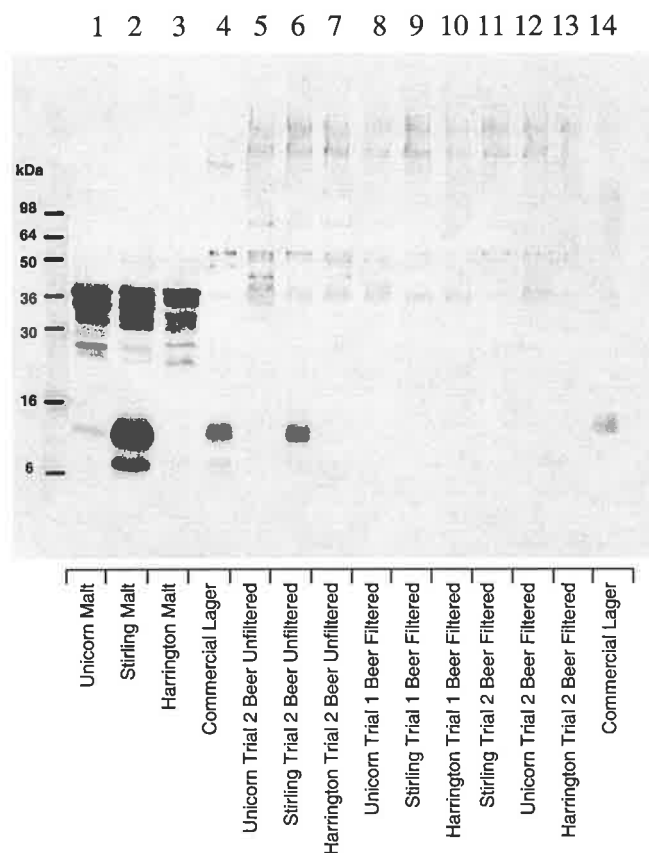
Figure 5: SDS-PAGE immunoblot using the anti-SE antibody (1/1000) dilution of total

protein extracts from malts (lanes 1-3); typical Australian lager beer (lanes 4 and 14);

pre filtration samples (lanes 5-7); trial 1 beers (lanes 8-10) and trial 2 beers (lanes 11-

13). The lanes were loaded at equivalent dilutions to allow for comparison.

To examine the predictability of the 5-day force test used in these brewing trials for the comparison of the haze stabilities obtained from SE +ve and SE –ve malt varieties, beer from trials 1 and 2 was stored for 12 months either in a cold room at 4°C or at room temperature (20°C). The permanent or room temperature (20°C) haze was then measured. The room temperature (20°C) haze values obtained from these 12 month stored (4°C or 20°C) beers displayed a similar trend to that of the forced haze results obtained in Figure 4, see Figure 6.
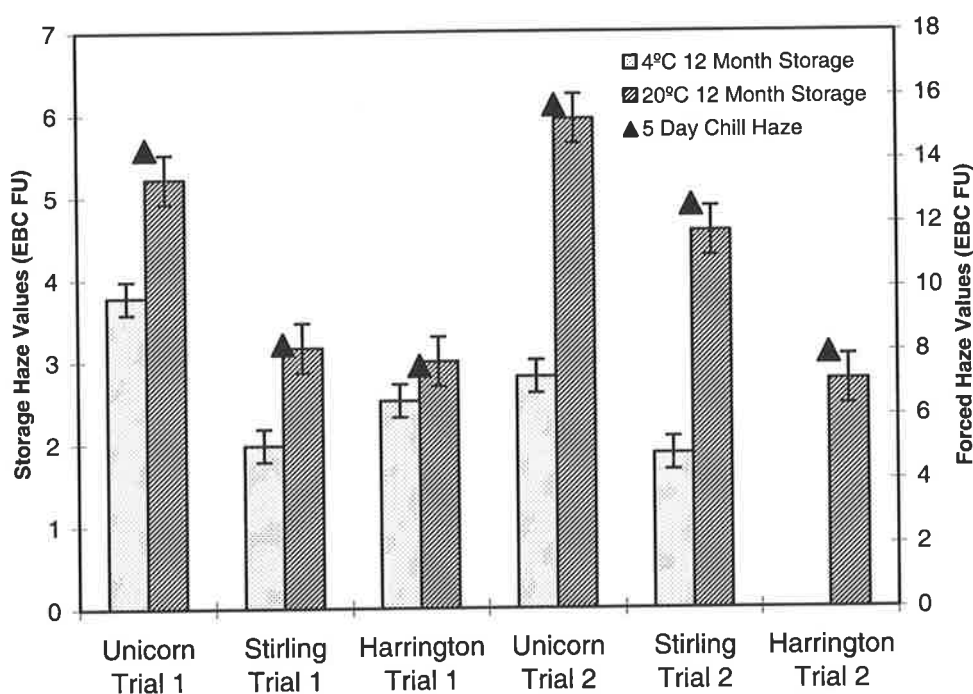
Figure 6: The colloidal stability of 12 month aged beer produced from 300 L brewing trials (experiment 2). Beer was aged at 4°C or 20°C for 12 months before the room temperature (20°C) haze was measured. The 5-day forced chill haze values used in this graph were obtained from Figure 4. A 4°C 12 month storage sample from Harrington (trial 2) was not available for analysis. Bars = Standard error of the mean, chill haze measurement.

3.3.1.3 Experiment 3 - 100 L Trials (VTT Biotechnology)

Eight 100 L pilot brews using four malt varieties, one variety (SE +ve; Scarlett) and three varieties (SE –ve; Barke, Kustaa and Saana) were conducted to follow the fate of the SE protein through the brewing process.

Initial analysis of the chill (0°C) haze data obtained in these 100 L brewing trials revealed a pattern of haze formation not commensurate with predicted haze stabilities of beers brewed with SE +ve or SE –ve malts. In these trials the haze stability data of the finished beer was not consistent, with variation observed between brews of the same malt, irrespective of whether the beer was brewed from a SE +ve or SE -ve malt variety. When the data was re-arranged in relation to the order of filtration a different pattern emerged. The chill haze data obtained in this trial suggested that the beer that was filtered first was more haze stable (5 days at 55°C 1 day at 0°C, force test) than the beer, which was filtered last (Figure 7). The initial chill hazes for all eight beers were <0.4 EBC FU (Figure 7).
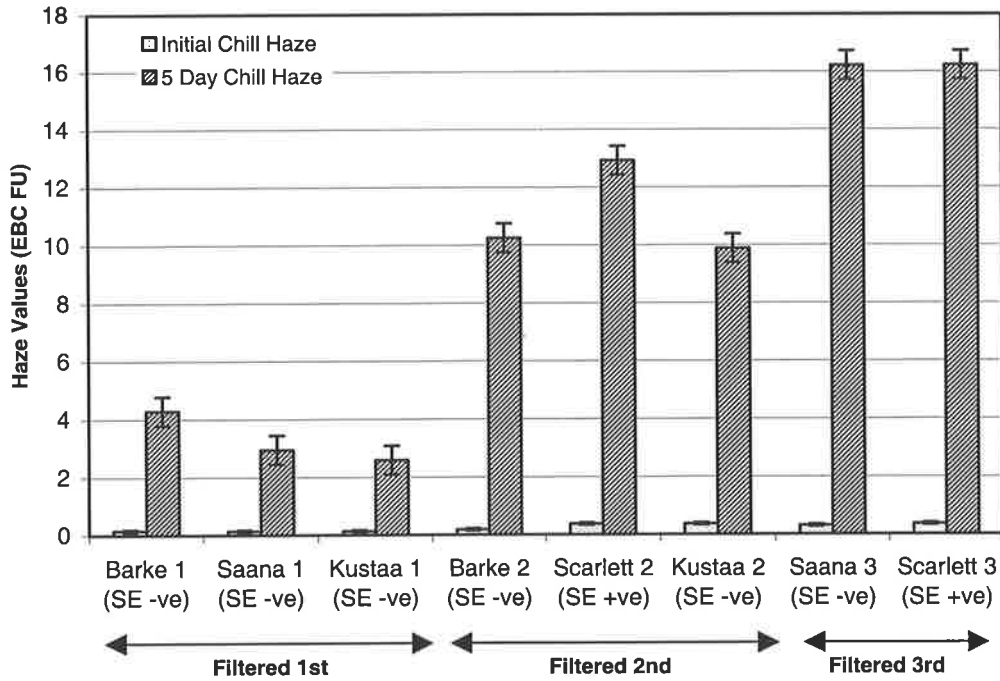
Figure 7: The colloidal stability of beer produced from 100 L pilot brewing trials conducted at VTT Biotechnology (Experiment 3) using a 5 day 55°C, 1 day at 0°C chill haze force protocol. The order of filtration is indicated below. Bars = Standard error of the mean, chill haze measurement.

High-nitrogen barleys have been suggested to contain higher levels of potentially troublesome material in relation to haze formation in beer (Bamforth, 1999a). However, the malt characteristics of the four malts used in these trials were all relatively similar, with respect to total protein (between 10.9% – 12%) and Kolbach indices (35.1 – 36.4) (Appendix B, Table 3). Wort analysis of these beers was consistent between beers brewed from the same variety and between varieties (Appendix B, Table 7).

The amount of total protein present in beer as measured by the Coomassie blue dye-binding assay (Bradford, 1976), was shown to increase as the amount of haze increased (Figure 8). The amount of protein/tannin (PT) present in the beer was measured using a PT standard analyser (2000PT). This PT measurement is used to predict haze stability. However, the PT values (mg/100 mL) obtained in this trial did not correlate with the haze values obtained (Figure 9).
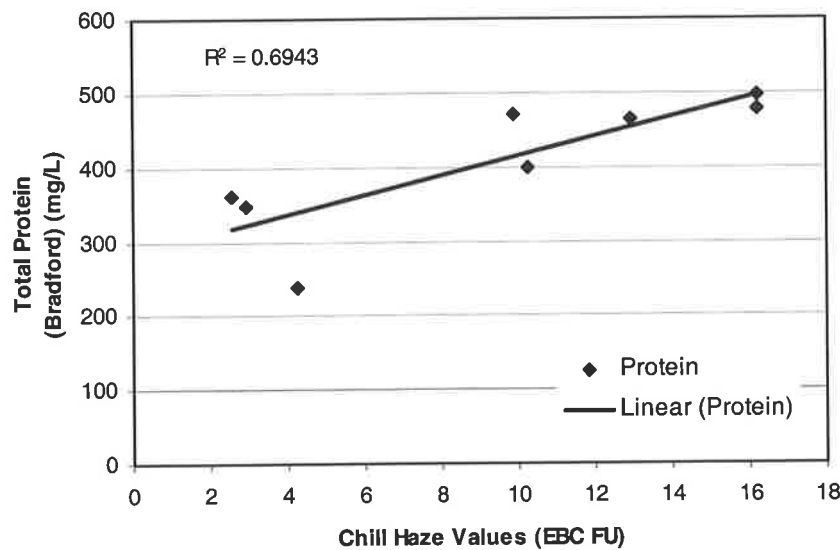
Figure 8: The relationship between 5-day chill haze and total protein as measured by Coomassie blue dye-binding. Eight 100 L pilot brews (from Experiment 3) were compared.
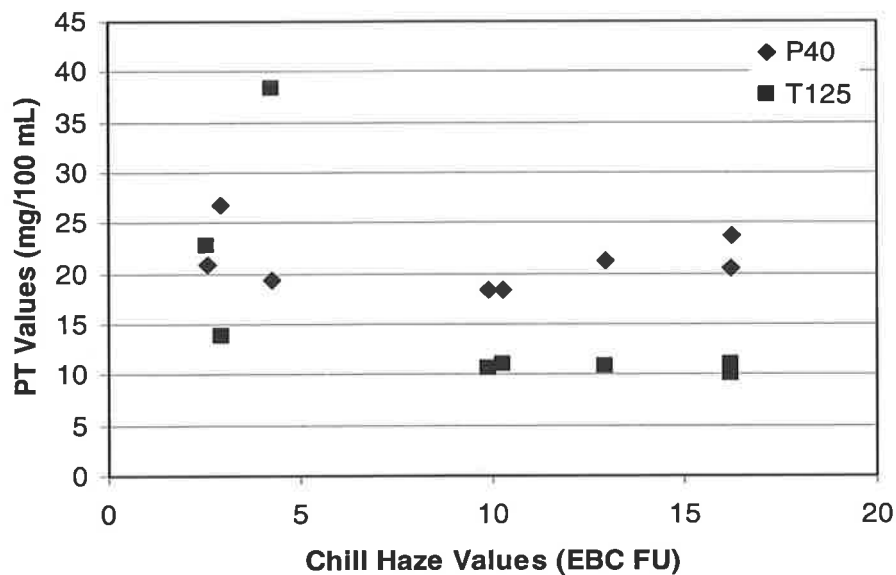
Figure 9: The relationship between PT values and 5-day chill haze determined from eight 100 L pilot brews (from Experiment 3). A high PT value equals a low predicted haze value and vice versa. The P40 value represents the protein component, the T125 value the tannin component.

SDS-PAGE immunoblot analysis with the SE antibody (1/1000 dilution), showed that the MW ~12000 SE band was absent in the SE −ve malts (Barke, Saana and Kustaa) and was not detected in either the unfiltered or filtered beer samples brewed from these malts. The SE protein was present in the SE +ve malt (Scarlett) and unfiltered beer, but was absent from the first filtered beer (Figure 10).
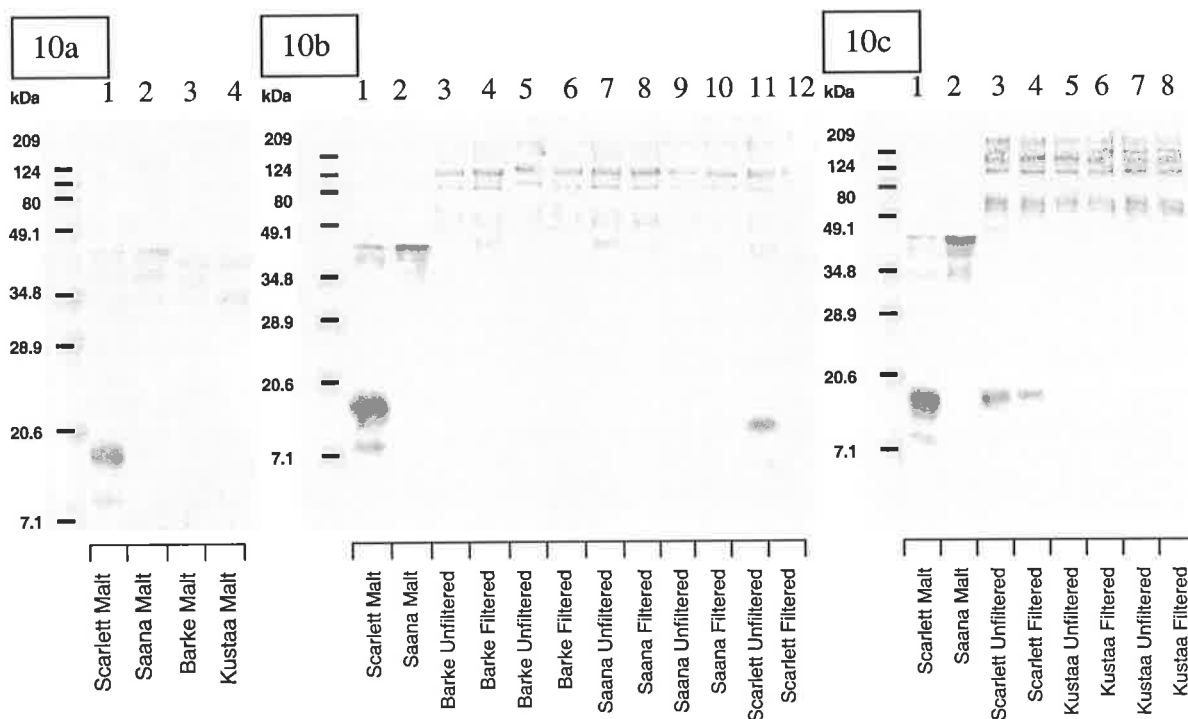
Figure 10: SDS-PAGE immunoblot using the anti-SE antibody (1/1000) dilution of total protein extracts from malts (figure 10a lanes 1-4; figure 10b lanes 1-2 and figure 10c lanes 1-2); pre filtration beer samples (figure 10b lanes 3, 5, 7, 9 and 11; figure 10c lanes 3, 5 and 7), and filtered beer samples (figure 10b lanes 4, 6, 8, 10 and 12; figure 10c lanes 4, 6 and 8). The lanes were loaded at equivalent dilutions to allow for comparison.

Two supplementary 100 L Scarlett (SE +ve) brews were completed at VTT Biotechnology (Espoo, Finland) by Mr Arvi Vilpola to re-assess the haze stability of an SE +ve variety, given that the previous results described for the Scarlett malt were from beer filtered 2nd and 3rd in the filtration run. The temperature of conditioning was reduced by Mr Arvi Vilpola from 0°C to -1°C, before filtering the beer at 0°C using a new set of the cellulose filter sheets for each beer. Combined the cold conditioning and filtration regime used with these two beers was successful and resulted in the over

stabilisation of the beer and thus improved colloidal stability (5 day at 55°C 1 day at 0°C, force test, Figure 11).
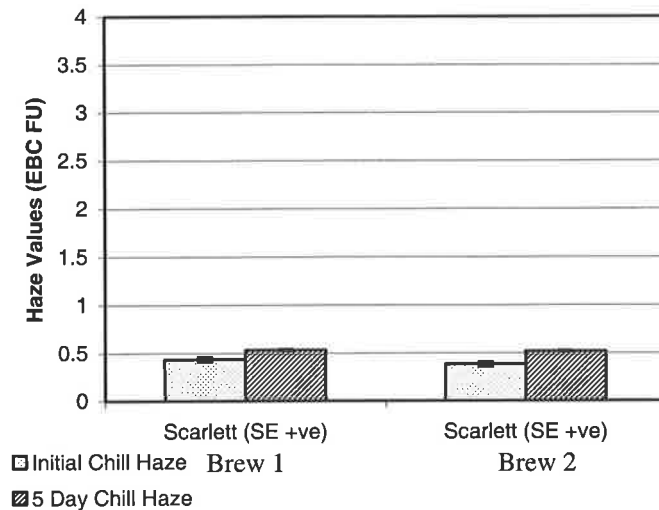
Figure 11: The colloidal stability of beer produced from two 100 L pilot brewing trials brewed with Scarlett (SE +ve) malt using a 5 day 55°C, 1 day at 0°C chill haze force protocol. The temperature of conditioning was -1°C, with filtration conducted at 0°C using a new set of filter sheets for each beer. Bars = Standard error of the mean, chill haze measurement.

SDS-PAGE immunoblot analysis with the SE antibody of these two supplementary Scarlett brews, showed that the MW ~12000 SE protein was present in the Scarlett (SE +ve) malt and unfiltered beer, but was absent from the filtered beer for both the Scarlett (SE +ve) brews (data not shown).

"Natural" storage of the Experiment 3 (100 L) beers was assessed by storing the beer in a cold room (6°C) and at room temperature (22°C) for 12 months. The permanent or room temperature (20°C) haze values were determined. The room temperature (20°C)

natural ageing haze values showed a similar trend to that of the 5-day haze force test results obtained for these beers (Figure 12).
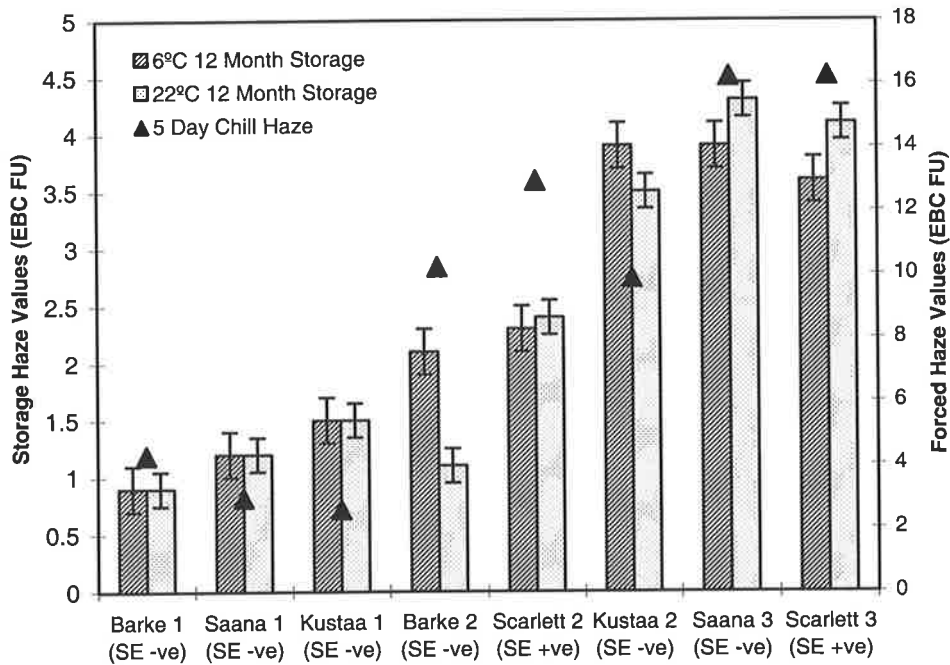
Figure 12: The colloidal stability of beer produced from 100 L pilot brewing trials (Experiment 3). Beer was aged at 6°C or 22°C for 12 months before the room temperature (20°C) haze was measured. Five day forced chill haze values used in this figure were obtained from data shown in Figure 7. Numbers listed next to the barley varieties indicates the order of filtration of these beers as shown previously in Figure 7. Bars = Standard error of the mean, chill haze measurement.

3.4 Discussion

The prediction of a beer's potential haze stability is an important quality control measurement for brewers to ensure that a beer maintains its quality to its 'best before' date. To gain a timely prediction of this quality parameter brewers use accelerated aging (force testing) protocols (holding the beer at elevated temperatures (i.e. 55°C or 37°C) for a number of days, before 1 day at 0°C, followed by clarity assessment), measurement of haze in line during filtration etc., beer protein content and, more recently, the PT analyser and PPT salt mediated precipitation analyser (Schneider and Raske, 2000) to monitor the stability of beer produced in the brew house. Breweries aim to package a product that will remain clear for a number of months, judging by 'best before' dates this is between 6-12 months.

The SSB method used in these experiments has been successfully applied in evaluating beer filtration (Stewart *et al.,* 1998). However in this investigation, where haze stability was being assessed, the method was not valid. With the small-scale method there are limitations with regard to the control of a number of factors important in haze stability compared to that of the pilot or the brew-house scale. Critical to the rate of haze formation in beer is the content of oxygen both during brewing and especially once the beer is packaged (Bamforth, 1988). After packaging oxygen should not be allowed to enter into the beer, as oxygen in beer after packaging promotes the polymerisation of polyphenols that can lead to greater colloidal instability problems.

Oxidation of the beer post fermentation was unavoidable in the SSB trial as the system does not allow for adequate control of oxidation. The beer was exposed after cold conditioning when the hops were added, during filtration (where it was centrifuged and

then filtered through Miracloth) and during transfer of the beer to the sample vials used in these haze stability trials. Permanent haze can form in beer after the polymerisation of polyphenols and their subsequent interaction with proteins. Five-day permanent haze values in these SSB trials ranged between 1.70 and 7.83 EBC FU (treatment 1 beers) (Appendix B, Table 5a) and 2.44 and 7.73 EBC FU (treatment 2 beers) (Appendix B, Table 5b) indicating that it was likely that there was polymerisation of polyphenols. The small sample size (10 mL beer) being force tested may also have been a factor for the high haze values obtained however, the 5-day chill hazes for those beers which were not force tested (treatment 3; beers filtered (0.45 μm) before pasteurisation, then stored at 20°C) were considered to be high for that test (*pers. comm.* Dr Marian Sheehan, based on initial haze values obtained from the 50 L trials using the same malts at Lion Nathan). The initial chill hazes in this trial ranged between 1.21 and 2.27 EBC FU (Appendix B, Table 5c). Consequently the SSB method was not found to be valid for comparing the haze stability of beer brewed from SE +ve or SE –ve malts.

The discrepancy in the haze stability of the 300 L Ballarat beers (Figure 4) is most likely to be a function of the sampling point during filtration for the beer used for haze stability testing. As a random keg was selected for bottling along with the kegs filled randomly it is unclear as to where in the filtration run each of the kegs was filled. Given the results observed in the 100 L trials (VTT Biotechnology) (Figure 7) it may be suggested that those beers with high colloidal instability were sampled from kegs filled during the later stages of filtration and vice versa. The absence of the SE protein from the Stirling (SE +ve) beer as shown by immunoblot (Figure 5) cannot be adequately explained by the extensive proteolysis that may occur during the brewing process removing the band because the MW ~12000 SE protein can be detected in typical

commercial beers (Chapter 4, section 4.3.2, Figure 8). Two distinct possibilities may explain the phenomena seen. These arise from the condition of the beer and of the filtration media at the time of bottling respectively.

En route to and through the filter, beer should be kept as cold as possible to ensure that precipitated material (haze precursors) does not return back into solution and re-enter the bright beer (Bamforth, 1999a). The chill haze values obtained in the 100 L trials (VTT Biotechnology Experiment 3) showed beers that were filtered earlier in a series were more haze stable (as assessed by haze force tests) than beer filtered last, irrespective of whether the beer was brewed from a SE +ve or SE –ve malt variety (Figure 7). During the filtration process it is possible there was an increase in the temperature of beer going through the filter and that this had an influence on the solubility of beer and haze proteins, including the MW ~12000 SE protein, which were as a consequence thereafter able to pass through the filter into the bright beer rather than remain as aggregates retained by the matrix of the filter material. Further testing of the temperature changes of beer during these stages of packaging is required to more fully investigate this possibility.

However, a more likely explanation is that the onset of chill haze was coincident with an increase in the level of total protein in the filtered beer, both within replicate brews and beers brewed from different malt varieties (Figure 8). The filter sheets used in the 100 L trial, (Seitz EK) were a mixture of a cellulose pad impregnated with kieselguhr, perlite and polyamineacetate. If the filterability of beer is poor, the differential pressure within the filter increases, resulting in a reduction in the flow volume and a delay in the filtration process. During the filtration run, the flow rate did not decrease nor did the

differential pressure increase which would have indicated that the filter sheets were blocked and required replacement. These results suggest that the filter sheet protein binding sites remove the SE and other proteins until saturated. Once the filter is saturated, these proteins again appear in the beer and beer colloidal stability decreases (Figures 7 and 10). The effect of both temperature and protein on the filterability of the beer is examined in further detail in Chapter 4 of this thesis.

When like beers are compared in terms of order of filtration (100 L trials), the haze data obtained from the first filtered Barke, Saana, and Kustaa beers (Figure 7) is similar to that reported by Evans *et al.,* (2003) for the Unicorn and Harrington beers (Figure 1). To compare the 2nd filtered haze data from the Scarlett (SE +ve) brew to that of the 2nd filtered beer of Barke (SE −ve) and Kustaa (SE −ve) the Scarlett brew had a haze value of 12.92 EBC FU as compared to the Barke and Kustaa brews, 10.25 and 9.88 EBC FU, respectively. When the 3rd filtered beers, Saana (SE −ve) and Scarlett (SE +ve) were compared the potential benefit of brewing with a SE −ve malt was not observed with both beers having poor colloidal stability (Figure 7).

In the commercial brewery where beer is filtered before being placed back into holding tanks and subsequently mixed before packaging, the possible effect of improved haze stability at the start of a filtration run as seen in these 300 L (experiment 2) and 100 L (experiment 3) brewing trials would not be observed.

Post fermentation, cold conditioning or lagering of beer is in part employed by brewers to remove by precipitation haze active proteins and polyphenols before packaging. The length of time and temperature of cold conditioning or lagering is very important in this

process. Brewers normally lager their beer at approximately 0°C (Lewis and Young, 2001) but what was observed with the second Scarlett brews was that if the lagering process was made more stringent using a lower temperature, <0°C, the SE protein was removed (most likely along with other proteins) and beer haze stability improved substantially compared to that expected for beer brewed from an SE -ve malt (Figure 11). Recently, Miedl and Bamforth (2004), have indicated that short periods of cold conditioning at very low temperatures (e.g., -2.5°C) are efficacious for haze stabilisation.

The chill haze values obtained in the 100 L and 300 L brewing trials (Figures 4 and 7) were higher than would be expected in the commercial brew-house. In part, this may have been due to the pilot brews being full malt beers, containing extra HA components whereas the majority of commercial beers are made with adjunct that dilutes the HA components. Also, in these 100 L and 300 L brewing trials there were no stabilisation treatments added (e.g. silica or PVPP) that are routinely used by the brewing industry to improve colloidal stability. On the evidence to date it appears that SE -ve malt varieties confer improved colloidal stability; this confirms a similar conclusion reported by Evans et al., (2003).

Both the VTT (100 L) and Ballarat (300 L) brewing trials revealed a number of important questions that may have significant impact on brewers' options for improving the colloidal stability of their beer. Firstly, the trials show that it is possible to remove some HA proteins as a result of the beer filtration process. Both trials also pose questions as to what role the MW ~12000 SE protein has in colloidal stability. Is it directly involved in the formation of haze or is it merely associated with other

presumably proteinaceous factors that influence haze stability? The following experiments as described in Chapter 4 of this thesis will attempt to provide answers to these questions.

3.5 Conclusions

Pilot brewing trials have shown that beers brewed from SE –ve malt varieties were substantially more haze stable when challenged by force testing (5 days 55°C, 1 day 0°C) than beer brewed from SE +ve malt varieties. Previous studies have looked at improving the colloidal stability of beer by brewing with proanthocyanidin free barley varieties (Fukuda *et al.*, 1999; Jende-Strid, 1997; von Wettstein *et al.*, 1977 and 1980), although these varieties showed improved colloidal stability, for a number of reasons they are not in wide spread use. Recently, it has also been reported that Scarlett malt which has been shown in this study to be SE +ve (Chapter 2, Table 1) and to have higher haze activity then its SE –ve counterparts (section 3.3.1.3), is involved in colloidal haze formation in wheat beers (Delvaux *et al.*, 2004). It has been suggested that permanent haze intensity in wheat beer is not only influenced by the wheat component but also by properties of the barley malt (Delvaux *et al.*, 2004). The observation in this study that SE -ve malt varieties have improved haze stability, indicates that selection of SE –ve varieties has the potential to improve haze stability presumably without negatively impacting on other beer processing or quality characteristics. The brewing experiments conducted in this chapter have also demonstrated the need to exercise rigorous experimental control in order to analyse the haze stability of beer brewed from quality malts.

Chapter 4

The impact on beer haze stability and the SE protein of different brewing conditions and filtration treatments

Abstract

The impact on beer haze stability of the interaction between brewing conditions and the SE protein was investigated in controlled atmosphere and filtration trials. The interaction between the presence or absence of the SE protein and controlled atmosphere brewing was investigated by brewing under nitrogen, oxygen or air. Controlled-atmosphere pilot brewing trials (10 L) indicated that beers produced in both oxygen and nitrogen rich atmospheres showed poorer colloidal stability compared to beer brewed under a normal atmosphere. Filtration trials showed that the colloidal stability of beer could be influenced by the filtration process. Filtration trials showed that the material used to filter the beer (cellulose sheets impregnated with DE) was capable of removing some haze protein from the beer, thus improving the colloidal stability of SE +ve beers. The removal of the SE protein and other proteins during filtration from beer brewed with a SE +ve malt variety, along with a reduction in the level of total protein, as measured by Coomassie blue dye binding, resulted in improved colloidal stability. These results suggest that the selection of SE -ve malt varieties for brewing, combined with optimised stabilisation and filtration treatments may provide an opportunity to improve the colloidal stability of beer. The data is discussed in terms of brewers' options for extending the colloidal stability of their beer and the optimisation of colloidal stabilisation treatments.

4.1 Introduction

The filtration process before the packaging of beer is designed to improve the brightness as well as the colloidal stability of the finished product. Before the packaging of beer, cold filtration, usually through diatomaceous earth (DE) sometimes followed by micro filters, removes solids (yeast and cold break) as well as the colloids (particles responsible for haze formation). There are a number of components present in beer that can reduce its filterability, including non-starch polysaccharides such as β-glucan (which tends to increase the viscosity of beer by forming large gel complexes) (Krüger et al, 1989; Sudarmana et al., 1996), arabinoxylans (which may behave similarly to β-glucans) (Stewart et al., 1998) and α-glucan (i.e. retrograde starch), and protein-polyphenol complexes (Bamforth, 1999a; Siebert, 1999), which may potentially block or clog the filter.

Temperature during filtration has been shown to affect the stability of the finished product (Hardwick, 1978). *En route* to and through the filter the beer should be kept as cold as possible to ensure that the precipitated material (haze precursors) does not return back into solution and re-enter the bright beer (Bamforth, 1999a). Critical to the rate of haze formation is the content of oxygen both during brewing and especially once the beer is packaged (Bamforth, 1988). However, little is known about the effect of non oxidising conditions on the brewing process in relation to haze formation. Potentially a brewing atmosphere that is non oxidative may produce beer with improved beer haze stability. Not surprisingly, brewers are particularly careful to minimise beer oxygen content in the process after the second day of fermentation and particularly in package to maximize both flavour and colloidal stability. It has been suggested that aggressive elimination of oxygen before fermentation may improve beer flavour (Back et al., 1999)

and possibly colloidal stability, however others are not convinced that this improvement is large (Bamforth, 1999a; Meilgaard, 2001).

In this chapter, the investigation of improved haze stability with SE –ve malts was extended by brewing under controlled atmospheric conditions (in nitrogen, oxygen and air). Immunological methods were used to identify and evaluate the fate of the MW ~12000 haze active SE protein throughout the brewing process. Micro-filtration, filtration temperature and filtration media were also examined for their impact on beer colloidal stability. The filtration investigation was a separate investigation from brewing under controlled atmospheres and was based on the questions posed from the haze stability results obtained in Chapter 3 of this thesis.

4.2 Materials and Methods

4.2.1 Malt and Beer Samples

Malt samples used in these trials were the same as those described in Chapter 3, section 3.2.1. Samples of commercial beer at different stages post fermentation to packaging were obtained from a commercial brewery. Commercial Australian and international beers for SE testing were obtained from Australian and international breweries.

4.2.2 Protein extraction, SDS-PAGE and immunoblotting

Protein extraction, SDS-PAGE and immunoblotting were completed as described in Chapter 2, section 2.2.2 and Chapter 3, section 3.2.2.

4.2.3 Pilot Brewing Trials (10 L)

Pilot brewing trials were conducted in a 10 L capacity pilot brewery at VTT Biotechnology (Espoo, Finland). These trials in Finland were conducted with the assistance of Mr Arvi Vilpola, Mr Eero Mattila and Dr Anu Kaukovirta-Norja. The 10 L brewing trials were conducted with specially constructed brewing equipment that allowed for the control of the atmosphere throughout the brewing process (Tankki Oy, Ähtäri, Finland). Scarlett malt, 2 kg, was dry milled (0.5 mm gap set point) then mashed in with 8 kg of water. Mashing salt $CaCl_2.2H_2O$ (3 g) was added and the pH was adjusted with 0.5 M H_2SO_4 to give mash pHs between 5.55-5.65 (as measured at 20°C).

In the trials to examine the effects of atmospheric composition during brewing, 'air' was excluded from the mash tun and O_2 or N_2 was added to the vessel prior to mashing. O_2 or N_2 was also bubbled through the mashing water prior to the addition of the grist. The grist however, was not milled under O_2 or N_2 as this was not feasible. The gases were maintained at a constant flow rate during mashing (4 L/min), lautering (6 L/min) and boiling (4 L/min), with total gas treatment approximately 2 kg per 10 L brew. Mashing in was at 48°C for 30 min (protein rest) followed by an increase in temperature (1°C/min) to 64°C, where the temperature was held for 30 min, then increased to 72°C over 30 min and finally held at 80°C for 10 min. The wort was separated using a lauter tun and boiled for 60 min; Saaz variety aroma hop pellets (α = 4%, dosing 35 g) were added at the beginning of the boil. Prior to fermentation all brews were aerated with O_2 (4 L/min) for 2 minutes to aid the yeast during fermentation.

Fermentation and conditioning and packaging of the beer was conducted as previously described in Chapter 3, section 3.2.5. One difference however, was that following conditioning the beers were filtered using 3, 20 x 20 cm Seitz-EK filter sheets (Seitz-Werke, Type 20z), not 7 as previously described for the 100 L trials.

4.2.3.1 Wort and Beer Analysis

Wort and beer analysis was completed as previously described in Chapter 3, section 3.2.5.1.

4.2.4 Filtration Trial

An industrial scale sample of green beer, brewed from a SE +ve malt variety (Scarlett) was obtained from a maturation vessel of a commercial brewery. The beer was diluted to 10°P prior to filtration. Filtration of the beer was conducted using a plate and frame filter (Seitzschenk Filtersystems) using 7 filter sheets (20 x 20 cm) (Seitz-EK - Seitz-Werke, Type 20z). The beer was passed through the filter under constant CO_2 pressure (0.5 bar) with 20 L of each beer filtered. The beer was filtered at three different temperatures: –1°C, 2°C and 5°C. The kegs were kept at the appropriate temperatures prior to filtration with the temperature of the beer monitored going in and out of the filter (PC logger – 2100, INTAB, Stenkullen, Sweden) along with the duration of filtration (Appendix B, Figures 1a and 1b). The bright beer was bottled using counter pressure as described in Chapter 3, section 3.2.5. Protein in unfiltered and filtered beer samples was measured using the Bradford method (1976) with the Coomassie blue protein assay reagent (Pierce), using BSA as the standard as previously described in Chapter 3, section 3.2.5.1. β-glucan in the beer was determined using the EBC method (8.13.1).

4.2.5 Micro-filtration (V_{max} Filterability Test)

Beer for the micro-filtration trial was brewed as described in Chapter 3, section 3.2.3. The trial used a membrane filtration procedure developed by Stewart *et al.* (1998), which was modified from a membrane filtration procedure previously described by Sudarmana *et al.* (1996). Briefly, an Amicon ultra-filtration system (Beverly, MA, USA) with a 1 L reservoir for degassed beer was connected to an Amicon unit with a volume of 10 mL, which housed the membrane (0.45 µm polyamide, 25 mm diameter, Sartorius AG, Germany). Beer flow from the reservoir was regulated using an in-line stopcock. The ultra-filtration system was held at 4°C and pressurised with nitrogen to 200 kPa.

V_{max} was calculated using the following formula:

$$V_{max} = \frac{3V_2}{\left[\dfrac{5V_2}{V_5}\right] - 2}$$

Where V_2 = Volume of beer collected after 2 min (mL); V_5 = Volume of beer collected after 5 min (mL). V_{max} is defined as the maximum volume of beer that can be filtered through the membrane, with a high V_{max} value indicating a beer that filters efficiently. Each beer sample was filtered in duplicate.

4.3 Results

4.3.1 Controlled Atmosphere Brewing

Pilot brews (10 L) with the SE +ve malt sample (Scarlett) in control (air) (2 brews), oxygen (1 brew) and nitrogen (2 brews) enriched atmospheres were conducted to investigate the influence of brewing conditions on colloidal stability. The Scarlett malt as a SE +ve variety was selected for these trials as it was shown to form haze under control conditions (Chapter 3, section 3.3.1.3). It was assumed that brewing under an oxygen rich atmosphere would result in very poor haze stability as this has previously been well established (Bamforth, 1988). However little is known about the effect of nitrogen on the brewing process.

Similar to the 100 L pilot brewing trials (Chapter 3, section 3.3.1.3), three beers were filtered in succession using the same set of filter sheets. From the haze data obtained, it was also observed that beer which was filtered first was more haze stable (5 day at 55°C 1 day at 0°C, force test) than the beer that was filtered last (Figure 1).
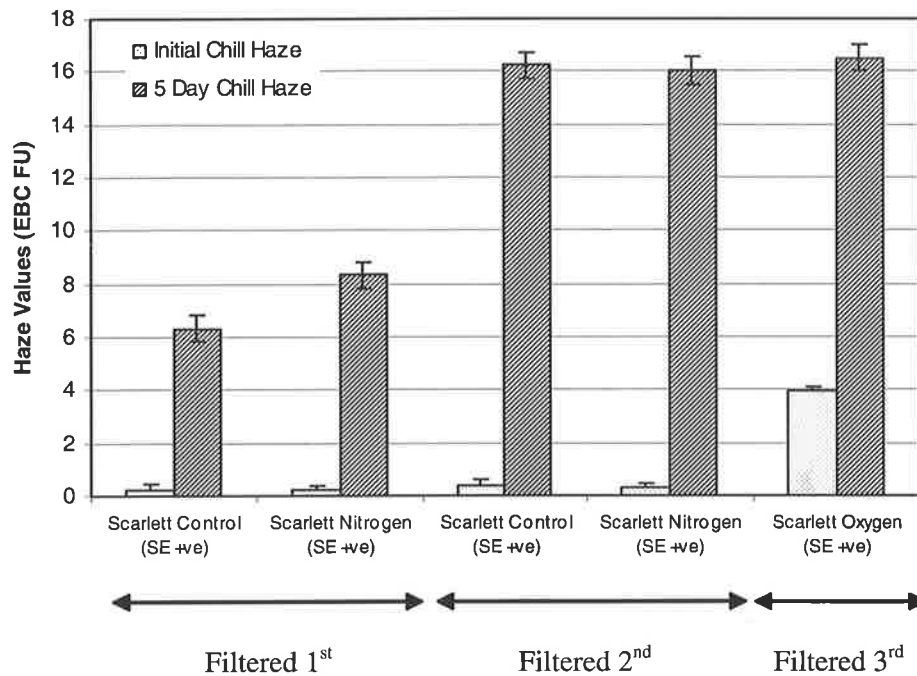
Figure 1: The colloidal stability of beer produced from 10 L controlled atmosphere pilot brewing trials using a 5 day 55°C, 1 day at 0°C chill haze force protocol. The order of filtration is indicated below the figure. Triplicate beer samples were analysed with bars = standard error of the mean chill haze measurement.

From the beers that were filtered first in this trial it was observed that the application of a nitrogen rich atmosphere produced beer with poorer haze stability compared to brewing under a normal atmosphere (Figure 1). The initial chill hazes for both beers were <0.3 EBC FU (Figure 1).

Oxidising conditions present in the mash lead to an increase in wort colour and a lowering of the level of proanthocyanidins in the wort (Bamforth, 1988). As a consequence, there is less polyphenol in the finished beer, which results in decreased colloidal stability (Bamforth, 1988). With the application of either N_2 or O_2 during

mashing, lautering and boiling, the levels of wort protein and fatty acids increased as the brewing conditions went from oxygen-depleted (N_2) to oxygen-rich (O_2) (Appendix B Table 9). The effect of oxygen during brewing in this trial was in agreement with that reported by Bamforth (1988), with increased wort colour and a decrease in the amount of polyphenol that could be detected in the finished beer (Appendix B, Table 10), as compared to the control (air) brews. Brewing under oxygen rich conditions resulted in very poor haze stability (Figure 1). Although the oxygen brew was filtered 3rd, the initial chill haze value obtained was quite high (3.94 EBC FU) compared to those beers also filtered 3rd in the 100 L trials (<0.4 EBC FU) (Figure 7, Chapter 3, section 3.3.1.3), probably indicative that brewing under oxygenated conditions is not advantageous for haze stability.

In common with results of earlier trials, Chapter 3, section 3.3.1.3 - Experiment 3, SDS-PAGE immunoblot analysis using the SE antibody (1/1000 dilution), showed that the MW ~12000 SE protein was present in the SE +ve malt (Scarlett) and unfiltered beer. However, it was absent from the control and nitrogen brews that were filtered using fresh filter sheets, and the nitrogen brew that was filtered using previously used filter sheets. The SE protein was present in the second filtered beer of the control brew along with the oxygen brew (Figure 2).
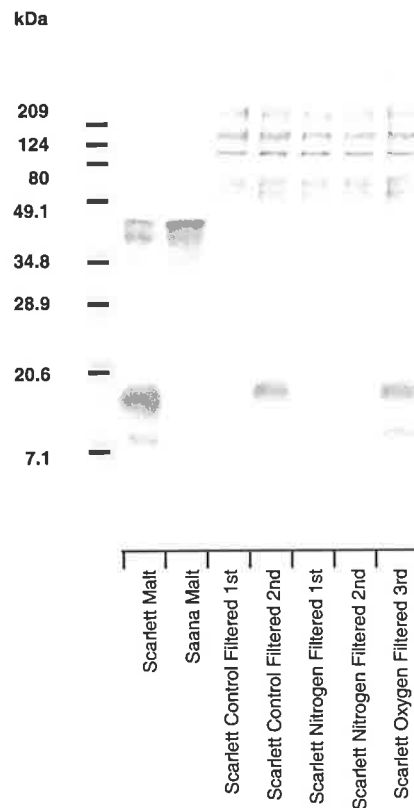
Figure 2: SDS-PAGE immunoblot using the anti-SE antibody (1/1000) dilution of total protein extracts from malt (lanes 1 and 2), and 10 L filtered samples (lanes 3, 4, 5, 6 and 7). Equivalent protein loadings were made in each lane to allow for comparison.

4.3.2 Filtration Trial

The filtration study was split into two parts, the first to examine the impact of temperature with particular emphasis on the fate of the SE protein, and the second to consider the effect of the filtration media in combination with the temperature on haze stability. Using a plate and frame filter, six filtration trials were conducted using commercially produced green beer (brewed from Scarlett, a SE +ve malt). The beer was filtered at three temperatures (-1°C, 2°C, and 5°C) and at a constant pressure (0.5 bar). The filter media used in this study were Seitz EK cellulose sheets impregnated with diatomaceous earth (DE). Sheet filters are made from cellulose fibers that are compressed into a thin mat. The filter mat is often impregnated with DE or perlite

including stabilisers such as PVPP in varying proportions to achieve various degrees of adsorption and retention. Generally, sheet filters have a positive electrostatic charge (referred to as the "zeta potential") to aid in filtration, as most beer contaminants are negatively charged (Goldammer, 2000). Negatively charged proteins have been implicated in the formation of haze (Savage and Thompson, 1972).

The haze stability of beers produced from the filtration trial showed that the haze stability (5 days at 55°C, 1 day at 0°C) was substantially reduced for those beers that were sequentially filtered using the same set of filter sheets (beers 1, 2 and 3) (Figure 3).
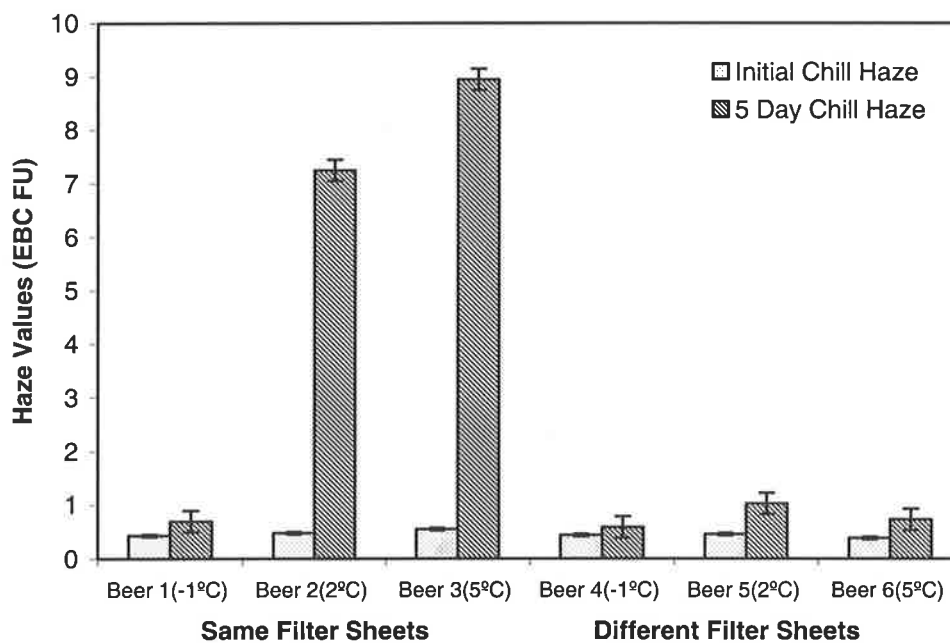
Figure 3: The colloidal stability of beer produced from beer filtered using the same set of filter sheets (beers 1-3) or using a different set of filter sheets for each beer (beers 4-6), using a 5 day 55°C, 1 day at 0°C chill haze force protocol. Triplicate beer samples were analysed with bars = standard error of the mean; chill haze measurement.

Beer 1 (-1°C) which was filtered first in the set was haze stable (0.7 EBC FU), but with beers 2 and 3 (2°C and 5°C) the stability was decreased with chill haze values increasing to greater than 7.0 EBC FU. For those beers that were filtered using new filter sheets at each temperature point (beers 4, 5 and 6), the colloidal stability was not substantially affected as result of the increased temperature during filtration (Figure 3). The colloidal stability of beers 4, 5 and 6 ranged between 0.6 and 1.0 EBC FU. Notably the haze stability of these commercially sourced beers was much better than that for the similarly treated pilot brewed beer (Figure 1, section 4.3.1 and Figure 7, Chapter 3, section 3.3.1.3). In part, this may have been due to the pilot brews being full malt beers, containing extra HA components whereas the commercial beer was made with adjunct thus diluting the HA components. In addition, the surface to volume ratio in the commercial brewery is much smaller than for the pilot brewery, this could contribute to improved haze stability, as there would be less oxidation of haze active materials and also a more vigorous roiling boil that would result in greater aggregation of haze precursors.

Anti-SE SDS-PAGE immunoblot analysis was conducted on unfiltered and filtered beer samples from each temperature point that were filtered using either the same filter sheets or changing the filter sheets between temperature points (Figure 4). The MW ~12000 SE protein was absent in the beer filtered with a new set of filtration sheets (beers 1, 4, 5, and 6), while with beers 2 and 3 that were filtered on a used filter sheet set, the SE protein was present.
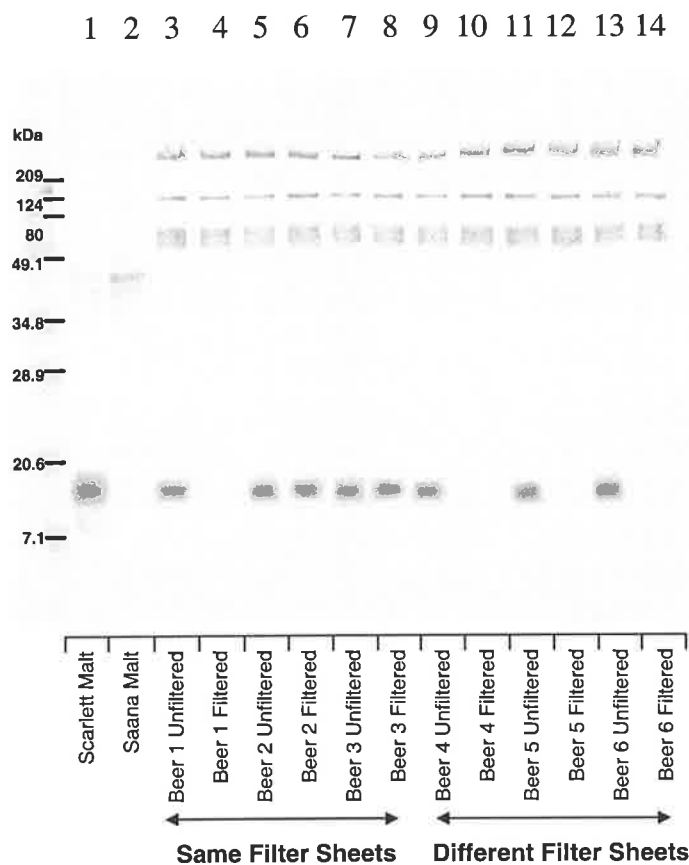
Figure 4: SDS-PAGE immunoblot using the anti-SE antibody (1/1000) dilution of total protein extracts from malts (lanes 1-2); pre filtration samples (lanes 3, 5, 7, 9, 11 and 13), filtered samples (lanes 4, 6, 8, 10, 12 and 14). Beer was filtered using the same filter sheets (beers 1-3) or using different filter sheets (beers 4-6). The lanes were loaded at equivalent dilutions to allow for comparison.

The extent of protein binding to the filter sheets was also assessed by measuring the level of total protein in beer pre and post filtration (Figure 5). It was observed that the total protein values obtained for the beers post filtration showed a similar trend to the haze values shown in Figure 3. With beer 1 (-1°C) that was filtered first, there was a 64% reduction in the amount of total protein in the filtered beer. However, as the filter sheets were unchanged, beers 2 (2°C) and 3 (5°C) did not show a substantial decrease in the level of protein post filtration compared to the corresponding pre filtration samples.

In these filtered samples there was only a 6-11% reduction in the amount of total protein. With beers 4, 5 and 6, filtered using different filter sheets, the amount of protein was substantially reduced (there was a 65, 50, and 45% reduction respectively) at each temperature point.
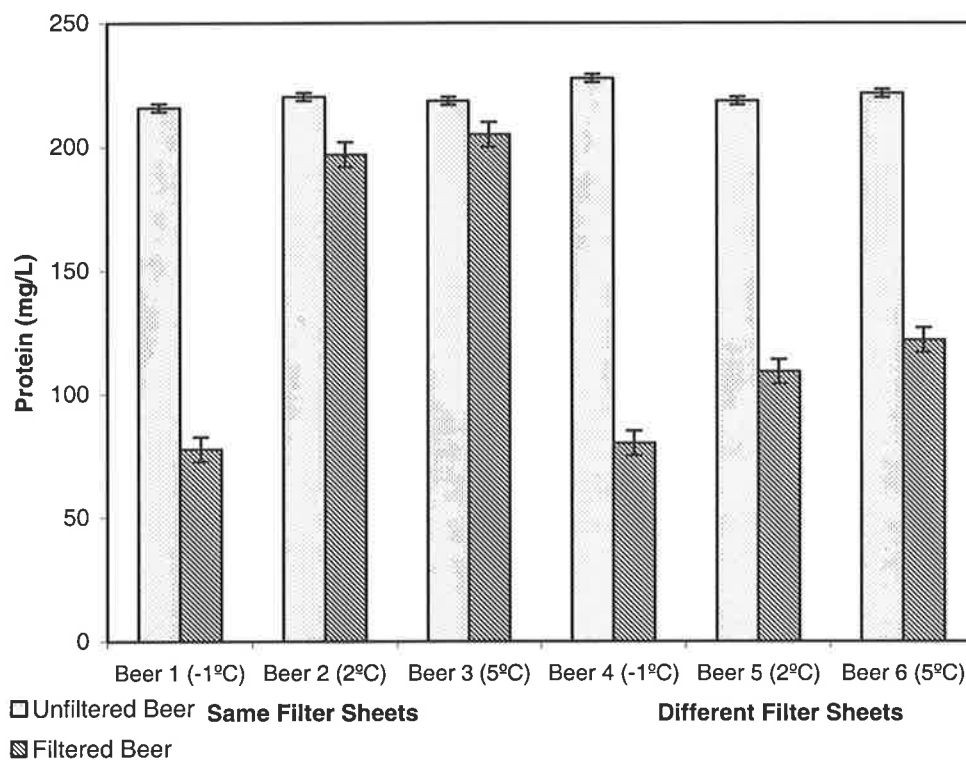
Figure 5: Total protein as measured by the Bradford (1976), Coomassie blue dye binding assay of unfiltered and filtered samples, beers filtered using the same filter sheets (beers 1-3) or using different filter sheets (beers 4-6). Bars = Standard error of the mean; total protein (mg/L).

Previous studies have shown that reduced beer filtration efficiency has been largely attributed to β-glucan (Fincher and Stone, 1986; Leedham *et al.*, 1975; Muts *et al.*, 1984; Siebert *et al.*, 1984). β-glucan, implicated in forming gels and precipitates at low temperature (<0°C) (Gjertsen, 1966; Takayanagi *et al.*, 1969), through hydrogen

bonding (Letters, 1977), potentially aggregating with protein-polyphenol complexes and thus contributing to haze formation, does not appear to be a factor as β-glucan levels in all six filtered beers was <20 mg/L and the filtration efficiency was not compromised.

If there were more protein/colloidal aggregates being removed during filtration, these would be likely to negatively influence beer filtration efficiency. At colder filtration temperatures there are likely to be larger aggregates formed, resulting in further reduced filtration efficiency. If filterability is poor, the differential pressure within the filter increases, resulting in a reduction in the flow rate and a delay in the filtration process. It was expected that the flow rate would have been slower at (-1°C) than at (5°C), but in these trials the flow rate was not retarded. The filtration times ranged between 10 min and 20 min, Beer 1 (-1°C), 18 min; Beer 2 (2°C), 10 min; Beer 3 (5°C), 18 min; Beer 4 (-1°C), 20 min; Beer 5 (2°C), 15 min; Beer 6 (5°C), 19 min (Appendix B, Figures 1a and 1b). Interestingly, for the beers that were filtered at 2°C (Beers 2 and 5), the time of filtration was shorter than at the other time points.

A follow up trial was completed to assess the rate at which the Seitz - EK filter sheets bound protein. Samples were collected post-filtration at 1-minute intervals during the filtration run at the three temperatures (-1°C, 2°C and 5°C) with a set of 7 fresh filter sheets used for each temperature point. With each of the temperature profiles, during the first 2 minutes, the levels of protein in filtered beers were low (<60 mg/L), then there was a sharp increase in the amount of protein eluting through the filter (between 3 and 8 minutes) (>150 mg/L) (2°C and 5°C) (>100 mg/L) (-1°C) (Figure 6). The amount of protein increased and then decreased slightly around the halfway point of filtration, then steadily increased until filtration was completed. In the final few minutes of

filtration, the amount of protein measured in the filtered samples was at a similar level to the same sample before filtration (>200 mg/L).
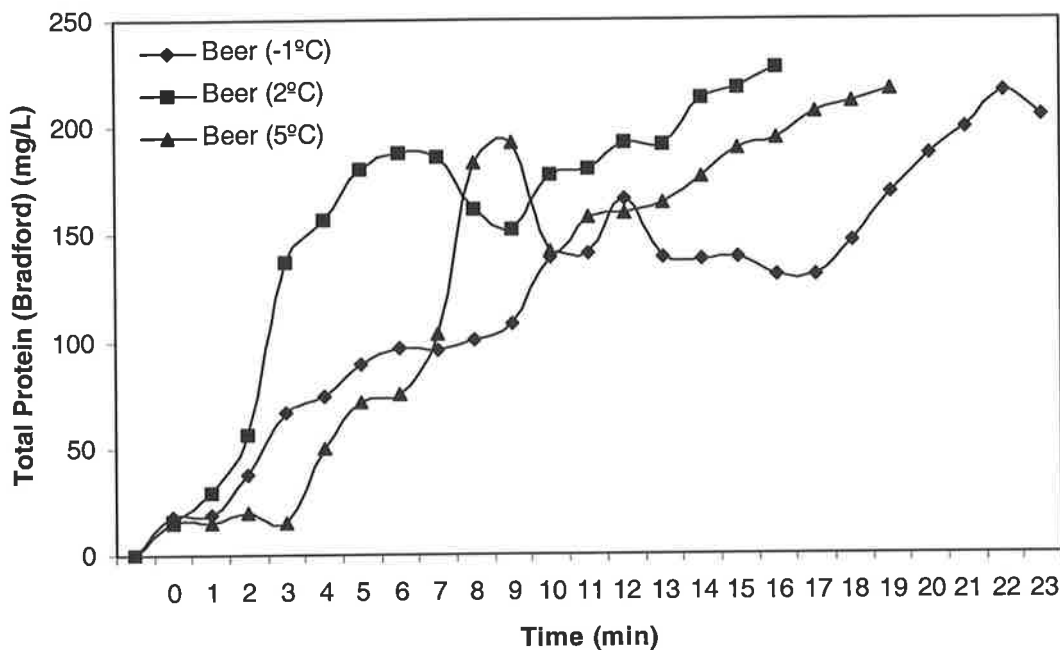
Figure 6: Total protein levels in filtered beers (as measured by the Bradford Coomassie blue dye binding assay) for beers 4 (-1°C), 5 (2°C) and 6 (5°C); samples were obtained at 1-minute intervals.

Interestingly, during the filtration run, the flow rate did not decrease nor did the differential pressure increase, which would have indicated that the filter sheets were becoming blocked and required replacement. These observations suggest that protein binding sites on the filter sheet remove the SE and other proteins until saturated. Once the filter is saturated, these proteins again appear in the beer and beer colloidal stability decreases. Moreover, observations suggest that the attainment of saturation levels of protein binding is not concurrent with immediate filter blockage. It would have been of interest to run SDS-PAGE immunoblots using the SE antiserum with these protein

profile samples to examine if there was a level of total protein that corresponded to the presence of the SE protein in filtered beer.

Previous studies have shown that only a small proportion of total beer protein is actually involved in the formation of haze (Siebert and Lynn, 1997b), with as little as 2 mg/L of protein sufficient to induce a haze of 1 EBC unit (Chapon, 1994). A constraint of the Bradford (1976) assay is that the quantity of protein which can be measured is primarily determined by the amount of arginine and the relative size of the protein, >5 kDa (Compton and Jones, 1985; Hii and Herwig, 1982; Lewis *et al.*, 1980). The SE protein fraction contains approximately 6% of arginine (mol/mol) so it has been shown to react relatively poorly with the Bradford (1976) Coomassie blue dye binding colorimetric assay for protein quality (Evans *et al.*, 2003). This indicates that the cellulose filter sheets are removing more than just HA protein, and in the initial stages of filtration, a substantial amount (Figure 6). It is well known that the Bradford test is a useful predictor of beer foam stability (Lewis and Lewis, 2003), and although foam stability was not tested in this investigation, the data from protein estimations of post-filter beers suggest that the cellulose filters may remove protein; this would negatively impact on beer foam stability.

Given the results observed in this filtration trial it was of interest to analyse commercial samples of beer at different stages post fermentation, namely during filtration and stabilising treatments, for the presence of the MW ~12000 SE protein. Samples of the same commercial beer at different stages post fermentation to packaging including beer from fermentation that was not yet fined, beer in storage that was fined, beer that was dosed with silica gel, after DE filtration, after PVPP treatment and a sample of the

packaged beer were obtained from a commercial brewery. SDS-PAGE immunoblot analysis of samples from the brewing process showed that the SE protein was present in the finished beer (Figure 7), even with the addition of stabilisation treatments (i.e. silica and PVPP), or after DE filtration. Its levels, however, were substantially decreased by these treatments, which were likely also to have removed other proteins also potentially implicated in haze formation. Clearly therefore, factors affecting filtration performance may impact significantly on the haze-forming potential of beers. A survey of Australian and international beers (Figure 8), primarily looking at the presence/absence of the MW ~12000 SE protein found that only 23 of the 40 beers assessed contained this protein (Table 1).
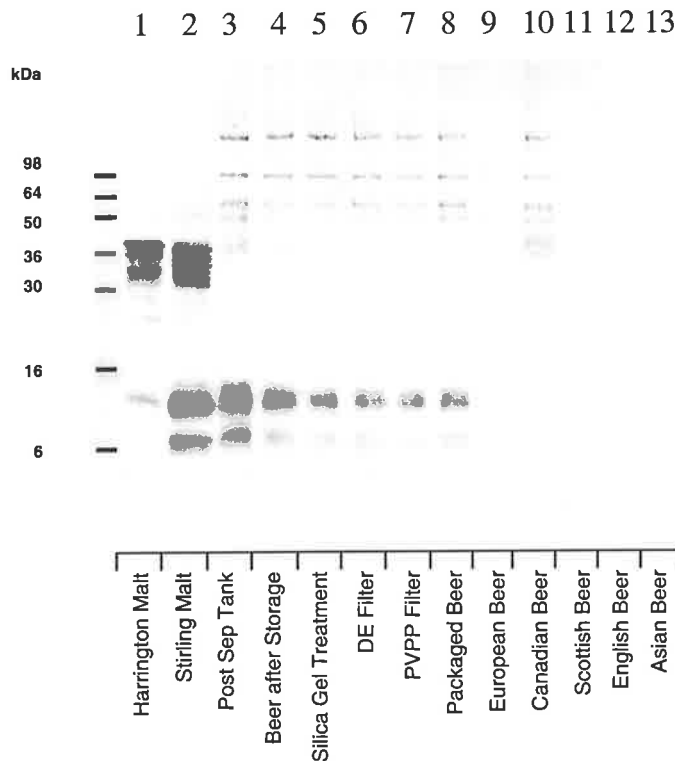
Figure 7: SDS-PAGE immunoblot using the anti-SE antibody (1/1000) dilution of samples of the same beer at different stages post fermentation (lanes 3-8), and a selection of international commercial beers (lanes 9-13); the country of origin is listed below the figure. Post Sep Tank = beer from fermentation that has not been fined. Equivalent protein loadings were made in each lane to allow for comparison.
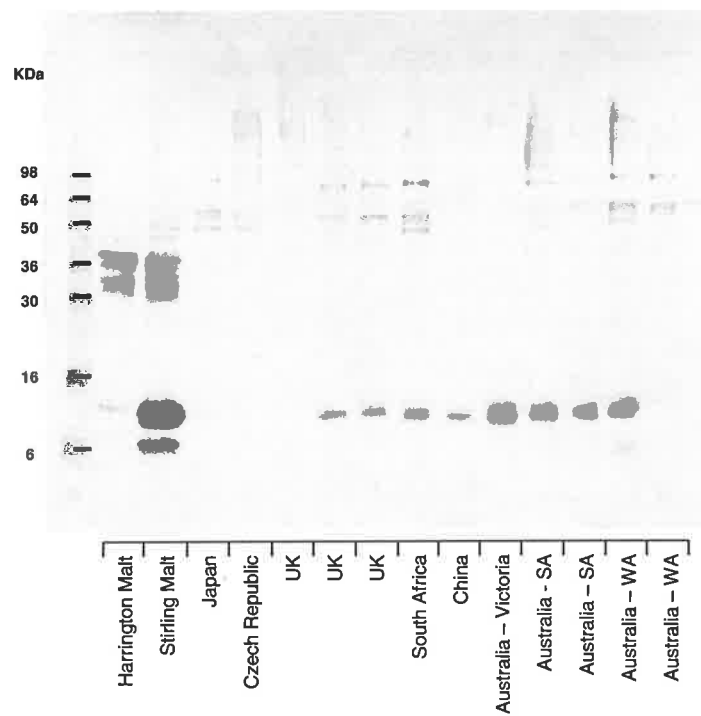
Figure 8: SDS-PAGE immunoblot analysis using the anti-SE antibody (1/1000) dilution of a selection of Australian and international commercial beers. The country of origin is listed below the figure. Equivalent protein loadings were made in each lane to allow for comparison.

Table 1: A survey of Australian and international beers examining the presence or absence of the MW ~12000 SE protein.

SE+ve	Brewery	SE -ve	Brewery
Boddingtons Pale Ale	Boddingtons - Interbrew	Asahi Super Dry	Asahi Breweries, Ltd
Cascade Light	Foster's	Bass	Bass - Interbrew
Castle Lager	SABMiller	Budweiser	Anheuser Busch Companies
Coopers Pale Ale	Coopers Brewery	Carlsberg Lager	Carlsberg Breweries
Eagle Blue	Lion Nathan	Hartwall Classic	Hartwall - Scottish and Newcastle
Hahn Premium	Lion Nathan	Heineken Lager[1]	Heineken
Heineken Lager[1]	Heineken	Karhu[1]	Sinebrychoff - Carlsberg Breweries
James Boag's Premium	J. Boag and Son - San Miguel	Karjala	Hartwall - Scottish and Newcastle
Karhu[1]	Sinebrychoff - Carlsberg Breweries	Labatt Blue Pilsner	Labatt Breweries - Interbrew
Koff	Sinebrychoff - Carlsberg Breweries	Lahden Erikois	Hartwall - Scottish and Newcastle
Little Creatures Pale Ale	Little Creatures Brewing	Lapin Kulta	Hartwall - Scottish and Newcastle
Matilda Bay	Matilda Bay Brewing Company	Legenda	Hartwall - Scottish and Newcastle
Moosehead Lager	Moosehead Breweries Limited	Miller Genuine Draft	SABMiller
Moreland 'Old speckled hen'	Greene King	Nokian Vaalea	Pirkanmaan Uusi Panimo
Nikolai	Sinebrychoff - Carlsberg Breweries	Olvi (CXX)	Olvi
Olvi	Olvi	Pilsner Urquell	SABMiller
Sandels	Olvi	Sapporo Premium	Sapporo Breweries
Tooheys New	Tooheys Brewery - Lion Nathan		
Tsingtao	Tsingtao Brewery		
Urho	Hartwall - Scottish and Newcastle		
Victoria Bitter	Foster's		
West End Draught	South Australian Brewing Company - Lion Nathan		
XXXX Bitter	Castlemaine Perkins Brewery - Lion Nathan		

[1]Two samples of Heineken and Karhu obtained from different breweries were tested. In the case of the Karhu samples the SE +ve and SE –ve difference observed was due to the variety of malt used. The SE +ve result obtained was from a brewery that predominately brewed with a SE +ve malt variety, whereas the SE –ve sample tested was from a brewery predominately brewing with a SE –ve malt variety. In regard to the

Heineken samples tested, the malt used is not known and as such comment cannot be made on why one sample was identified as SE +ve compared to the other which was identified as SE −ve.

4.3.3 V_{max} Filterability

A small-scale micro-filtration experiment (V_{max} filterability test) was carried out to compare beer filterability with colloidal stability, as improved beer filtration efficiency may be directly related to improved colloidal stability. Also, as filters retain beer protein, the experiment would test if this has an influence on beer micro-filtration efficiency. V_{max} values were determined from beer that was brewed from the three malts (Unicorn (SE −ve), Harrington (SE −ve) and Stirling (SE +ve)) used in the Ballarat 300 L pilot brewing trials (Chapter 3, section 3.3.1.2) and those same described malts used in the SBB trials (Chapter 3, section 3.2.1). V_{max} values ranged from 243 mL to 545 mL (Appendix B, Table 11). No improved filtration efficiency was observed with the SE −ve malts as compared to the SE +ve malt. Filtration efficiency as measured by V_{max} was shown not to relate to haze stability (Figures 9a and 9b).
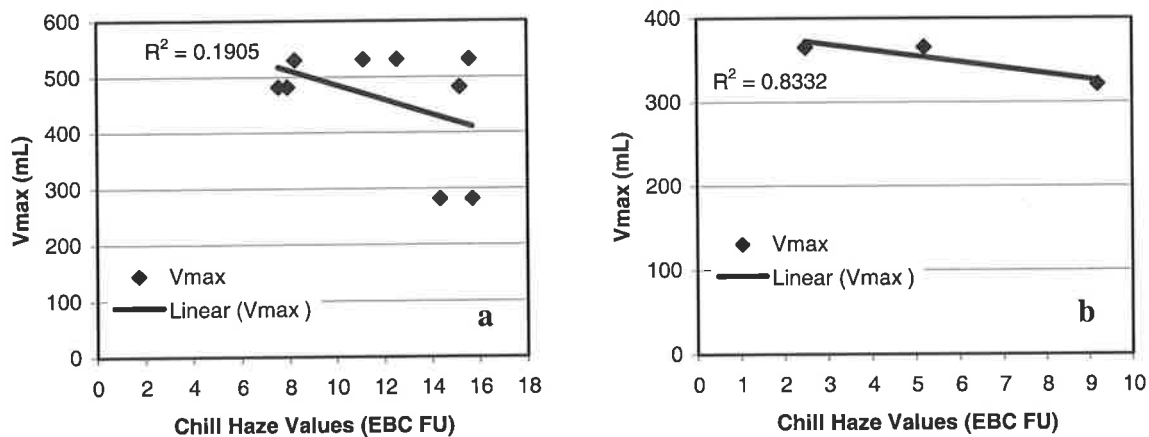
Figures 9a and 9b: Filtration efficiency as measured by V_{max} (mL) compared to the 5-day chill haze stability from beer brewed using the SSB method. Haze values used in Figure 9b were obtained from the 50 L pilot brewing trials conducted at Lion Nathan (Evans *et al.*, 2003) (Chapter 3, section 3.3.1.1, Figure 1).

4.4 Discussion

Pilot or small scale brewing trials are routinely employed by brewers and researchers to predict the effect of manipulation of raw materials, additives, treatments and the brewing procedure in the full scale brewing plant. For colloidal stability, this investigation has focused attention on oxidation during brewing, the filtration media used and the raw material source.

In terms of oxidation during brewing, it was assumed that increased oxidation during brewing would decrease colloidal stability (Bamforth, 1988). However, it was found that aggressive elimination of oxygen by purging the brewing process prior to fermentation with nitrogen gas did not improve colloidal stability and probably reduced

stability. It was expected that brewing under nitrogen would improve beer colloidal stability but the N_2 beer showed no improvement. A possible explanation may be that some oxidation is needed during brewing to ensure that a critical proportion of beer haze active proteins and polyphenols are precipitated during boiling and maturation so that they do not carry through into the finished beer to be present to allow for more rapid haze formation. The oxidation of polyphenols during wort production is known to lead to the polymerisation of these compounds and binding with protein, forming large insoluble complexes which precipitate during boiling, benefiting overall colloidal stability (Bamforth, 1999a). The results of the 10 L pilot brewing trials completed in this study suggests that there is an optimal degree of oxidation during brewing, at least in terms of the resultant haze stability rather than the total exclusion of oxygen. These results are consistent with the conclusions of Back *et al.* (1999). In the interests of colloidal stability in isolation it would appear that there is an optimum level of oxidation required during brewing to reduce the load of HA protein and polyphenol in the beer, thus improving colloidal stability.

This investigation highlighted the importance of both stabilisation and filtration processes on beer colloidal stability. The filtration medium was shown to have a substantial influence on beer colloidal stability. In general, the brewing industry relies primarily on DE for beer filtration, sometimes followed by filtration through nylon, ceramic or cellulose micro-filters for particular beer styles. The cellulose filtration sheets used in these trials or their equivalent are used routinely and widely for small scale brewing investigations, primarily because of their ease of use, consistency and cost effectiveness. However, as shown in these trials, the sheets are capable of removing HA protein (Figures 4, 5, and 6 and Figure 5; Chapter 3, section 3.3.1.2) that

impacts on haze stability (Figure 3) and possibly foam stability, given that foam-associated protein measured by the Coomassie blue assay (Lewis and Lewis, 2003) is also removed from the beer (Figures 5 and 6). The cellulose filtration sheets used in these pilot brewing trials are most likely the reason for the removal of the MW ~12000 SE protein (Figure 5; Chapter 3, section 3.3.1.2), which produced the contradictory results observed for the Stirling (SE +ve) brews (Figure 4; Chapter 3, section 3.3.1.2) in the 300 L Ballarat trial.

The seemingly inconsistent haze stability results with some of the trials described in Chapter 3 of this thesis can now be rationally explained. A follow up pilot brewing trial with Scarlett (SE +ve) also showed that cold conditioning and filtration conditions could remove the MW ~12000 SE protein and improve beer colloidal stability (Figure 11; Chapter 3, section 3.3.1.3). In the 300 L Ballarat trial, the size of the plate and frame filter (40 x 40 cm) device, and the number filter sheets (20 sheets) was substantially greater than those for the VTT trials (20 x 20 cm, 3 sheets (10 L); 7 sheets (100 L)), resulting in a greater protein binding capacity and the removal of the MW ~12000 SE band (Figure 5; Chapter 3, section 3.3.1.2), than was possible in the VTT filtration trial (Figure 4). The discrepancy observed in the colloidal stability of the Ballarat Stirling beers (Figure 4; Chapter 3, section 3.3.1.2) is most likely to be a function of the sampling point during filtration for the beer used for stability testing. This conclusion is based on an extrapolation of the beer filtration protein profile data shown in Figure 6. These observations and conclusions suggest greater consideration should be made to matching the characteristics of the filter media used for small scale filtration as on the commercial scale filtration that is being emulated. For DE filtration the use of a Walton filter (i.e. VEL, Leuven, Belgium) or a similar device, which doses

DE throughout the run to achieve filtration, maybe a worthwhile solution. Finally, these trials also suggest that a test allowing detection of the presence or absence of the MW ~12000 SE protein could be developed that would allow the brewer to optimise the stabilisation of beer. If test results indicated that the MW ~12000 SE protein was removed during filtration, this may mean that no further stabilisation treatments are required on that particular batch, thus avoiding potential over-stabilisation of beer and resulting in large cost savings to the brewery.

In terms of commercial brewing, the trials highlight a number of points for consideration. Firstly, in small scale brewing trials where haze and probably foam stability are being investigated, all care must be taken to ensure that all the beers are treated by the same procedure during filtration to allow for valid comparison. At this stage there is no evidence to suggest that other beer parameters are influenced by filtration through cellulose filter sheets. As such, it can be stated with confidence that the comparisons between the SE -ve and SE +ve brews presented here (Figure 4, Chapter 3, section 3.3.1.2 and Figure 7, Chapter 3, section 3.3.1.3) and previously by Evans *et al.,* (2003) (Figure 1, Chapter 3, section 3.3.1.1) correctly identify that SE -ve malts confer an advantage in improved colloidal stability. However, data presented in the current trial emphasizes that the absence of the MW ~12000 SE protein is not the only component that can influence colloidal stability. The removal of protein measurable with the Coomassie blue assay (Figures 5 and 6) is correlated with a substantial improvement in colloidal stability (Figure 3).

The trials suggest that filtration using cellulose filter sheets is capable of improving beer colloidal stability. However, this improvement is unlikely to be cost effective as this

property is relatively fleeting (as the majority of protein in beer is removed in the first 5 min of filtration) (Figure 6) and is also likely to remove desirable foam stabilizing proteins as Coomassie blue protein decreases and this is correlated with foam stability (Lewis and Lewis, 2003). Although it is most likely that the positively charged cellulose is removing the HA protein along with and other proteins (Goldammer, 2000), it would be of interest to establish if and to what extent the DE component of the filter sheets makes a contribution to the removal of HA protein, as other grades of silica are used as colloidal stabilisation treatments (Siebert and Lynn, 1997a).

A survey of Australian and international beers (Figure 8), primarily looking at the presence or absence of the MW ~12000 SE protein found that only 23 of the 40 beers assessed contained this protein (Table 1). Given that more than 80% of barley varieties tested were found to be SE +ve (Chapter 2, section 2.3.1.1, Table 1), this suggests that some brewers have the capability to remove the HA SE protein from their beer. It is unclear as to how this achieved because the conventional silica and PVPP treatments only appear to remove a portion of the SE protein (Figure 7). However, given the results observed in the filtration trial (section 4.3.2), this removal of the SE protein may be indicative of different filtration regimes and cold conditioning employed by the breweries.

Given the results obtained in the aforementioned filtration trial (section 4.3.2), a small-scale micro-filtration experiment was carried out to examine beer filterability in relation to haze stability, as it was thought that beer that filters efficiently may relate directly to improved haze stability of the same beer. Protein-polyphenol complexes, important in haze formation, may also potentially block or clog the filter, reducing filtration

efficiency. In general, most breweries rely on DE for primary filtration of beer. As a predictor of filterability, micro-filtration has been shown to be both highly correlated and predictive of DE filtration performance (Eyben and Duthoy, 1979; Siebert *et al.*, 1984; Stewart *et al.*, 2000). Siebert and colleagues also demonstrated the same relationship of micro-filtration with cellulose pulp filtration (Siebert *et al.*, 1984). In the current trial no improved filtration efficiency was observed with the SE –ve malts when compared to SE +ve malts (Figures 9a and 9b) and filtration efficiency was not shown to relate to colloidal stability.

Interestingly, the two brews using the barley variety Unicorn (SE –ve), that showed very poor haze stability (14.36 and 15.69) in the 300 L Ballarat trials (Figure 9a, section 4.3.3 and Figure 4, Chapter 3, section 3.3.1.2), also showed poor filtration efficiency when compared to the Unicorn malt used in the Lion Nathan (50 L trials) and SSB trials, and also compared to the Harrington and Stirling malts used in both trials (Appendix B, Table 11). Siebert and colleagues showed using micro-filtration tests that clear beers had good filterability, and hazy beers very poor filterability (Siebert *et al.*, 1984), and in the range of forced haze stabilities that were observed between SE –ve and SE +ve malts in this trial (Chapter 3, section 3.3.1) (range between 2 EBC FU up to 16 EBC FU) they demonstrated that filtration efficiency was severely reduced (from >400 to <100 g) (Siebert *et al.*, 1984). With the exception of the Unicorn haze stability performance in the 300 L pilot brewing trials, discrimination on the basis of improved haze stabilities observed between quality malts of both SE –ve and SE +ve varieties and improved filtration efficiency was not observed. The SE protein which has been demonstrated to influence haze stability (Chapter 3, section 3.3.1) does not appear to influence micro-filtration efficiency.

4.5 Conclusions

Controlled atmosphere pilot brewing trials (10 L) showed that a nitrogen rich atmosphere produced beer with poorer colloidal stability compared to brewing under a normal atmosphere. Filtration trials showed that the material used to filter the beer (cellulose sheets impregnated with DE) was capable of removing some haze protein from the beer, thus improving the haze stability of SE +ve beers. This suggests that the cellulose, DE or a combination of both are capable of removing some haze active protein. These results suggest that the selection of SE -ve malt varieties for brewing, combined with optimised stabilisation and filtration treatments may provide an opportunity to improve the colloidal stability of beer, thus providing an alternative option to the use of traditional colloidal stabilisation treatments in the brewery.

Chapter 5

The SE protein is a Barley Trypsin Inhibitor of the chloroform/ methanol type

Abstract

The previously identified SE protein was characterised using comparative two-dimensional (2-D) gel electrophoresis immunoblots of barley seed extracts from both SE +ve and SE -ve varieties. The SE protein spot identified was excised and its partial sequence determined, after in-gel cleavage using trypsin and the separation of the resulting fragments by reversed-phase HPLC. N-terminal sequence analysis of the tryptic peptides from SE +ve and SE -ve varieties identified the SE protein as the barley trypsin inhibitor CMe precursor (BTI-CMe). The mature BTI-CMe protein is 13.3 kDa and the functional gene is located on chromosome 3H, consistent with the information available on the SE protein (Chapter 2, Figures 1 and 3). DNA sequence analysis of BTI-CMe genes cloned from two SE –ve varieties, Harrington and Haruna Nijo and two SE +ve varieties, Sloop and Proctor, revealed that the SE –ve varieties had the highest sequence identity with variant BTI-CMe3.1 (Harrington and Haruna Nijo 98% homology), while the SE +ve varieties aligned with variant BTI-CMe1 (Sloop 96%; Proctor 97% homology). Cloning of the DNA encoding the BTI-CMe protein demonstrated that both SE -ve and SE +ve barley varieties contain a BTI-CMe protein family member that is similar but consistently different, primarily in the last 30 amino acid residues of their C-termini. Specific primers were designed to amplify the full length BTI-CMe DNA as well as DNA encoding a truncated protein (C-terminal region) in both BTI-CMe1 and BTI-CMe3.1 variants. Four BTI-CMe constructs were made including full length BTI-CMe1 and BTI-CMe3.1 constructs, as well as shortened

constructs of 174 bp (58 amino acids) BTI-CMe1 (SE +ve) and 177 bp (59 amino acids) BTI-CMe3.1 (SE -ve). BTI-CMe was expressed in *E.coli*, purified and polyclonal antibodies raised to the recombinant protein. The recombinant BTI-CMe proteins proved to be poorly immunogenic, thus this experiment was not conclusive.

5.1 Introduction

Proteinaceous protease inhibitors in plants may be important in regulating and controlling endogenous proteases and in acting as protective agents against insect and/or microbial proteases (Ryan, 1973 and 1989). Inhibitors of proteinases can be divided into four families based on their amino acid sequences and their target enzymes (serine, thiol-, metallo-, and acid proteases) (Shewry and Darlington, 2002; Shewry, 1999; Shewry, 1993).

During the malting and brewing process, the proteinases of barley and malt partially hydrolyze hordeins (Jones and Marinac, 2002). In particular, protein degradation in barley begins with these insoluble storage proteins being hydrolysed by the endopeptidases (proteases) that then generate 'soluble protein' and peptides, upon which the exopeptidases (carboxypeptidases) act to release amino acids (Bamforth and Barclay, 1993). The solubilisation of malt storage proteins can affect many aspects of beer quality made from these worts, including their clarity. Forty-two proteases have been isolated and characterised from malted barley using two-dimensional gel electrophoresis (Zhang and Jones, 1995) and they are representative of all four protease classes (aspartic, cysteine, metallo- and serine). Serine proteinase inhibitors that are present in barley include the chymotrypsin/subtilisin inhibitors 1 and 2 (CI – 1 and 2), a bifunctional barley α-amylase/subtilisin inhibitor (BASI), a barley Bowman-Birk type

trypsin inhibitor (BBBI), trypsin α-amylase inhibitors (chloroform/methanol - soluble proteins (CM proteins)) and serpins (protein Z) (Carbonero and García-Olmedo, 1999; Shewry, 1999).

CM proteins belong to the trypsin/α-amylase inhibitor family. Enzymes belonging to this family of inhibitors target exogenous α-amylases, trypsin and other serine proteases (Fincher and Stone, 1993; Shewry, 1993). These inhibitors have low molecular weights ranging from 9,000 to 16,000 (Shewry, 1993). The properties of these inhibitors have been previously reviewed extensively (Carbonero and García-Olmedo, 1999; García-Olmedo *et al.*, 1987 and 1992). In barley, the first trypsin inhibitor from this family was isolated from the endosperm by Mikola and Suolinna (1969). This inhibitor was active against trypsin and inactive against the endogenous proteinases in green malt. Barley trypsin inhibitors have been found to be endosperm specific (Kirsi and Mikola, 1971). Genetic studies show that the synthesis of CM proteins is controlled by a disperse multi-gene family (García-Olmedo *et al.*, 1992; Lazaro *et al.*, 1985), with genes located on chromosomes 2H (2), 3H (3), 4H (4), 6H (6) and 7H (1) (Mena *et al.*, 1992; Nielson and Hejgaard, 1985; Salcedo *et al.*, 1984).

CM protein genes are expressed in the developing endosperm before the deposition of the majority of the storage proteins and starch (Kirsi, 1973; Paz-Ares *et al.*, 1983b). Within the barley endosperm, one of the most abundant members of the family is the 13 kDa monomer CMe (García-Olmedo *et al.*, 1987 and 1992). A barley trypsin inhibitor (BTI) that was initially sequenced at the protein level by Odani *et al.* (1983), has been subsequently shown to be identical to the CMe protein (Lazaro *et al.*, 1985; Salcedo *et*

al., 1984), and the corresponding gene for BTI-CMe has been shown to be located on chromosome 3H (3) of barley (Hejgaard *et al.,* 1984; Salcedo *et al.,* 1984).

In this chapter, the SE protein identified previously (Chapter 2), that was subsequently shown to have an influence on beer haze stability (Chapter 3), was identified as the barley trypsin inhibitor - CMe precursor (chloroform/methanol - soluble protein) (BTI-CMe). BTI-CMe was cloned, expressed in *E.coli*, and purified, with polyclonal antibodies raised to the recombinant preparations of BTI-CMe.

5.2 Materials and Methods

5.2.1 Identification of the SE Protein

5.2.1.1 2-D Electrophoresis and *In Situ* Digestion

Ground barley seed for 2-D electrophoresis was extracted in 1M urea/1% (v/v) 2-mecapto-ethanol. First-dimension separation was achieved using IPG strips, Immobiline DryStrip gels (Amersham Biosciences, Uppsala, Sweden) (3-10 pH, non-linear, 18cm). The IPG strips where rehydrated in a rehydration solution containing IPG Buffer (*pI* 3-10) (Appendix D) for 12 h, prior to focusing for 32000 Volt hours (Vh) using an IPGphor™ isoelectric focusing system (Amersham Biosciences) (Table 1). The IPG strips were loaded with 350 μL of rehydration solution containing ~0.8 mg of total protein.

Table 1: First dimension isoelectric focusing protocol. This protocol was derived from the 2-D electrophoresis manual of Berkelman and Stenstedt (1998).

IPG strip length	Step	Voltage	Step Duration (h:min)	Volt hours (Vh)	Gradient Type
18 cm	rehydration	-	12.00	-	-
	1	500	1.00	250	Step-n-hold
	2	1000	1.00	1000	Step-n-hold
	3	8000	4.00	32000	Step-n-hold

Equilibration of the IPG strips with DTT and iodoacetamide in SDS equilibration buffer (Appendix D) was performed prior to second dimension separation. The second dimension was resolved using SDS-PAGE (15% (w/v) gels), gels were prepared and run as previously described (Chapter 2, section 2.2.2), using a Hoefer DALT electrophoresis unit (Amersham Biosciences). The IPG strip was sealed in the polyacrylamide gel using an agarose solution (Appendix D). For in-gel digestion, gels were stained overnight with a 0.05% G-250 colloidal Coomassie stain (Appendix D), modified from (Neuhoff *et al.*, 1988), de-staining was in 1% acetic acid (v/v). The appropriate spots were excised from the gel, washed, digested with trypsin and eluted according to Hellman *et al.*, (1995). Briefly, the appropriate spots were excised with a clean scalpel and cut into pieces 2 mm^2 and placed into 2 mL round bottom Eppendorf tubes. The excised gel pieces were further de-stained with two 30 min, 500 µL washes of 100 mM NH$_4$HCO$_3$ in 30% (v/v) acetonitrile followed by an extraction in 30% (v/v) acetonitrile. Residual acetonitrile was evaporated (10-15 min) in a SpeedVac (Savant Instruments Inc., Farmingdale, NY, USA). Protein was digested with trypsin (250 ng/25 µL) (sequencing grade, modified, Promega Corporation, Madison, WI, USA), overnight at room temperature with 33.33 mM NH$_4$HCO$_3$ in 10% (v/v) acetonitrile. Peptides were extracted from the gel pieces in 300 µL of 10% (v/v) acetonitrile/0.1% TFA for 60 min using a rotary suspension mixer (Ratek Instruments, Melbourne, Australia).

5.2.1.2 Reversed Phase-HPLC

The eluted peptides were separated using reversed-phase (RP)-HPLC (Hewlett-Packard 1090 LC, Rockville, IL, USA). Separations were achieved using a 2.1 x 250 mm Vydac C18 protein column (Vydac Separations Group, Hesperia, CA, USA). Eluent A was aqueous 0.05% trifluoroacetic acid (TFA), eluent B was 0.04% TFA in acetonitrile, with a flow rate of 0.2 mL min^{-1}. Elutions were monitored at 280 and 214 nm and the fractions were collected manually for N-terminal sequencing.

5.2.1.3 Amino acid sequence analysis

N-terminal sequencing using automated Edman chemistry was performed using a Hewlett-Packard G1000A protein sequencer with an on-line 1090 LC for phenylthiolhydantoin (PTH) – amino acid analysis. N-terminal sequencing was completed by Mr Jelle Lahnstein (University of Adelaide). Sequence analysis and comparison of results were completed using the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and SWISSPROT/TrEMBL protein databases (http://www.expasy.org/sprot). Sequences were aligned using the ClustalW program (European Molecular Biology Laboratory (EMBL) - European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/clustalw/).

5.2.2 DNA analysis - Cloning of BTI-CMe

5.2.2.1 Plant Material for DNA Extraction

Barley (*Hordeum vulgare* L. cv.) seed was obtained from the Australian Winter Cereals Collection, (Tamworth, Australia). Twenty varieties (14 SE +ve; 6 SE –ve) (Table 2), were selected with 4 seeds from each variety germinated in 500 μL dH$_2$O in 2 mL Eppendorf tubes, placed at 20°C in the dark. All plants were grown and maintained for 28 days in a controlled environment growth chamber with a 12 h photoperiod, 20°C day and 15°C night temperature. Light intensity was 380 μmol/m^2/sec on average.

Table 2: Varieties grown for DNA extraction

#	Variety	SE +/-	Origin
1	Harrington	-	Canada
2	Sloop	+	Australia
3	Betzes	-	Unknown
4	Zephyr	+	Norway
5	Proctor	+	UK
6	Franklin	+	Australia
7	Haruna Nijo	-	Japan
8	Galleon	+	Australia
9	Chebec	+	Australia
10	Steptoe	+	USA
11	Morex	-	USA
12	Amagi Nijo	+	Japan
13	Alexis	+	Germany
14	Blenheim	+	UK
15	Calicuchima	+	Mexico
16	Kustaa	-	Finland
17	Risø 1508	-	Denmark
18	Plaisant	+	France
19	Arapiles	+	Australia
20	Bowman	+	USA

5.2.2.2 DNA extractions

The method used was modified from Rogowsky *et al.* (1991). Briefly, a 10-15 cm long piece of leaf tissue from a 3-week old seedling was folded in four lengthways and placed in the bottom of a 10 mL round-bottom screw cap collection tube. Four small (3 mm) and one large (9 mm) ball bearings were added to each collection tube and the

samples were frozen in liquid nitrogen for 5 min. The samples were vortexed for 30 sec to grind the tissue and then returned to liquid nitrogen until use. Samples were allowed to thaw slightly at room temperature for 2-5 min before the addition of 700 μL of DNA extraction buffer (Appendix D). The tubes were vortexed for 5 sec before 700 μL of phenol/chloroform/isoamyalcohol (25:24:1) (Appendix D) was added and the tubes mixed for a further 20 min on a shaking platform. Silica gel (0.5 mL) was added to the top of each tube with a syringe and the tubes were centrifuged at 4000 rpm for 10 min. The upper aqueous phase was transferred to a new 1.5 mL Eppendorf tube. Isopropanol (600 μL) and 60 μL of 3M sodium acetate (pH 4.8) were added and the DNA precipitated by inversion using an orbital mixer for 5 min. Samples were centrifuged for 30-60 sec with the supernatant discarded. One mL of 70% (v/v) ethanol was added, and the supernatant removed. The samples were left at room temperature for 15-20 min to air dry to remove the residual ethanol. The pellet was resuspended in 50 μL of R40 (40 μg/mL Rnase A in TE buffer, see Appendix D) at 4°C overnight before being stored at -20°C.

5.2.2.3 PCR Primer Design

To clone BTI-CMe, forward and reverse primers were designed using the Vector NTI Suite (InforMax, Bethesda, MD, USA) software. The primers were designed based on the published genomic sequence for *H. vulgare Itr1* gene (HVITR1) (for expressed protein CMe this was between nucleotides 2394 and 2877 of the 5463 nucleotide sequence) (Diaz *et al.*, 1995; Royo, 1992). The primers were obtained from Invitrogen (Melbourne, Victoria, Australia) and were as follows: Forward primer 5'- AAC AAT GGC GTT CAA GTA CCA- 3', reverse primer 5'- CAT GCT TAT TCA TGA TCG AGC TAG - 3'.

5.2.2.4 PCR

The reaction mixture contained 2.5 μL of the DNA template, 0.3 μM of each primer, 0.2 mM of dNTP (2'-deoxynucleoside 5'-triphosphate) (Invitrogen), 1.5 mM MgCl$_2$, 0.5 U *Taq* DNA polymerase (Invitrogen), and a 1/10 volume of 10X reaction buffer, (Invitrogen) made up to a final volume of 30 μL in H$_2$O. The reaction was mixed well and reagents collected in the bottom of the reaction tube by brief centrifugation before thermocycling (PTC-100 Programmable Thermal Controller, MJ Research, MA, USA). The PCR amplification profile was 2 cycles at 95°C for 5 min, 60°C for 1 min and 72°C for 1.5 min followed by 10 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1.5 min, 20 cycles at 95°C for 1 min, 56°C for 1 min and 72°C for 1.5 min, then 72°C for 3 min. The PCR reaction was terminated at 12°C.

5.2.2.5 DNA electrophoresis

The PCR products (5 μL) were mixed with 1 μL of 6x DNA loading buffer (ficoll dye) and separated using 2% (w/v) agarose gels. A 100 bp and/or 1 kb DNA marker was also loaded alongside the PCR products. Agarose gels were prepared from a 2% (w/v) solution of agarose in 1X TAE buffer (Appendix D). Gels were electrophoresed in 1X TAE buffer at a constant voltage of 80V. Following electrophoresis, the gels were stained in an ethidium bromide solution (10 μg/mL in water) for 10-15 min, and then de-stained in water before analysis under short wave UV light.

5.2.2.6 Purification of PCR products

A QIAquick PCR purification kit (Qiagen, Melbourne, Victoria, Australia) was used to purify the DNA fragments from the PCR reactions according to the manufacturer's instructions. Briefly, 5 volumes of Buffer PB (binding buffer) to 1 volume of the PCR

sample was applied to a QIAquick column placed into a 2 mL collection tube. To bind the DNA, the columns were centrifuged at 10,000 x g for 30 – 60 sec. The flow-through was discarded and the column washed with 0.75 mL Buffer PE (wash buffer) then centrifuged at 10,000 x g for 30–60 sec. The flow-through was removed and the column was centrifuged for an additional 1 min at 10,000 x g. The column was placed into a clean 1.5 mL microcentrifuge tube and the DNA eluted by adding 30 μL of H_2O to the centre of the membrane and centrifuging the column for 1 min. The purified products were run on a 2% (w/v) agarose gel with 2 μL of DNA loaded per well as described in section 5.2.2.5.

5.2.2.7 DNA Quantification

DNA was diluted into nano-pure H_2O. Absorbance was determined at 260 nm, with the DNA concentration (μg/mL) calculated using OD_{260} x 50 x dilution factor.

5.2.2.8 DNA Sequencing

DNA sequencing was performed using the forward primer described in section 5.2.2.3. A Big Dye Terminator mix (ABI PRISM® BigDye™ Terminator v 3.0 Cycle Sequencing Kit; Perkin Elmer Applied Biosystems Division, Foster City, CA, USA) was used in the dideoxy-mediated sequencing reactions. The reaction mix contained 5 μL of the DNA template (50-100 ng), 4 μL of 3.2 $p$mole primer, 4 μL of the Big Dye III mix, and H_2O (7 μL) to a final reaction volume of 20 μL. The PCR amplification profile was 95°C for 1 min, then 30 cycles at 95°C for 20 sec, 50°C for 10 sec and 60°C for 2 min. The PCR reaction was terminated at 11°C.

The sequencing runs were transferred to 1.5 mL Eppendorf tubes and the reaction products purified/precipitated by adding 80 µL of 75% (v/v) isopropanol. The tubes were vortexed for 5 - 10 sec, and then incubated at room temperature for 15 min. The samples were centrifuged at 10,000 x g for 20 min and the supernatant removed. Isopropanol (300 µL of 75% (v/v)) was added and the tubes centrifuged at 10,000 x g for 10 min. The supernatant was removed and the pellet was left to air-dry or the tube was placed in a heating block at 37°C for 5-10 min. The samples were sent for sequencing to the Institute of Medical and Veterinary Science (Adelaide, South Australia, Australia). Analysis of the sequencing results was performed using the freeware software package, Chromas 1.45 (http://www.technelysium.com.au/chromas.html). Sequence analysis and comparison of results was completed using the NCBI (http://www.ncbi.nlm.nih.gov) database. Sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/).

5.2.2.9 DNA sequence analysis and PCR primer design for recombinant BTI-CMe protein expression

Following analysis of the two SE +ve and two SE –ve varieties sequenced, more specific primers were then developed to both SE +ve and SE –ve varieties. These included primers designed to the C-terminal region in both SE +ve and SE -ve varieties. Primers were designed based on the nucleotide sequences for BTI-CMe1 and BTI-CMe3.1 (Royo, 1997). Primers were designed to the full length sequence of BTI-CMe1, 444 bp (148 amino acids), BTI-CMe3.1 447 bp (149 amino acids) and a shortened fragment of 174 bp (58 amino acids) BTI-CMe1 SE +ve; 177 bp (59 amino acids) BTI-CMe3.1 (SE -ve). Using the Vector NTI Suite (InforMax) software the following four primer sequences were designed:

Forward Primer (F1) (SE +ve and SE –ve varieties), whole protein

5'- GCG TTC AAG TAC CAG CTC - 3'

Forward Primer (F2) (SE +ve and SE –ve varieties), truncated protein

5'- GGT GCC TAC TTC AAG GAC - 3'

Reverse Primer (R1) (SE –ve variety), whole protein and truncated protein

5'- GAT CGA GCT AGT CGT TAT AAC - 3'

Reverse Primer (R2) (SE +ve variety), whole protein and truncated protein

5'- CGA GCT AGT CGT TAG AAC TTA - 3'

The SE –ve variety Harrington and the SE +ve variety Sloop were used to clone 4 fragments (2 long, 2 short) that were amplified and purified as previously described in sections 5.2.2.4 and 5.2.2.6. The products were designated, Harrington Long (HL), Harrington Short (HS), Sloop Long (SL) and Sloop Short (SS). The amplified and purified products were examined by electrophoresis as described in section 5.2.2.5. With the amplified products, 5 µL of the sample was loaded per well, of the purified products 2 µL of the sample was loaded.

5.2.2.10 Ligation

Purified PCR products from Harrington (SE –ve) and Sloop (SE +ve) were ligated into a *His*- tagged expression vector, pQE-30 UA (50 ng/µL) (Qiagen). The pQE-30 UA expression vector has been designed for direct cloning of PCR products. UA-cloning technology exploits the fact that *Taq* DNA polymerase and other non-proofreading

DNA polymerases add a 3'-end A overhang to PCR products. The prelinearised pQE-30 UA vector, which has a U overhang on each 3' end allows for the direct insertion of such PCR products. This eliminates the need for restriction digestion of the vector or insert, primers with built-in restriction sites, or specially designed adapters, resulting in a much more efficient and robust cloning procedure (Qiagen, The QIAexpressionist™). The ligation mix consisted of 1 µL of pQE-30 UA vector, 2 µL of the purified PCR product, adjusted to 5 µL with nano-pure H_2O and 5 µL 1X ligation master mix. The ligation-reaction mixture was incubated in a water bath at 16°C overnight then kept at 4°C before transformation.

5.2.2.11 Transformation of competent XL1-Blue cells with BTI-CMe

For transformation, individual aliquots of compotent XL1-Blue cells (Appendix D) were thawed on ice. The *E. coli* strain, XL1 Blue was chosen for transformation as it contains the *lacI^q* mutation which produces enough lac repressor to efficiently block transcription, and is ideal for storing and propagating pQE plasmids (Qiagen, The QIAexpressionist™). The ligation mix (2 µL) was mixed with 100 µL of compotent cells and incubated on ice for 45 min. The cells were heat shocked at 42°C for 90 sec then allowed to recover by incubation at 37°C for 60 min with mixing after the addition of 900 µL of SOC (SOB medium containing 20 mM glucose) (Appendix D). The transformation suspensions were plated out onto Luria-Bertani (LB) agar (Appendix D) containing amplicillin (50 µg/mL) and tetracycline (12 µg/mL) and incubated overnight at 37°C. Positive and negative controls were also plated to check transformation efficiency and the antibiotic activity. Single white colonies were selected for plasmid DNA analysis.

A single colony, (carrying the recombinant plasmid) was inoculated into 5 mL of LB media (Appendix D) containing 50 µg/mL ampicillin and 12 µg/mL tetracycline and was grown at 37°C overnight with vigorous shaking.

5.2.2.12 Miniprep of plasmid DNA

A QIAprep Spin Miniprep Kit (Qiagen), was used to purify the plasmid DNA from the overnight *E. coli* cultures according to the manufacturer's instructions. Cells were centrifuged at 8,000 x *g* for 5 min, the suspension discarded and the pellet resuspended in 250 µL Buffer P1 (resuspension buffer containing RNase A). To lyse the cells, 250 µL of Buffer P2 (lysis buffer) was added and the tubes gently inverted 5 times. Buffer N3 (350 µL) was added and the tubes gently inverted several times. The tubes were centrifuged at 10,000 x *g* for 10 min at room temperature, with the supernatants applied to the QIAprep columns and then centrifuged for 45 sec. The flow-through was discarded and the column was washed by adding 750 µL of Buffer PE (wash buffer) and centrifuging for 30-60 sec. The flow-through was discarded and the column centrifuged for an additional 1 min to remove residual ethanol. The column was placed into a clean 1.5 mL microcentrifuge tube and the DNA eluted by adding 50 µL Buffer EB (elution buffer) (10 mM Tris-Cl, pH 8.5) to the centre of the membrane. The column was allowed to stand for 1 min, and was then centrifuged again for 1 min. Minipreps were stored at -80°C.

5.2.2.13 Orientation determination

The four constructs (HL, HS, SL and SS) were sequenced in order to determine their cloning orientations and to check that the inserts were in frame, using pQE vector sequencing primers (Qiagen). Sequencing runs for all four constructs were completed

using both the forward and reverse pQE sequencing primers. The reaction mix contained 5 µL (300 ng) plasmid DNA, 1 µL of 10 μM primer, 2 µL of Big Dye III mix and 3 µL of buffer made up to a final volume of 20 µL with H_2O. The amplification profile was 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The PCR reaction was terminated at 12°C. The PCR products were purified, sequenced and analysed as described in section 5.2.2.8. Plasmids with the correct orientations and in frame were selected for expression.

5.2.2.14 Transformation of competent M15 cells (with the BTI-CMe plasmid)

The *E. coli* M15 [pREP4] host strain was used for the production of recombinant BTI-CMe proteins as it is a high expression vector (Qiagen, The QIAexpressionist™). The plasmid (1 µL) was placed into a sterile 1.5 mL Eppendorf tube and kept on ice. Competent M15 [pREP4] cells (50 µL) (Appendix D) were added to the plasmid, gently mixed, and kept on ice for 20 min. The cells were heat shocked at 42°C in a water bath for 60 sec, then 400 µL of Psi broth (Appendix D) was added and the cells were incubated for 60 min at 37°C. The cells (100 µL) were plated out on LB-agar plates containing 100 µg/mL amplicillin and 25 µg/mL kanamycin. The plates were incubated at 37°C overnight. A single white colony, (carrying the recombinant plasmid) was inoculated into 5 mL of LB media containing 50 µg/mL ampicillin and 25 µg/mL kanamycin and grown at 37°C overnight with vigorous shaking.

5.2.2.15 Expression of BTI-CMe

5.2.2.15.1 Small-Scale Expression

Small-scale expression was completed to check the expression of the recombinant BTI-CMe proteins. Pre-warmed LB media (10 mL), including antibiotics was inoculated

with 500 μL of the 5 mL overnight cultures, and grown at 37°C with vigorous shaking for approximately 30 min until an OD_{600} of 0.5-0.7 was reached. A non-induced control was kept at this time. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG), to a final concentration of 1 mM. Induction continued at 37°C for an additional 4-5 h, a second (induced control) was collected at this time and then the cells were harvested by centrifugation (4,990 x g for 10 min). The cells were frozen in liquid nitrogen, before being stored at –80°C.

The thawed cells were resuspended in 1 mL lysis buffer for native purification (Appendix D) along with 10 μL of lysozyme and incubated on ice for 15 min. The cells were sonicated using a Sonifer B-12, (Branson Ultrasonic, Danbury, CT, USA) at 40 W (5 times x 5 sec) with 10 sec pauses between sonications. Between sonications the lysate was kept on ice. The lysate was centrifuged at 10,000 x g at 4°C for 5 min, with 900 μL of the supernatant (soluble protein) (crude extract A) transferred to a new 1.5 mL Eppendorf tube and kept on ice. The cell pellet was resuspended in 100 μL of lysis buffer (insoluble fraction) (crude extract B). SDS-PAGE (2x) sample buffer (5 μL) was added to 5 μL aliquots of crude extracts A and B. The samples were boiled along with the induced (IPTG) and non-induced (no IPTG) controls (cells were resuspended in 50 μL of 5x SDS-PAGE sample buffer) for 5 min at 95°C. The samples were centrifuged at 10,000 x g for 1 min. SDS-PAGE (proteins were visualised using a R250 Coomassie stain) (Appendix D) and immunoblot analysis with the SE antiserum was completed as previously described in Chapter 2, section 2.2.2 to examine the over-expression and solubility of BTI-CMe in *E. coli* and its cross-reactivity with the SE antiserum.

5.2.2.15.2 Large-Scale Expression

BTI-CMe proteins could be expressed and were contained within the soluble fraction. Large-scale expression was performed in order to purify the recombinant BTI-CMe protein. Cell cultures (500 mL) were prepared as previously described for the small-scale expression (section 5.2.2.15.1), with appropriate scaling of the overnight cultures (from 10 mL overnight cultures, 5 mL were inoculated into 100 mL cultures grown overnight then 25 mL of these overnight cultures were inoculated into the 500 mL cultures). The cells were harvested by centrifugation at 4,000 x g, 4°C for 10 min (Beckman Coulter, Avanti J-E rotor JLA16-250) and then the pellet was resuspended in 10 mL lysis buffer (Appendix D), frozen in liquid nitrogen and stored at –80°C.

5.2.2.16 Purification of BTI-CMe

Cells were defrosted on ice and then 100 μL of lysozyme, and 20 μL of both DNase and RNase were added. The cells were left on ice for 30 min and then sonicated (Sonifer B-12), at 40 W (5 times x 5 sec) with 10 sec intervals between sonications. The cells were placed into liquid nitrogen prior to sonication. The lysate was sonicated to reduce viscosity and insoluble material was removed by centrifugation. The cells were centrifuged at 4°C for 15 – 20 min (4,000 rpm) with the cleared cell lysate stored on ice. Ni-NTA (nitrilotriacetic acid) resin (500 μL) (Qiagen), pre-equilibrated by washing 3X in 1 mL lysis buffer, centrifuging at 700 x g between washes, and finally by resuspending in 1 mL lysis buffer, was added to 100 μL of the cleared lysate. The soluble recombinant protein was allowed to bind to the Ni-NTA resin for 30 min at 4°C using a rotary suspension mixer (Ratek Instruments). The resin was spun down at 600 x g for 1 min and the supernatant was kept. The resin was washed at room temperature, 3X each with wash buffer 1, followed by wash buffer 2 and finally with wash buffer 3

(Appendix D). With each wash, 5 mL of buffer was used and between washes the resin was centrifuged for 1 min at 700 x g, with each of the washes kept for analysis. The recombinant protein was eluted with 400 μL of elution buffer (Appendix D). The eluent was titrated back to pH 7.0 with 50 μL of titration buffer (Appendix D). The concentration of protein in the purified preparation was determined by measuring its absorbance at 260 and 280 nm using the following equation: [Protein] (mg/mL) = $1.55 * A_{280} - 0.76 * A_{260}$ (Stoscheck, 1990).

With the large-scale expression and purification, 1 mL of non-induced cells, cells induced with IPTG, the cleared lysate, the flow through, each of the wash steps, and the eluates were collected. SDS-PAGE (2x) sample buffer (5 μL) was added to 5 μL aliquots of both the cleared cell lysate and flow through, and 3 μL of 2x SDS-PAGE sample buffer was added to 15 μL aliquots of the washes and the eluates. The samples were boiled along with the induced and non-induced controls (cells resuspended in 50 μL of 5x SDS-PAGE sample buffer) for 5 min at 95°C. The samples were centrifuged at 10,000 x g for 1 min. SDS-PAGE (proteins were visualised using a R250 Coomassie stain) (Appendix D) of these samples and immunoblot analysis with the SE antiserum of the purified protein was completed as previously described in Chapter 2, section 2.2.2. Mass spectrometry analysis of the purified HL and SS proteins was completed by Mr Yoji Hayasaka at the Australian Wine Research Institute, Adelaide, South Australia.

5.2.2.17 Antibodies raised against the recombinant BTI-CMe protein

Antibodies to the purified protein preparations designated as HL and SS were raised as follows. Two female semi-lop rabbits were injected subcutaneously with the HL and SS preparations (200 μg in 500 μL of PBS) emulsified with Freund's complete adjuvant

145

(total 500 µL, Sigma, St Louis, MO, USA) at 10 sites (i.e. 10 x 0.1 mL) on the neck and

back regions. One further booster injection of these proteins (200 µg in 500 µL of PBS)

was administered with Freund's incomplete adjuvant (500 µL, Sigma) at 4 weeks after

the initial injection. This mixture was injected subcutaneously as described above.

Blood samples were collected by bleeding the marginal ear vein just prior to the first

immunisation (pre-immune serum) and at two weekly intervals (20 mL maximum)

starting at 6 weeks after the initial immunization (three, two week cycles in total).

The antibodies were raised by Dr Evan Evans at the University of Tasmania (Hobart,

Tasmania, Australia). SDS-PAGE immunoblot analysis with the HL and SS antiserum

was completed as previously described in Chapter 2, section 2.2.2. Serum dilution was

1/100.

5.2.2.18 SDS-PAGE immunoblot analysis with the SE antiserum of known BTI-CMe1 and BTI-CMe3/3.1 variants for the presence or absence of the SE protein

Seventeen varieties identified in the literature or by their sequence data (section

5.3.2.2), as either BTI-CMe1 or BTI-CMe3/3.1 variants were screened for the presence

or absence of the SE protein by SDS-PAGE immunoblot analysis using the SE

antiserum. Seed was sourced for 16 of the 17 varieties from the Australian Winter

Cereals Collection, (Tamworth, Australia), Pirkka seed was sourced from the

Brinkworth site (South Australian Barley Improvement Program). SDS-PAGE and

immunoblot analysis was conducted as previously described in Chapter 2, section 2.2.2.

5.3 Results

5.3.1 Protein Analysis

5.3.1.1 Identification of the SE protein

Two-dimensional (2-D) SDS-PAGE immunoblot analysis of the 1M urea/1% (v/v) 2-mecapto-ethanol crude protein extracts using the SE antiserum detected a range of protein spots. The MW ~12000 SE protein, which was observed in the previously described one-dimensional SDS-PAGE immunoblot analysis (Chapter 2, section 2.3.1.1) was evident in the 2-D immunoblot of the SE +ve variety, Sloop, but was not present in the SE −ve variety, Harrington (Figure 1). Additional protein spots between MW 32000-98000 were also identified. 2-D gel electrophoresis (*pI* 3-10) of the crude protein extracts identified approximately 100 spots as detected by colloidal Coomassie blue staining (Figure 2). Approximately 20 protein spots were identified between MW 7000-20000.

The SE protein was identified by comparison of the Coomassie blue stained gels with the SE immunoblot results. The SE protein was clearly separated from other proteins on the gel. A protein of MW ~15000 (*pI* 6.5-7.0) was identified in the SE +ve variety (Sloop), however a matching protein was also identified in the SE −ve variety (Harrington), in the same region of the gel (Figure 2). In the SE −ve variety Harrington, this protein appeared to consist of two partially coalesced spots. The lower of the two spots in the Harrington sample was selected for further analysis. These SE protein spots from Sloop (SE +ve) and Harrington (SE −ve) were extracted and digested with trypsin for N-terminal sequencing (Figure 2).
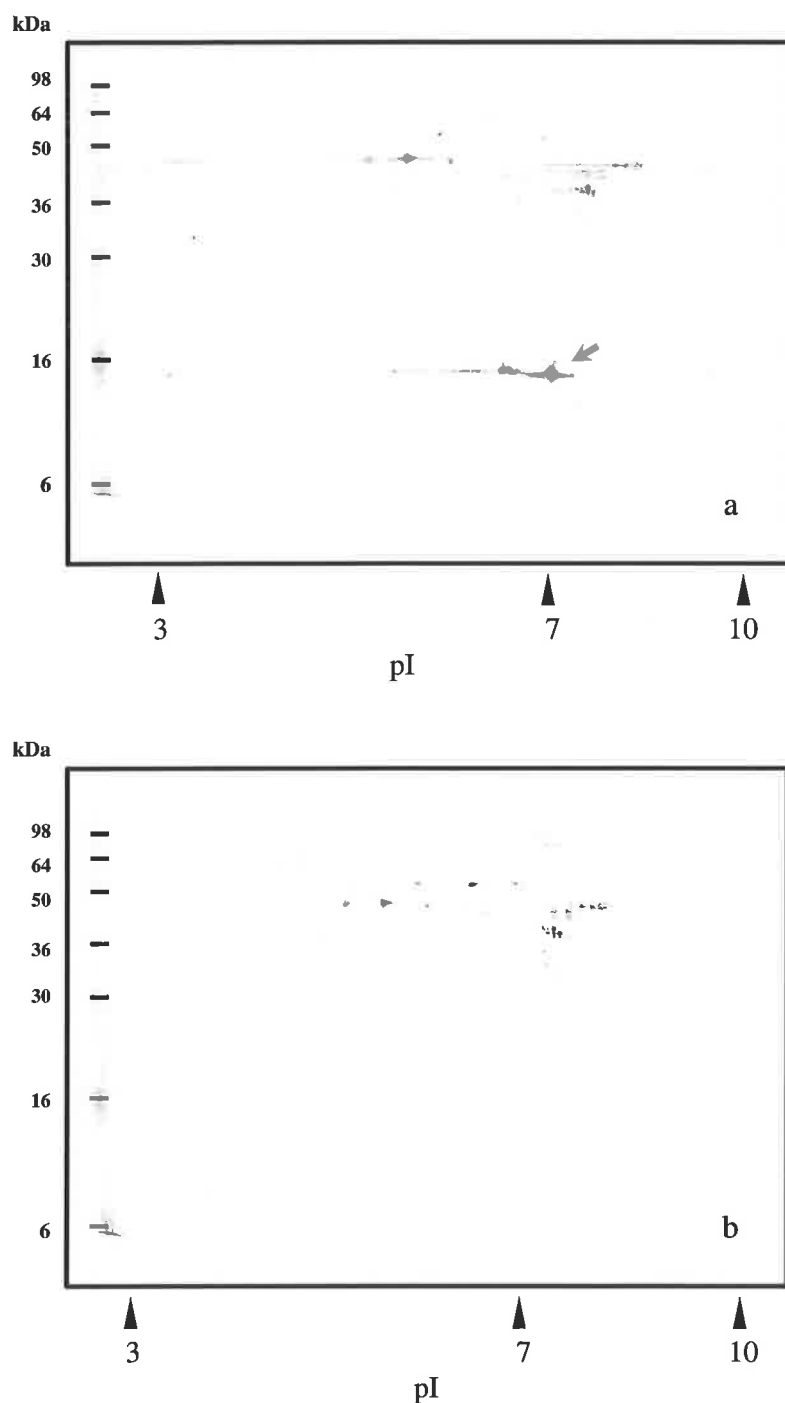
Figure 1: 2-D immunoblots using the anti-SE antibody (1/1000) to probe the soluble

protein fractions extracted from ground barley of an SE +ve (Sloop) (a) and an SE -ve

(Harrington) (b) variety. Proteins were separated in the first dimension using a *pI* 3-10

IPG strip and in the second dimension using 15% (w/v) SDS-PAGE.   The arrow

indicates the SE protein. Per gel (~0.8 mg) of total protein was loaded.
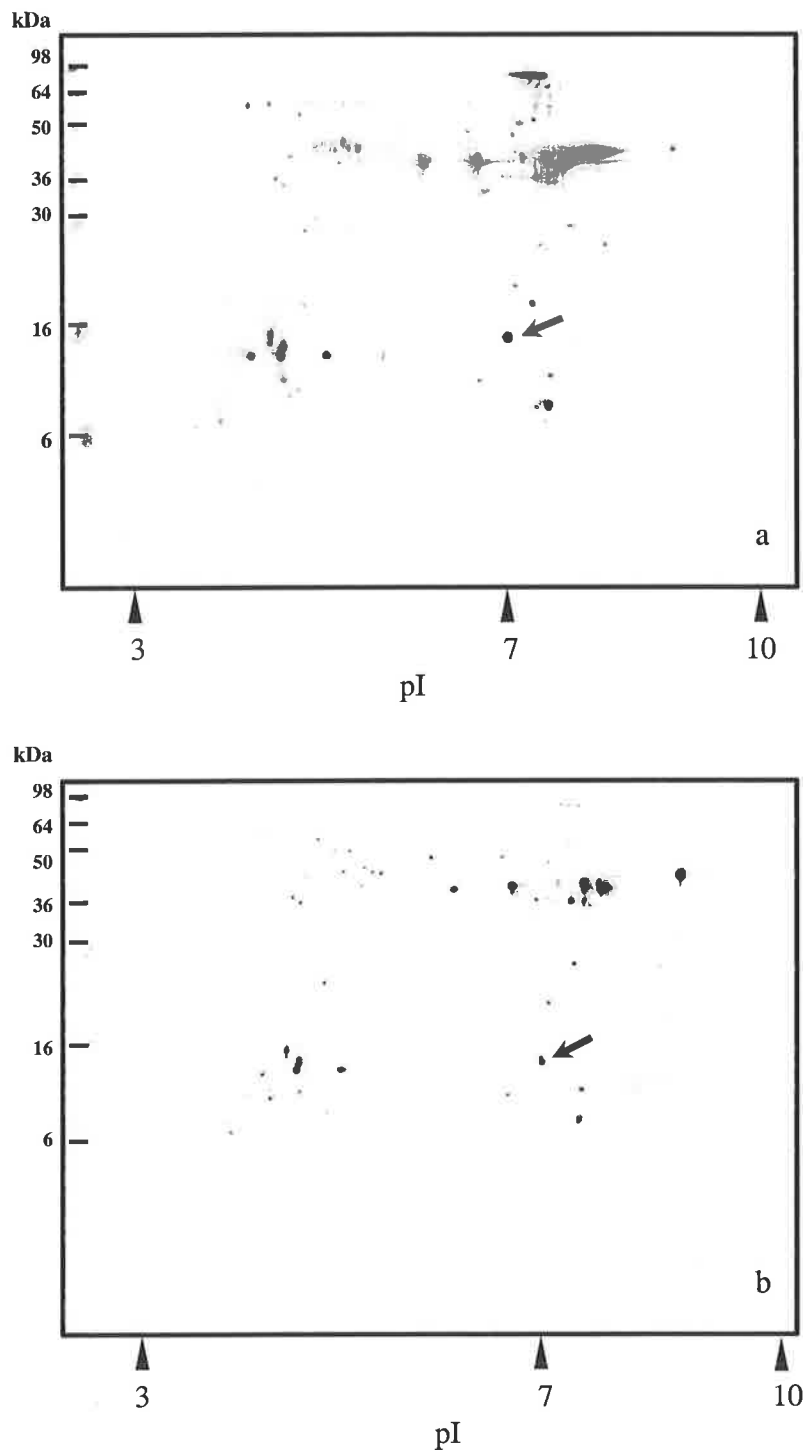
Figure 2: 2-D electrophoresis of the 1M urea/1% (v/v) 2-mecapto-ethanol soluble protein fractions extracted from ground barley from an SE +ve (Sloop) (a) and a SE –ve (Harrington) (b) variety. Proteins were visualised by colloidal Coomassie blue staining. The spots labelled with an arrow were those identified as the SE protein and were excised from the gel for tryptic digests. Per gel (~0.8 mg) of total protein was loaded.

149

5.3.1.2 Amino acid sequence analysis

N-terminal and internal amino acid sequences of the tryptic peptides derived from the SE +ve (Sloop) and SE –ve (Harrington) varieties were determined. Tryptic digestions of the SE –ve and SE +ve preparations produced at least 12 peptide fractions, which were resolved by reversed-phase HPLC. Three of these fractions were selected for N-terminal sequencing (Figure 3). These included peak 7 from both the SE +ve variety Sloop and the SE –ve variety Harrington. It was noted that peak 7 displayed a difference of ~4 min in the retention time between the Sloop and Harrington fractions. Peak 5 from the SE +ve variety Sloop was also selected as it was predicted, using MS-Digest in ProteinProspector 4.0.4. (http://prospector.ucsf.edu/ucsfhtml4.0/msdigest. htm), to be the N-terminal fragment of the undigested proteins.
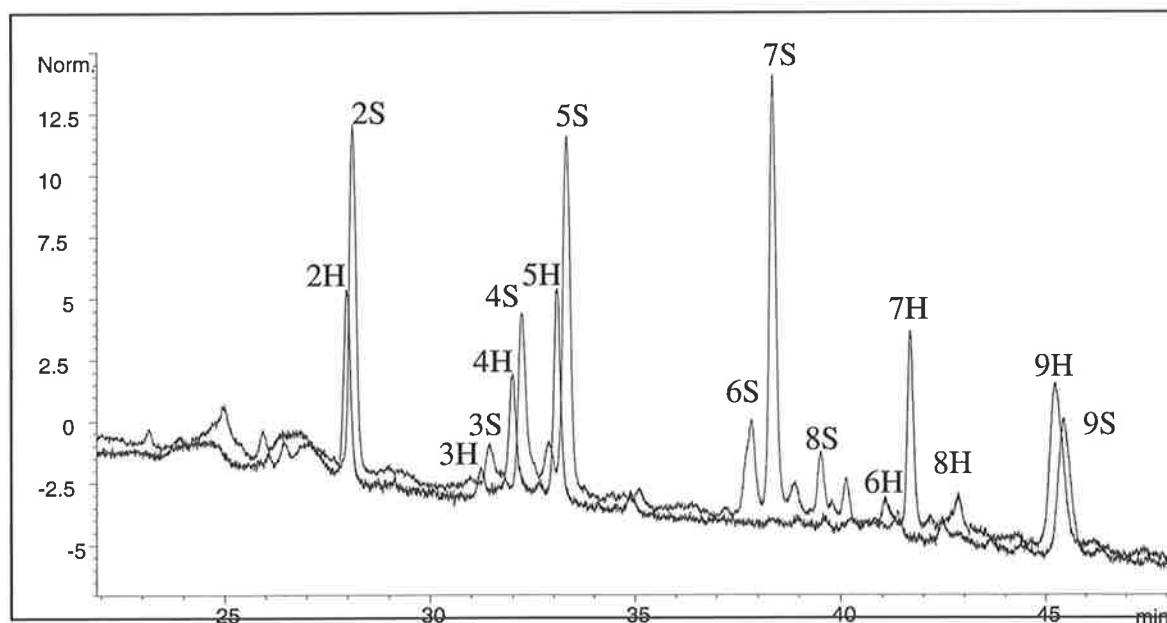
Figure 3: Peptide map of the tryptic digests of the SE protein from Harrington (SE –ve) and Sloop (SE +ve). H = Harrington, S = Sloop. Fractions 5 and 7 from the SE +ve Sloop variety were selected for sequencing, along with fraction 7 from the SE –ve variety Harrington.

The peak fractions collected (Figure 3) were subsequently sequenced. The SE +ve fraction (peak 7) was sequenced for 25 cycles with the following sequence obtained; QTSYAANLVTPQECNLGTIHGSAY, and the sequence for a second (SE +ve) fraction (peak 5) was also obtained; FGDSCAPGDALPH. The SE –ve fraction (peak 7) sequence was found to be: QTSYAANLVT-QE-NL--. As fraction 7 from both the SE +ve (Sloop) and SE –ve (Harrington) varieties was identified as the same protein sequence, the difference in retention time of ~4 min in the RP-HPLC observed for this fraction in both varieties indicate differences in the amino acid sequence (see that peaks 4, 5 and 9 have similar retention times). It is possible that additional amino acids were present in the SE –ve fraction that were not sequenced. This may have been due to a limited amount of protein present in the sample. Results of non-redundant BLAST searches of these three sequences revealed 100% sequence identity to the *Hordeum vulgare* L. trypsin inhibitor CMe precursor (Chloroform/methanol-soluble protein - CMe) (Table 3).

Table 3: Characteristics of the sequenced peptides and matches to the databases.

N-terminal * or internal sequence +	pI [(a)]	MW [(a)]	Matching polypeptide from SWISSPROT	Identity (%)[(b)]	pI [(c)]	MW [(c)]
QTSYAANLVTPQECNLGTIHGSAY + starting from aa 89 (SE +ve)	6.5-7.0	~15000	IAAE_HORVU P01086	100	6.95	13258.2
FGDSCAPGDALPH * (SE +ve)	6.5-7.0	~15000	IAAE_HORVU P01086	100	6.95	13258.2
QTSYAANLVT-QE-NL-- + starting from aa 89 (SE -ve)	6.5-7.0	~15000	IAAE_HORVU P01086	83.33	6.95	13258.2

a. Determined from Figures 1 and 2.

b. The % identity was calculated using FASTA (http://www.ebi.ac.uk/fasta33/) (Pearson and Lipman, 1988), based on the number of residues that were sequenced.

c. Calculated from the sequence of the mature protein using MS-Digest in ProteinProspector 4.0.4. http://prospector.ucsf.edu/ucsfhtml4.0/msdigest.htm.

5.3.2 Cloning, expression and purification of BTI-CMe

5.3.2.1 DNA sequencing

Using the forward and reverse primers designed from the published sequence of the *H. vulgare Itr1* gene (HVITR1), a fragment of approximately 450 base pairs (bp) could be observed in 18 of the 20 barley varieties screened following amplification of genomic DNA by PCR (Figure 4). Of these varieties, four varieties, two previously identified as SE –ve (Harrington and Haruna Nijo) and two as SE +ve (Proctor and Sloop) were selected, the PCR products purified (Figure 4), and then sequenced.
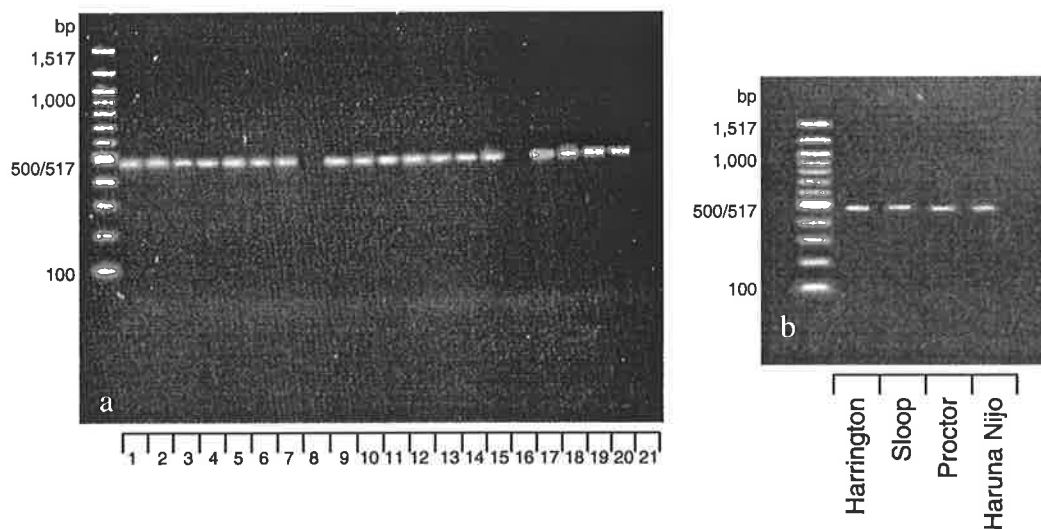
Figure 4: (a) Agarose gel electrophoresis of PCR amplified products of BTI-CMe from genomic DNA preparations of 20 barley varieties (lanes 1-20), lane 21 is a control (H$_2$O). Lanes 1 to 20 are the 20 varieties listed in Table 2, section 5.2.2.1 and have been run in the order listed. (b) Agarose gel electrophoresis of the purified PCR products used in sequencing reactions (Harrington, Sloop, Proctor and Haruna Nijo).

5.3.2.2 DNA sequence analysis

BTI-CMe has been found to be polymorphic (Moralejo *et al.*, 1993; Salcedo *et al.*, 1984), with 5 allelic variants so far being identified in both *Hordeum vulgare* and *Hordeum spontaneum*. They include: (1.) BTI-CMe1, (2.) BTI-CMe2; which consists of three components BTI-CMe2.1, BTI-CMe2.2 and BTI-CMe2.3, (3.) BTI-CMe3; which consists of two components BTI-CMe3.1 and BTI-CMe3.2 (Ladogina, 1997; Moralejo *et al.*, 1993; Salcedo *et al.*, 1984), (4.) BTI-CMe4 and (5.) BTI-CMe5 (Ladogina, 1997). Additionally, on the NCBI database (http://www.ncbi.nlm.nih.gov), the following unpublished variants of BTI-CMe are listed: BTI-CMe1 (HVAJ2977) (*H. vulgare*), BTI-CMe2.1 (HVU251931 and HSAJ2974) (*H. vulgare* and *H. spontaneum*, respectively), BTI-CMe2.2 (HVAJ3458 and HSAJ2975) (*H. vulgare* and *H. spontaneum*, respectively), BTI-CMe3.1 (HVAJ2978) (*H. vulgare*), and BTI-CMe4 (HSAJ2976) (*H. spontaneum*) (Royo, 1997, 1998 and 1999).

BLAST searches of the BTI-CMe DNA sequences obtained in this study were performed to determine the homology between the sequences with those previously reported. The nucleotide sequences obtained from the two SE −ve varieties, Harrington and Haruna Nijo, and the two SE +ve varieties Sloop and Proctor with those available on the NCBI database, revealed that the SE −ve varieties aligned with the BTI-CMe3.1 variant (Harrington and Haruna Nijo: 98% homology), while the SE +ve varieties aligned with the BTI-CMe1 variant (Sloop 96%; Proctor 97% homology). Proctor has been previously identified as a BTI-CMe1 variant (Ladogina, 1997). Analysis of the sequence data from Harrington and Haruna Nijo (SE −ve) revealed 98% sequence identity between these SE −ve varieties, between the SE +ve varieties Sloop and Proctor, 98% identity was also observed. Comparison of the SE −ve varieties

(Harrington and Haruna Nijo) and the SE +ve varieties (Proctor and Sloop), revealed 95% sequence identity. A phylogram was created using the ClustalW program (http://www.ebi.ac.uk/clustalw/) to show the relationship between the SE –ve and SE +ve varieties cloned in this study and known BTI-CMe variants obtained from the NCBI database (Figure 5).
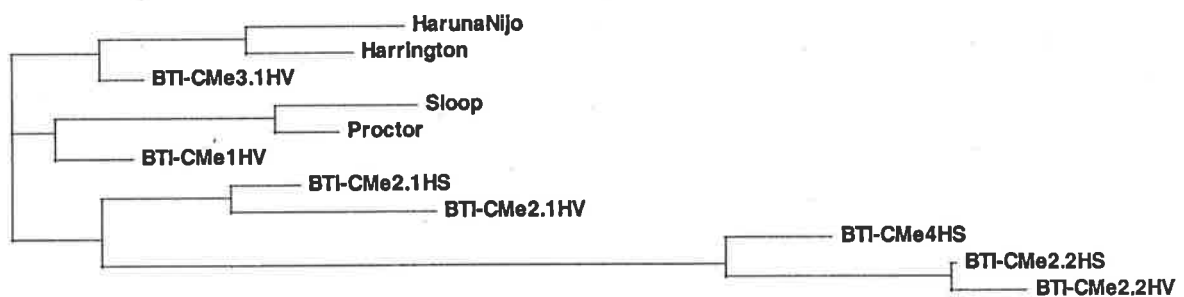
Figure 5: A phylogenetic tree created using the ClustalW program (http://www.ebi.ac.uk/clustalw/) to show the relationship between the SE –ve (Harrington and Haruna Nijo) and SE +ve (Sloop and Proctor) varieties cloned in this study and known BTI-CMe variants obtained from the NCBI database. HV after the variant indicates BTI-CMe sequences from *H. vulgare*, HS after the variant indicates sequences from *H. spontaneum*.

Comparison of the sequence data obtained from these two variants, revealed differences in the DNA encoding the C-terminal region of the protein between SE +ve (BTI-CMe1) and SE –ve (BTI-CMe3.1) variants (Figure 6). These nucleotide differences in the C-terminal region of the protein amount to six amino acid substitutions between the SE

154

+ve (BTI-CMe1) and SE -ve (BTI-CMe3.1) variants (Figure 6). The amino acid substitutions are at positions 105, 129, 135, 136, 148, and 149 of the expressed BTI-CMe proteins. There were a further three nucleotide changes observed across the full-length sequence of BTI-CMe. Two of these changes do not result in amino acid changes, while the third change did result in an amino acid substitution of Ser (S) in the SE +ve varieties to Phe (F) in the SE –ve varieties at position 4 (Figure 6). The amino acid Phe (F) is not shown in any of the published variants of BTI-CMe on the NCBI database, however BTI-CMe has been shown to be polymorphic (Ladogina, 1997; Moralejo *et al.,* 1993; Salcedo *et al.,* 1984) and as a limited number of barley varieties have been sequenced to date, this data may represent the identification of a different form of BTI-CMe in the SE –ve varieties Harrington and Haruna Nijo.

Figure 6: Nucleotide sequences of the SE +ve (Sloop and Proctor) and the SE −ve (Harrington and Haruna Nijo) varieties incorporating the published BTI-CMe1 and BTI-CMe3.1 sequences. An * indicates a match between SE −ve and SE +ve varieties and the published BTI-CMe1 and BTI-CMe3.1 sequences. **Bold** and <u>underlined</u> text indicates nucleotide changes between SE +ve and BTI-CMe1 sequences and SE −ve and BTI-CMe3.1 sequences; **Bold** text indicates nucleotide changes between SE +ve and SE −ve varieties but not BTI-CMe1 and BTI-CMe3.1 sequences except with regard to the nucleotide changes of G (BTI-CMe1) to C (BTI-CMe3.1) at position 261 and from GC (BTI-CMe3.1) to CG (BTI-CMe1) at position 298 in the BTI-CMe1 and BTI-CMe3.1 sequences; *Italic* text indicates no match. The amino acid sequence listed below was deduced from the nucleotide sequences obtained and is shown for the mature BTI-CMe protein. This multiple sequence alignment was performed using ClustalW (1.82).

```
SE +ve Sloop       ----------------GCNTCTCTGGTCGGCCGCCGTCATGCTCGCCATTTTT-TCGCC 42
SE +ve Proctor     ------------------CTCTCCTGTCGGCCGCCGTCATGCTCGCCATTCTT-TCGCC 40
BTI-CMe1           ATGGCGTTCAAGTACCAGCTCATCCTCTCGGCCGCCGTCATGCTCGCCATTCTCGTCGCC 60
SE -ve Harrington  ----------------GCNNCCTCTCGGCCGCCGTCATGCTCGCCATTTTTTTTCGCC 41
SE -ve Haruna Nijo ------------------GCTATCTCTCGGCCGCCGTCATGCTCGCCATTCTTT-CGCC 40
BTI-CMe3.1         ATGGCGTTCAAGTACCAGCTCATCCTCTCGGCCGCCGTCATGCTCGCCATTCTCGTCGCC 60
                                       ********************** *    ****

SE +ve Sloop       ACTGCCACCAGTTTCGGGGATTCGTGTGCTCCAGGGGATGCGTTGCCACACAACCCTCTC 102
SE +ve Proctor     ACTGCCACCAGTTTCGGGGATTCGTGTGCTCCAGGGGATGCGTTGCCACACAACCCTCTC 100
BTI-CMe1           ACTGCCACCAGTTTCGGGGATTCGTGTGCTCCAGGGGATGCGTTGCCACACAACCCTCTC 120
SE -ve Harrington  ACTGCCACCAGTTTCGGGGATTTTTGTGCTCCAGGGGATGCGTTGCCACACAACCCTCTC 101
SE -ve Haruna Nijo ACTGCCACCAGTTTCGGGGATTTTNGTGCTCCAGGGGATGCGTTGCCACACAACCCTCTC 100
BTI-CMe3.1         ACTGCCACCAGTTTCGGGGATTCGTGTGCTCCAGGGGATGCGTTGCCACACAACCCTCTC 120
                   *******************    **********************************
                         F  G  D S/F C  A  P  G  D  A  L  P  H  N  P  L

SE +ve Sloop       AGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACC 162
SE +ve Proctor     AGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACC 160
BTI-CMe1           AGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACC 180
SE -ve Harrington  AGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACC 161
SE -ve Haruna Nijo AGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACC 160
BTI-CMe3.1         AGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACC 180
                   ************************************************************
                     R  A  C  R  T  Y  V  V  S  Q  I  C  H  Q  G  P  R  L  L  T

SE +ve Sloop       TCGGACATGAAGAGGCGGTGTTGCGACGAGCTGTCGGCCATCCCGGCGTACTGTAGGTGC 222
SE +ve Proctor     TCGGACATGAAGAGGCGGTGTTGCGACGAGCTGTCGGCCATCCCGGCGTACTGTAGGTGC 220
BTI-CMe1           TCGGACATGAAGAGGCGGTGTTGCGACGAGTTGTCGGCCATCCCGGCGTACTGCAGGTGC 240
SE -ve Harrington  TCGGACATGAAGAGGCGGTGTTGCGACGAGTTGTCGGCCATCCCGGCGTACTGCAGGTGC 221
SE -ve Haruna Nijo TCGGACATGAAGAGGCGGTGTTGCGACGAGTTGTCGGCCATCCCGGCGTACTGCAGGTGC 220
BTI-CMe3.1         TCGGACATGAAGAGGCGGTGTTGCGACGAGTTGTCGGCCATCCCGGCGTACTGCAGGTGC 240
                   ****************************** ********************** ******
                     S  D  M  K  R  R  C  C  D  E  L  S  A  I  P  A  Y  C  R  C

SE +ve Sloop       GAAGCGCTGCGTATCATCATGCAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTGCC 282
SE +ve Proctor     GAAGCGCTGCGTATCATCATGCAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTGCC 280
BTI-CMe1           GAAGCGCTGCGTATCATCATGGAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTCGC 300
SE -ve Harrington  GAAGCGCTGCGTATCATCATGCAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTGCC 281
SE -ve Haruna Nijo GAAGCGCTGCGTATCATCATGCAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTGCC 280
BTI-CMe3.1         GAAGCGCTGCGTATCATCATGCAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTGCC 300
                   ********************* ************************************* *
                     E  A  L  R  I  I  M E/Q G  V  V  T  W  Q  G  A  F  E  G R/A

SE +v Sloop        TACTTCAAGGACTCGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCCGCCAACCTC 342
SE +ve Proctor     TACTTCAAGGACTCGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCCGCCAACCTC 340
BTI-CMe1           TACTTCAAGGACACGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCCGCCAACCTC 360
SE -ve Harrington  TACTTCAAGGACACGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCCGCCAACCTC 341
SE -ve Haruna Nijo TACTTCAAGGACACGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCCGCCAACCTC 340
BTI-CMe3.1         TACTTCAAGGACACGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCCGCCAACCTC 360
                   ************ ***********************************************
                     Y  F  K  D S/T P  N  C  P  R  E  R  Q  T  S  Y  A  A  N  L

SE +ve Sloop       GTCACCCCGCAGGAGTGCAACCTAGGGACCATCCACGGCAGCGCGTACTGCCCCGAACTG 402
SE +ve Proctor     GTCACCCCGCAGGAGTGCAACCTAGGGACCATCCACGGCAGCGCGTACTGCCCCGAACTG 400
BTI-CMe1           GTCACCCCGCAGGAGTGCAACCTATGGACCATCCACGGCAGCGCGTACTGCCCCGAACTG 420
SE -ve Harrington  GTCACCCCGCAGGAGTGCAACCTATGGACCATCCACGGCAGCCCGTCCTGCCCCGAACTG 401
SE -ve Haruna Nijo GTCACCCCGCAGGAGTGCAACCTATGGACCATCCACGGCAGCCCGTCCTGCCCCGAACTG 400
BTI-CMe3.1         GTCACCCCGCAGGAGTGCAACCTATGGACCATCCACGGCAGCCCGTCCTGCCCCGAACTG 420
                   ************************ ***************** **** ************
                     V  T  P  Q  E  C  N  L G/W T  I  H  G  S A/P Y/S C  P  E  L

SE +ve Sloop       CAGCCCGGATATGGAGTGGTCTTTGTAATAAGTTCTAACGACTAGCTCG 450
SE +ve Proctor     CAGCCCGGATATGGAGTGGTCTTTGTAATAAGTTCTAACGACTAGCTCG 448
BTI-CMe1           CAGCCCGGATATGGAGTGGTCTTTGTAATAAGTTCTAACGACTAGCTCG 468
SE -ve Harrington  CAGCCCGGATATGGAGTGGTCTCGTCATAAGTTATAACGACTAGCTCG 449
SE- ve Haruna Nijo CAGCCCGGATATGGAGTGGTCTCGTCATAAGTTATAACGACTAGCTCG 448
BTI-CMe3.1         CAGCCCGGATATGGAGTGGTCTCGTCATAAGTTATAACGACTAGCTCG 468
                   ********************* ** ******* ***************
                     Q  P  G  Y  G  V  V L/S Y/S
```

156

5.3.2.3 Cloning of BTI-CMe1 and BTI-CMe3 variants

Specific primers were designed to amplify the genomic DNA of the full length BTI-CMe protein, as well as to amplify truncated C-terminal ends in both SE +ve and SE –ve varieties. Four constructs to recombinant BTI-CMe proteins (2 full length, 2 truncated) were made using DNA obtained from SE +ve (Sloop) and SE –ve (Harrington) varieties. The primers were designed based on the nucleotide sequences for BTI-CMe1 (HVAJ2977) and BTI-CMe3.1 (HVAJ2978) (Royo, 1997). Primers were designed to the full-length sequence of BTI-CMe1, 444 bp (148 amino acids), BTI-CMe3.1 447 bp (149 amino acids) and shorter fragments of 174 bp (58 amino acids BTI-CMe1 SE +ve) and 177 bp (59 amino acids BTI-CMe3.1 SE -ve); in total 4 BTI-CMe fragments were amplified.

The fragment amplified by forward primer F1 and reverse primer R1 (section 5.2.2.9) began at nucleotide 4 and ended at nucleotide 471, encoding the full length of BTI-CMe3.1. The second fragment amplified with forward primer F1 and reverse primer R2 (section 5.2.2.9) was 464 bp long, from nucleotide 4 to 468, encoding the full length of BTI-CMe1. The third fragment amplified with forward primer F2 and reverse primer R1 (section 5.2.2.9) began at nucleotide 295 and ended at nucleotide 471, encoding the shortened fragment of BTI-CMe3.1. The fourth fragment amplified with forward primer F2 and reverse primer R2 (section 5.2.2.9) was 173 bp long, from nucleotide 295 to 468, encoding the shortened fragment of BTI-CMe1. The primers and their fragments amplified are shown in Figure 7. The amplified and purified products were examined using agarose gel electrophoresis, as shown in Figure 8.

```
                              ┌──────────────────────────┐
                              │         F1               │
                              └──────────────────────────┘
BTI-CMe3.1 (SE -ve)  ATGGCGTTCAAGTACCAGCTCATCCTCTCGGCCGCCGTCATGCTCGCCATTCTCGTCGCC 60
BTI-CMe1 (SE +ve)    ATGGCGTTCAAGTACCAGCTCATCCTCTCGGCCGCCGTCATGCTCGCCATTCTCGTCGCC 60
                     ******************************************************* ****
                      M  A  F  K  Y  Q  L  I  L  S  A  A  V  M  L  A  I  L  V  A

BTI-CMe3.1 (SE -ve)  ACTGCCACCAGTTTCGGGGATTCGTGTGCTCCAGGGGATGCGTTGCCACACAACCCTCTC 120
BTI-CMe1 (SE +ve)    ACTGCCACCAGTTTCGGGGATTCGTGTGCTCCAGGGGATGCGTTGCCACACAACCCTCTC 120
                     ************************************************************
                      T  A  T  S  F  G  D  S  C  A  P  G  D  A  L  P  H  N  P  L

BTI-CMe3.1 (SE -ve)  AGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACC 180
BTI-CMe1 (SE +ve)    AGAGCCTGCCGCACCTACGTGGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCCTCACC 180
                     ************************************************************
                      R  A  C  R  T  Y  V  V  S  Q  I  C  H  Q  G  P  R  L  L  T

BTI-CMe3.1 (SE -ve)  TCGGACATGAAGAGGCGGTGTTGCGACGAGTTGTCGGCCATCCCGGCGTACTGCAGGTGC 240
BTI-CMe1 (SE +ve)    TCGGACATGAAGAGGCGGTGTTGCGACGAGTTGTCGGCCATCCCGGCGTACTGCAGGTGC 240
                     ************************************************************
                      S  D  M  K  R  R  C  C  D  E  L  S  A  I  P  A  Y  C  R  C

BTI-CMe3.1 (SE -ve)  GAAGCGCTGCGTATCATCATGCAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTGCC 300      ┌──────┐
BTI-CMe1 (SE +ve)    GAAGCGCTGCGTATCATCATGGAAGGGGTAGTAACTTGGCAGGGTGCGTTCGAGGGTCGC 300 ──▶  │  F2  │
                     *********************  ***********************************  *       └──────┘
                      E  A  L  R  I  I  M  Q/E G  V  V  T  W  Q  G  A  F  E  G  A/R

BTI-CMe3.1 (SE -ve)  TACTTCAAGGACACGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCCGCCAACCTC 360
BTI-CMe1 (SE +ve)    TACTTCAAGGACACGCCCAACTGCCCTAGGGAGAGGCAAACGAGCTACGCCGCCAACCTC 360
                     ************************************************************
                      Y  F  K  D  T  P  N  C  P  R  E  R  Q  T  S  Y  A  A  N  L

BTI-CMe3.1 (SE -ve)  GTCACCCCGCAGGAGTGCAACCTATGGACCATCCACGGCAGCCCGTCCTGCCCCGAACTG 420
BTI-CMe1 (SE +ve)    GTCACCCCGCAGGAGTGCAACCTATGGACCATCCACGGCAGCGCGTACTGCCCCGAACTG 420
                     ******************************************  *** **********
                      V  T  P  Q  E  C  N  L  W  T  I  H  G  S  P/A S/Y C  P  E  L

BTI-CMe3.1 (SE -ve)  CAGCCCGGATATGGAGTGGTCTCGTCATAAGTTATAACGACTAGCTCGATCATGAATAAG 480     ┌──────┐
BTI-CMe1 (SE +ve)    CAGCCCGGATATGGAGTGGTCTTGTAATAAGTTCTAACGACTAGCTCGATCATGAATAAG 480 ──▶ │  R1  │
                     **********************  **  *****  *********************          └──────┘
                      Q  P  G  Y  G  V  V  S/L S/-          ┌──────────────────────────┐
                                                           │           R2             │
BTI-CMe3.1 (SE -ve)  CATG 484                              └──────────────────────────┘
BTI-CMe1 (SE +ve)    CATG 484
                     ****
```

Figure 7: Cloning of BTI-CMe1 and BTI-CMe3 variants, showing the primers designed F1, F2, R1 and R2 and their amplified products. Primers are in **bold** and are <u>underlined</u>. Primers were designed based on the BTI-CMe1 and BTI-CMe3.1 variants available on the NCBI database. With the sequence data obtained from the NCBI database there are nucleotide changes at positions 298 and 299 between BTI-CMe3.1 (GC) and BTI-CMe1 (CG), with this data within the designed F2 primer region. From the sequence data obtained for two SE –ve (Harrington and Haruna Nijo) and two SE +ve (Sloop and Proctor) varieties, the BTI-CMe3.1 (GC) nucleotide combination was observed (Figure 6) and thus the F2 primer designed was based on the BTI-CMe3.1 data and was used in the amplification of both BTI-CMe3.1 (SE –ve) and BTI-CMe1 (SE +ve) variants.
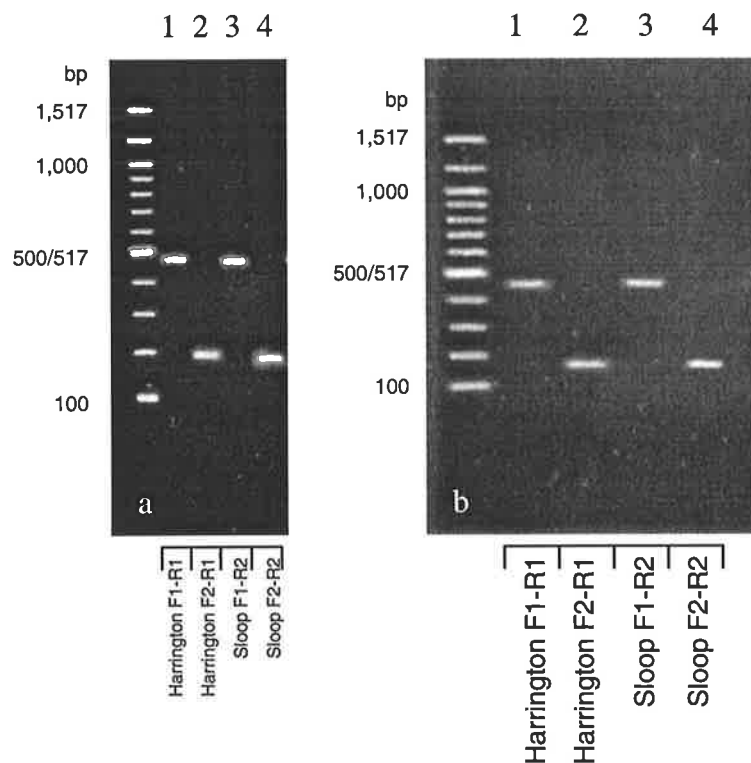
Figure 8: (a) Agarose gel electrophoresis of the PCR amplified products of BTI-CMe from Harrington and Sloop (lanes 1-4). (b) Agarose gel electrophoresis of the purified PCR products (Harrington and Sloop). Lane 1 (Harrington F1-R1), a fragment amplified by forward primer F1 and reverse primer R1, encoding the full length of BTI-CMe3.1. Lane 2 (Harrington F2-R1), a fragment amplified with forward primer F2 and reverse primer R1, encoding a shortened fragment of BTI-CMe3.1. Lane 3 (Sloop F1-R2), a fragment amplified with forward primer F1 and reverse primer R2, encoding the full length of BTI-CMe1. Lane 4 (Sloop F2-R2), a fragment amplified with forward primer F2 and reverse primer R2, encoding a shortened fragment of BTI-CMe1.

5.3.2.4 Expression of BTI-CMe1 and BTI-CMe3 variants

The four PCR fragments amplified (and then purified), two from Harrington (SE –ve) and two from Sloop (SE +ve), were then sub-cloned into a *His-* tagged bacterial expression vector (pQE-30 UA). This vector is designed to allow for the production of recombinant proteins with N-terminal 6-*His* tags. The recombinant proteins were designated as HL, HS, SL and SS based on the variety used (Harrington (H) or Sloop (S)) and the length of the fragment amplified (full length protein (L) or the shortened protein (S)). The BTI-CMe pQE constructs HL, HS, SL and SS were transformed into *E. coli* [XL1-Blue] and single white colonies were selected for further analysis. The plasmid DNA was purified and the constructs were analysed by agarose gel electrophoresis (Appendix C – Figure 1), with two constructs from HL and SL, three constructs from SS and four from HS successfully made.

The four constructs (HL, HS, SL and SS) were sequenced in order to determine their cloning orientations and to check that the inserts were in the open reading frame. HL, HS and SS were in frame and in the correct orientation. The SL construct was found to be in frame but not the correct orientation (data not shown) and was not used any further in the expression studies. The BTI-CMe pQE plasmids (HL, HS and SS) were transformed into competent *E. coli* [pREP4] cells. The products were all expressed in *E.coli* as soluble proteins (Figure 9), however the BTI-CMe protein was also observed in the insoluble fraction (Figure 9). It was shown that BTI-CMe was able to be expressed under native conditions. SDS-PAGE immunoblot analysis with the original SE antiserum showed that a protein of ~49 kDa was recognised in both the soluble and insoluble fractions, along with a ~20 kDa protein which was recognised by the SE antiserum in the insoluble fractions (Figure 9).

160
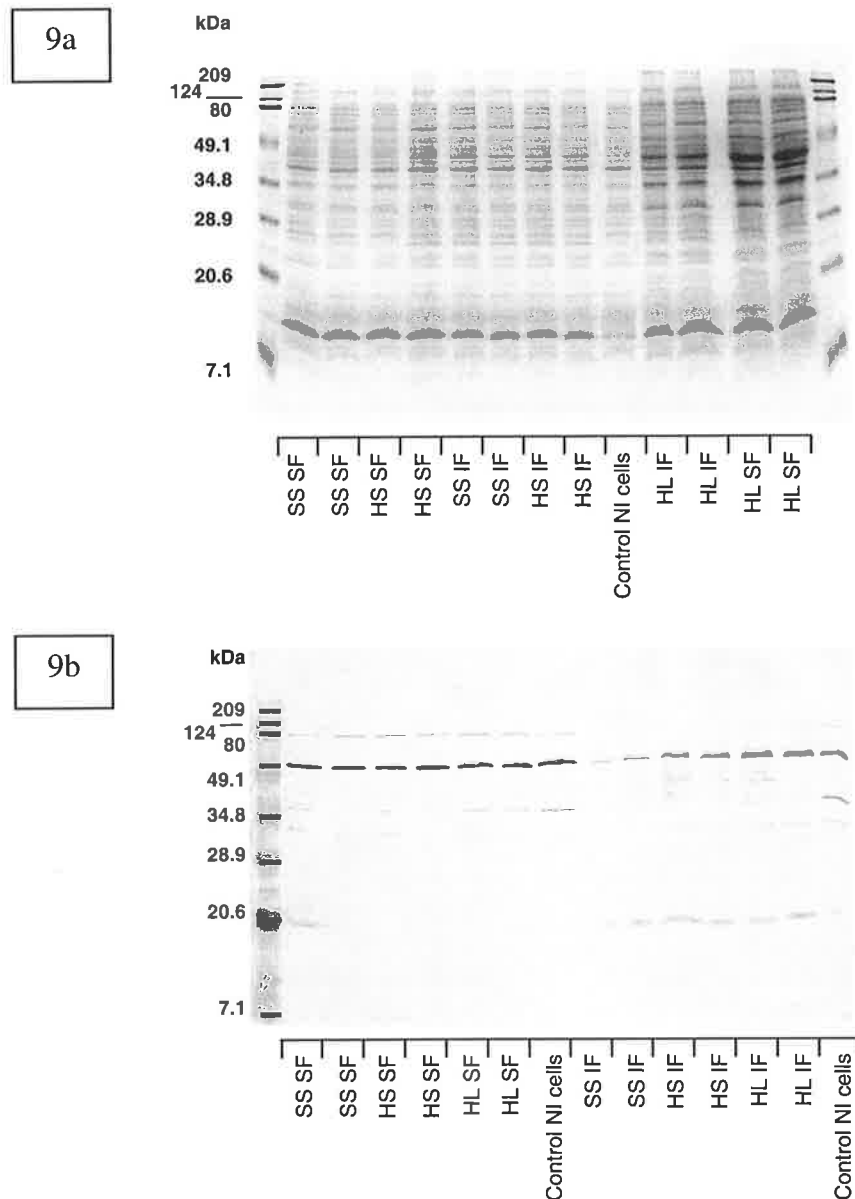
Figure 9: Expression analysis of recombinant BTI-CMe proteins. (a) SDS-PAGE Coomassie blue stained gels of crude soluble and insoluble expressed proteins from BTI-CMe preparations HL, HS, and SS. (b) SDS-PAGE immunoblot analysis with the SE antiserum (1/1000 dilution), of crude soluble and insoluble expressed proteins from BTI-CMe preparations HL, HS, and SS. HL = Harrington Long (full length protein), HS = Harrington Short (truncated protein), and SS = Sloop Short (truncated protein). SF = Soluble Fraction, IF = Insoluble Fraction, and NI = Non-induced cells.

5.3.2.5 Purification of BTI-CMe1 and BTI-CMe3 variants

The three constructs (HL, HS and SS) were expressed in *E. coli* as 6-*His* tagged soluble proteins and purified using a Ni-NTA agarose matrix. The HL and SS recombinant proteins were purified to apparent homogeneity as determined by Coomassie blue staining (Figure 10). No protein could be purified from the cells expected to express the HS construct. The purified BTI-CMe1 (SS) and BTI-CMe3.1 (HL) had apparent molecular masses of ~10 or 11 kDa and ~9 or 10 kDa respectively, and were observed as single major bands on the SDS-PAGE gels (Figure 10).

It was perhaps surprising that the purified SS protein showed lower mobility than the corresponding protein for the HL preparation. Given that in both cases the proteins were expressed with N-terminal 6-histidine tags, and were individually affinity purified, it is unlikely that the protein bands observed on SDS-PAGE gels represented major contaminants in each preparation. With the HS preparation after protein purification no protein band was detected (Figure 10). The crude protein banding patterns of HL, HS and SS (Figure 10) were similar to those observed in the initial expression analysis in Figure 9. The expressed protein along with other protein bands could be observed in the IPTG induced cells, the cleared cell lysate, the flow-through and first washes. With the 2nd and 3rd washes no protein banding was visible until the detection of the purified BTI-CMe protein in the eluants (Figure 10). The concentration of protein in the purified preparations (HL and SS) was determined by measuring the absorbance at 280 nm. This method was based on that described by Stoscheck (1990). Protein concentration in the HL preparation was (~0.8 mg), in the SS preparation (~0.5 mg). Mass spectrometry analysis of the purified HL and SS proteins confirmed the presence of the purified BTI-CMe protein in the eluted fraction from HL, with a fraction

corresponding to the HL protein observed at MW 11,036. However, with the SS protein no fraction could be detected by mass spectrometry at the expected molecular weight (data not shown). Given the SDS-PAGE data for the SS protein and the successful recovery of the HL fraction by mass spectrometry, the non-result for the SS sample may have been as a result of a problem with the sample (i.e. contaminants from the buffer the protein was in) used for mass spectrometry analysis.
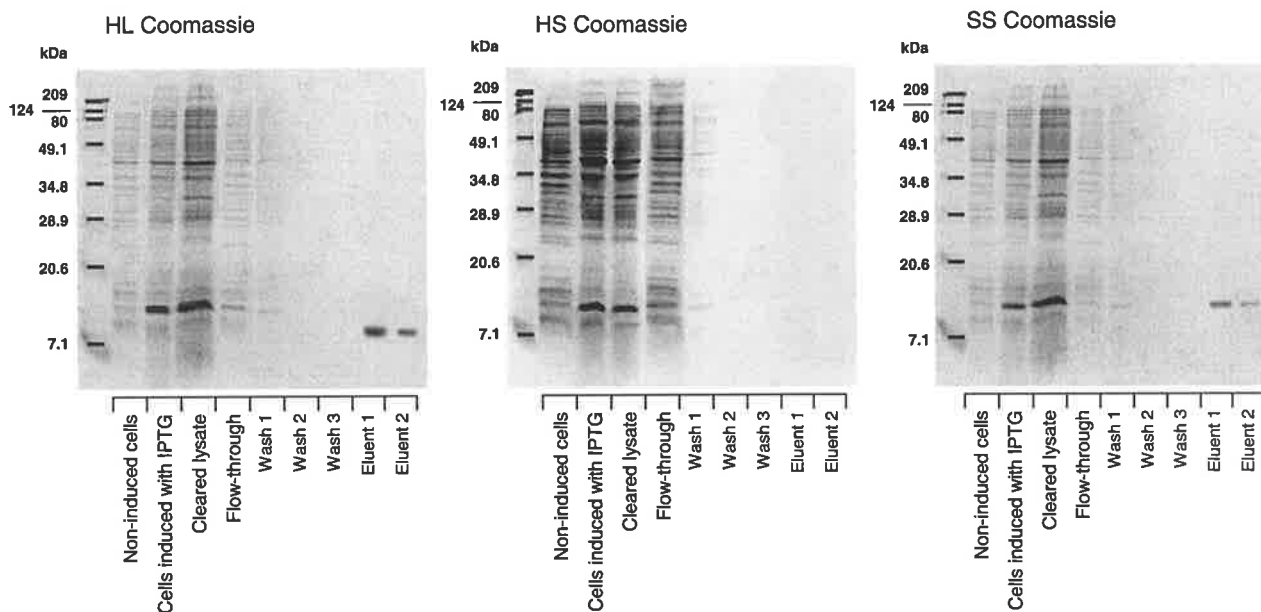
Figure 10: Purification of expressed native BTI-CMe proteins. BTI-CMe proteins were purified using Ni-NTA agarose. SDS-PAGE (15% w/v) of the purified BTI-CMe preparations of the HL, HS and SS fragments. Samples of the non-induced cells, cells induced with IPTG, the cleared lysate, the flow through, the wash and elution steps are shown in this figure. Proteins were visualised by Coomassie blue staining. HL = Harrington Long (full length protein), HS = Harrington Short (truncated protein) and SS = Sloop Short (truncated protein).

5.3.2.6 Polyclonal antibodies raised against the recombinant BTI-CMe protein

Polyclonal antibodies to the purified recombinant HL and SS protein preparations were raised in rabbits. SDS-PAGE immunoblot analysis with the HL and SS antisera of their respective crude and purified proteins resulted in no detection of any bands in the purified preparations, including the primary band and only higher molecular weight bands than expected for BTI-CMe in the crude protein preparations (Figure 11). Although the purification of the HL and SS protein appears to have been successful (Figure 10), the proteins appear to be poorly immunogenic, thus antibodies to the BTI-CMe protein were not produced.

The original SE antiserum identified an intense protein band with an apparent MW of ~12000 in crude malt and barley protein extracts with additional faint bands located between 32000 and 98000 Chapter 2, section 2.3.1.1, Figure 1. The SE antiserum did not cross-react with the purified HL (SE –ve) protein as anticipated, however, it also did not cross-react with the SS (SE +ve) preparation (Figure 11). The SS and SE antisera both detected common bands at ~49 kDa and ~80 kDa (Figure 11). No clear bands were observed in the immunoblot using the HL antisera (Figure 11). This observation reinforces the conclusion that the purified recombinant SS and HL proteins were poorly immunogenic.
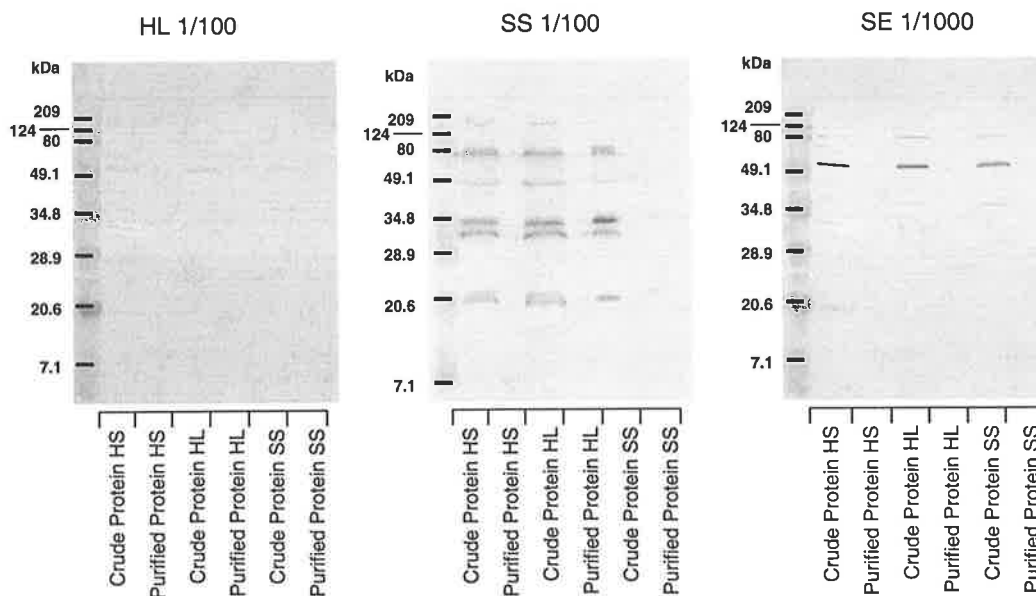
Figure 11: SDS-PAGE (15% w/v) immunoblot analysis with the HL and SS (1/100 dilution) and SE antisera (1/1000 dilution), of both crude (cleared lysate) and purified expressed proteins from the recombinant SS and HL preparations. HS = Harrington Short (truncated protein), HL = Harrington Long (full length protein) and SS = Sloop Short (truncated protein).

5.3.3 SDS-PAGE immunoblot analysis with the SE antiserum of known BTI-CMe1 and BTI-CMe3/3.1 variants for the presence or absence of the SE protein

Barley varieties that have been previously attributed as either BTI-CMe1 or BTI-CMe3/3.1 variants, in the literature or by their sequence data (section 5.3.2.2), were screened by immunoblot to verify that these types matched the SE +ve and SE –ve types respectively. Of the 17 varieties screened, five varieties (or 29%) were found to be the opposite form of the SE protein to their expected SE classifications (Table 4). The BTI-CMe1 varieties in Betzes and Albacete and the BTI-CMe3/3.1 varieties in Trebi (*H. spontaneum*), Omskii 13709 and Valticky were identified as SE –ve and SE +ve

respectively (Table 4). Interestingly, with the Trebi samples analysed the SE protein was present in the *H. spontaneum* sample but was not present in the *H. vulgare* sample. Eglinton and Evans (1997) found similar discrepancies for the assignment of β-amylase type (Sd1 and Sd2) when comparing present day varietal samples with their historical assignment (10 conflicts in 70 samples assessed). They concluded that these "differences are most likely explained by outcrossing, miss-labelling or varietal heterozygosity that had occurred in the intervening time period". This is most likely to be the case in this study too, although it could also be indicating that the SE antibody detected a difference in the relative abundance of the BTI-CMe protein within the grain.

Table 4: SE classification of varieties identified as either BTI-CMe1 or BTI-CMe3 variants. Varieties in **bold** text are those identified as the opposite SE form as compared to their expected classifications. SE = Silica Eluent, Hv = *H. vulgare*, Hs = *H. spontaneum*.

Variety	Expected SE +/-	Actual SE +/-	Variant	Reference
Bomi	+	+	BTI-CMe1	Ladogina (1997)
Bonus	+	+	BTI-CMe1	Ladogina (1997)
Viking	+	+	BTI-CMe1	Ladogina (1997)
Albacete	+	-	**BTI-CMe1**	Royo (1997)
Betzes	+	-	**BTI-CMe1**	Ladogina (1997)
Carlsberg II	+	+	BTI-CMe1	Ladogina (1997)
Zephyr	+	+	BTI-CMe1	Ladogina (1997)
Sloop	+	+	BTI-CMe1	Sequence data (section 5.3.2.2)
Proctor	+	+	BTI-CMe1	Sequence data (section 5.3.2.2)
Harrington	-	-	BTI-CMe3.1	Sequence data (section 5.3.2.2)
Haruna Nijo	-	-	BTI-CMe3.1	Sequence data (section 5.3.2.2)
Pirkka	-	-	BTI-CMe3	Ladogina (1997)
Domen	-	-	BTI-CMe3	Ladogina (1997)
Omskii 13709	-	+	**BTI-CMe3**	Ladogina (1997)
Trebi (Hs)	-	+	**BTI-CMe3**	Ladogina (1997)
Trebi (Hv)	-	-	BTI-CMe3	Ladogina (1997)
Valticky	-	+	**BTI-CMe3**	Ladogina (1997), Royo (1997)

5.4 Discussion

5.4.1 Identity of the SE protein

In this chapter, the identity of the SE protein was shown to be a member of the BTI-CMe family of proteins. The SE protein, identified in Chapter 2 (section 2.3.1.1) was separated by 2-D gel electrophoresis, identified by immunoblot, cleaved in-gel by trypsin with the resulting fragments being separated by reversed-phase HPLC (Figures 1, 2 and 3). A non-immuno reacting spot with similar electrophoretic mobility (MW ~15000, *pI* 6.5-7.0) was also identified in the SE –ve variety, Harrington (Figure 2). N-terminal sequence analysis of selected tryptic peptides from both SE –ve (Harrington) and SE +ve (Sloop) varieties identified the SE protein as the barley trypsin inhibitor CMe precursor (BTI-CMe) (Table 3). The SE protein spot was clearly separated from other proteins in the 2-D gel (Figure 2) and the identified BTI-CMe protein's size, *pI* and gene location on barley chromosome 3H (Chapter 2, section 2.3.2.2) all confirm that it is the SE protein.

The partial amino acid sequences for BTI-CMe from Sloop and Harrington were used to design PCR primers to clone the respective genes for these proteins in two SE +ve varieties (Sloop and Proctor) and two SE –ve varieties (Harrington and Haruna Nijo). Based on the sequence homology, the SE protein identified in Sloop and Proctor was most similar to the BTI-CMe1 variant. The allelic variant BTI-CMe3 has been demonstrated to consist of two components BTI-CMe3.1 and BTI-CMe3.2 (Ladogina, 1997; Moralejo *et al.,* 1993; Salcedo *et al.,* 1984). In Harrington and Haruna Nijo the genes were shown to be homologous to the BTI-CMe3.1 variant. Consistent with this assignment, the Harrington protein isolated in the 2-D gels appeared to consist of two partially resolved spots (Figure 2).

5.4.2 Antibody validation of SE phenotype identification

Polyclonal antibodies were raised against BTI-CMe to validate that BTI-CMe is the SE protein and for the potential to develop useful quantitative (ELISA) assays for the detection of the haze forming potential in both quality malts and in beer. Purification of the recombinant HL and SS proteins was successful (Figure 10). SDS-PAGE immunoblot analysis with the HL and SS antiserum of their respective crude and purified proteins showed no reactivity with the purified preparations and only higher molecular weight bands than expected in the crude protein preparations of the recombinant BTI-CMe protein. Therefore the recombinant proteins were poorly immunogenic (Figure 11), suggesting that antibodies to the BTI-CMe protein were not raised. It may well be that the constructs, SL (not able to be cloned in the right orientation) and the HS construct (which was not able to be purified) could have been more informative than the HL and SS constructs used. Although unlikely, it can not at this stage be ruled out that the *His* tags attached to the recombinant proteins did not interfere with the raising of the antibodies. The original SE antiserum did not bind with the purified HL protein which was to be expected given that the SE antiserum did not recognise a band in SE –ve varieties, however neither did it bind with the purified SS protein (SE +ve fragment) preparation. As there was no cross-reactivity observed, the antibodies raised to the HL and SS preparations are unfortunately inconclusive with regard to the validation of the BTI-CMe protein being the SE protein. However, the evidence that the gene encoding BTI-CMe is expressed on chromosome 3H (Hejgaard *et al.*, 1984; Salcedo *et al.*, 1984), the clear resolution of the candidate spots by 2-D electrophoresis, the appropriate size and *pI* makes BTI-CMe the most likely candidate for the SE protein.

Confirmation that the BTI-CMe protein is the SE protein would be obtained by the following investigations. Nucleotide sequence data from other SE +ve and SE –ve varieties would further confirm that SE –ve varieties correspond to the BTI-CMe3 variant and that SE +ve varieties correspond to the BTI-CMe1 variant. Additional cloning of the BTI-CMe protein from further but genetically unrelated SE -ve and SE +ve varieties may confirm the divergence at the C-terminal end of these proteins. Examination of the varieties that were used in brewing trials (Chapter 3, section 3.3.1) and were shown to have improved (SE -ve) or decreased (SE +ve) haze stability would be an obvious place to begin. Extraction of BTI-CMe proteins from SE +ve and SE –ve barley varieties and subsequent 2-D SDS-PAGE analysis to determine the allelic form of BTI-CMe present in more varieties could also answer this question, as a difference in the C-terminal could explain the mechanism by which BTI-CMe contributes to haze formation.

5.4.3 Barley Proteinase Inhibitors

Proteinase inhibitors from the trypsin/α-amylase inhibitor family (CM proteins) inhibit exogenous α-amylase from many different sources, and can also inhibit bovine and insect trypsins and other serine proteases (Fincher and Stone, 1993; García-Olmedo *et al.*, 1987; Shewry, 1993). BTI-CMe has been shown to inhibit trypsin-like proteases and the α-amylase of *Spodoptera frugiperda* (fall armyworm) (Alfonso *et al.*, 1997), and transgenic wheat producing BTI-CMe has been shown to have increased insect resistance (Altpeter *et al.*, 1999).

A barley trypsin inhibitor (BTI) that was initially sequenced at the protein level by Odani *et al.*, (1983), has been subsequently shown to be identical to barley protein CMe

169

(Lazaro *et al.*, 1985; Salcedo *et al.*, 1984). Both proteins are apparently active as single subunits and the reactive or inhibitory site in CMe has been identified as the Arg-Leu peptide bond at amino acid position 57 (Odani *et al.*, 1983) and they also contain five disulfide bonds, which are essential for the inhibitor activity. The corresponding gene for BTI-CMe has been shown to be located on chromosome 3H (3) of barley (Hejgaard *et al.*, 1984; Salcedo *et al.*, 1984).

The mature protein is 13.3 kDa and the gene that codes for trypsin inhibitor BTI-CMe in barley, *Itr1* is located on chromosome 3H. This strongly supports the claim for the identification of the SE trait on the short arm of chromosome 3H in the Chebec (SE +ve)/Harrington (SE -ve) mapping population (Chapter 2, section 2.3.2.2, Figure 3). The gene for trypsin inhibitor CMe (*Itr1*) is regulated in *trans* by the *lys 3a* locus in the endosperm of barley (Rodriguez-Palenzuela *et al.*, 1989). The *lys 3a* locus which is located on chromosome 5H, regulates in *trans* the expression of the *Itr1* gene, which is located on chromosome 3H. The barley *lys 3a* gene is thought to also control the expression of several genes in the barley endosperm, such as those that encode for B and C hordeins, β-amylase and protein Z (Brandt *et al.*, 1990; Entwistle, 1988; Hopp *et al.*, 1983; Kreis *et al.*, 1984 and 1987; Lazaro *et al.*, 1985; Rodriguez-Palenzuela *et al.*, 1989). The accumulation of protein CMe1 is totally blocked in the "high lysine" mutant Risø 1508 and partially blocked in the high lysine barley Hiproly (Salcedo *et al.*, 1984). This is consistent with the SE antiserum not detecting the MW ~12000 SE protein in the Risø 1508 variety (Chapter 2, Section 2.3.1.1, Table 1).

170

It is of interest to note the diversity of BTI-CMe1 (homology with SE +ve) and BTI-CMe3 (homology with SE –ve) in barley. Of the 80 *H. vulgare* samples analysed by Ladogina (1997), 49 varieties were identified as CMe1 (61.25%) and 23 varieties CMe3 (28.75%) and these frequencies were similar to those previously reported by Moralejo *et al.*, 1994. Interestingly, in *H. spontaneum* the frequency was reversed with 9 out of 20 varieties screened found to be BTI-CMe3, with only 2 identified as BTI-CMe1. This may explain the high number of varieties identified as SE +ve (~83%) by the SE antiserum as compared to SE –ve (~17%) (Chapter 2, Section 2.3.1.1, Table 1).

The roles and potential mechanisms of the SE/BTI-CMe protein and its influence on beer haze stability will be discussed in following chapter (Chapter 6).

5.5 Conclusions

The SE protein was characterised as the barley trypsin inhibitor CMe (BTI-CMe), a protein that has been demonstrated in this thesis (Chapter 3, section 3.3.1) to have an influence on the haze stability of beer. The proteomic identification (Figure 2 and Table 3), genetic mapping (Chapter 2, section 2.3.2.2) and cloning (section 5.3.2) of the BTI-CMe protein have demonstrated that both SE -ve and SE +ve varieties contain a BTI-CMe protein family member that is similar but consistently different, primarily in the last 30 amino acid residues of their C-termini (Figure 6). Further research with BTI-CMe is required to elucidate the role BTI-CMe has in the formation of haze in beer. The improved understanding of the impact of malt and brewing processes on BTI-CMe will potentially provide further scope for optimising both malt characteristics and beer quality. The future successful raising of polyclonal antibodies to recombinant BTI-CMe proteins will allow for the development of quantitative (ELISA) assays to

ascertain the haze potential of beer (irrespective of the malt variety brewed with), enable the monitoring of haze potential through the brewing process, which will give brewers greater control and optimisation of stabilisation and filtration regimes during brewing. The end result will be improved haze stability of the finished product.

Chapter 6

General Discussion

The influence of barley protein on both malt and beer quality characteristics is complex because of the diversity of protein groups that are present in the barley grain and their functional roles during malting and brewing (Shewry and Darlington, 2002). The work presented in this thesis has shown that a barley trypsin inhibitor - CMe (chloroform/methanol - soluble protein) (BTI-CMe) is almost certainly involved with the formation of haze in bright beer. This is the first time that a specific barley protein has been identified that can be shown to have an influence on beer haze stability. Individual barley varieties can be discriminated for their haze forming potential based on the SE/BTI-CMe variant that is present within the grain.

6.1 The role of protease inhibitors during malting and brewing

During the brewing process there are a number of proteinases that are active, particular during malting and mashing (Jones and Marinac, 2002). These proteinases partially degrade the barley storage proteins into amino acids and peptides that are critical for producing high quality beer (Jones and Marinac, 2002). A number of these protease inhibitors also survive the brewing process to end up in the finished beer. A boiled preparation of lipid transfer protein 1 – probable amylase/protease inhibitor (LTP1-PAPI), has been shown to survive the brewing process relatively intact, and has been suggested to control the enzymatic breakdown of storage proteins by controlling cysteine protease activity during malting and mashing (Jones and Marinac, 1997). As such, it has been proposed that it can play an important role in determining beer quality by influencing yeast nutrition (Free Amino Nitrogen) and the extent that proteins are

hydrolysed, thus influencing beer foam and haze stability. The claim that LTP1 is a protease inhibitor has however been disputed by Davy *et al.,* (1999), Jones (*pers comm.*) however, maintains that his original interpretation is valid and the original investigation (Jones and Marinac, 1997), appears sound. Perhaps the main source of contention is the potential effect that boiling of the barley preparation during purification (Jones and Marinac, 1997) may have had on the structure of LTP1 and its potential thereafter to inhibit proteases.

The serpin, Protein Z, was the first characterised protein in beer to have roles attributed for it in foam stability and/or haze formation (Hejgaard, 1977; Kaersgaard and Hejgaard, 1979; Yokoi *et al.,* 1989). Protein Z survives the brewing process due to its heat stability and its protease inhibitory properties, retaining its size and immunological identity (Dahl *et al.,* 1996; Hejgaard, 1977; Lundgard and Svensson, 1989). However, a target barley protease has yet to be identified for protein Z with more recent investigations suggesting that it may act in grain defence by inhibiting insect proteases (Hejgaard, 2001).

Serine proteinase inhibitors accumulate during seed maturation suggesting that they facilitate storage protein accumulation by attenuating the activities of proteases (Koiwa *et al.,* 1997). The α-amylase/trypsin inhibitors have also been shown to rapidly disappear with the onset of germination suggesting that these proteins do not play a specific role during germination (Kirsi and Mikola, 1971; Pace *et al.,* 1978). BTI-CMe although active against trypsin has been shown to be inactive against other proteases in chymotrypsin, papain, subtilopeptidase A, pepsin, bacterial and fungal proteinases, as well as against the endogenous proteinases from green malt (Mikola and Suolinna,

1969). An understanding of the potential roles of BTI-CMe may provide insights into

how this protein could potentially influence beer haze stability.

6.2 How does the SE/BTI-CMe protein influence beer haze stability?

In beer, haze active proteins isolated to date have been found to be derived primarily

from the barley storage proteins or the hordeins, are comprised of fragments of several

different molecular weights, and are relatively rich in proline (Asano et al., 1982). An

important feature of the hordeins in terms of their haze forming potential is their rod-

shape, composed of a repetitive domain rich in β-turns, with a loose spiral configuration

based on the repetitive β-turns (Shewry, 1993) that increases both hydrophobic

interactions and hydrogen bonding.   Hordeins or their fragments, particularly the

hydrophobic domains which survive into finished beer, have been suggested to

influence beer haze forming potential (Smith, 1990).   The hydrophobic domains of

hordeins are also potentially valuable in improving foam stability (Evans and Sheehan,

2002).   As the repetitive domains of the B, C and D hordeins are also resistant to

protease activity, they have also been suggested to be involved in haze formation

(Smith, 1990). However, there must be a distinction between foam and haze active

hordein fragments as silica removes haze active proteins without noticeably reducing

foam stability (Evans et al., 2003; Siebert and Lynn, 1997a).

In contrast to the hordeins, the SE/BTI-CMe protein is a low molecular weight protein

(~13000) with relatively low proline content (Chapter 2, Figure 1; Chapter 5, Figures 1,

2 and 6, Table 3; Appendix C, Table 1; Odani et al., 1983). BTI-CMe belongs to the

CM protein family, which are salt soluble, hydrophobic proteins (Paz-Ares et al.,

1983a; Salcedo et al., 1980). It is possible that like their hordein counterparts, these

hydrophobic proteins or their fragments can survive the protease action during malting and brewing to appear in the finished beer and subsequently be involved in haze formation. Furthermore, the SE antibody that originally identified the BTI-CMe protein, was raised to a protein fraction eluted from silica used for the colloidal stabilisation of beer, indicating that this protein is most likely haze active (Evans *et al.,* 2003).

To date there has not been any clear demonstration that high molecular weight proteins have a greater tendency to form haze than do low molecular weight proteins. Based on the conceptual mechanism of protein-polyphenol interactions proposed by Siebert *et al.,* (1996b), it could be argued that relatively low molecular weight proteins with proline residues could enter into a more convoluted and presumably larger-sized network than a few very high molecular weight proteins. In addition, it has been suggested that the position of the proline residues within a proteins structure is most important in defining a proteins haze activity (Outtrup *et al.,* 1987; Outtrup, 1989).

6.2.1 An SE/BTI-CMe model for haze formation

The SE/BTI-CMe protein was detected in barley, malt and beer (Chapter 2, Figure 1) using an antiserum raised against a silica eluent (SE) protein fraction (obtained from silica gel, used for the colloidal stabilisation of beer) (Evans *et al.,* 2003). The addition of silica to beer has been suggested to improve beer haze stability through binding of silica to the proline residues of HA proteins that otherwise have the capacity to bind to HA polyphenols (Siebert and Lynn, 1997a). It is reasonable to argue therefore that the silica gel is most likely removing proteins involved in haze formation. However, in this case the silica may have also removed the low proline SE protein from the beer because

of the position of the proline residues within the SE protein's structure. The SE BTI-CMe protein may be less HA than proteins conforming to the generic pattern of HA-active properties (higher molecular weight; high proline content; hordein derived (Siebert, 1999). The two types of putative HA proteins may bind by polyphenol-mediated cross-linking between the proline sites. This suggests that the SE/BTI-CMe protein may initiate or act as a nucleation site for haze precursors (Evans *et al.,* 2003). Acting as a catalyst, SE/BTI-CMe could sequester other components (including possibly other non-generic haze active proteins) that also survive the brewing process to be involved in haze formation. This model could be critical for describing the mechanism by which the SE/BTI-CMe protein binds to polyphenols and other proteins to form haze.

The question arises why the SE antiserum strongly detects the SE +ve/BTI-CMe1 protein while not binding to the SE -ve/BTI-CMe3 variant. One suggestion is that the SE -ve protein does not make it into the finished beer to be bound to the silica, and thus was not present in the protein fraction from which the SE antibody was raised. Silica gels have been found to have high specificity for HA protein because the silica gel binds to the same HA proline residues as do the HA polyphenols (Siebert and Lynn, 1997a). Conversely, the SE -ve protein may have been present in the finished beer but did not bind to the silica gel because it was not as 'haze active' as other proteins including the SE +ve protein. A further possibility is that the BTI-CMe protein is modified during the brewing process, resulting in the SE -ve/BTI-CMe3 protein becoming less 'haze active', while the SE +ve/BTI-CMe1 protein becomes more 'haze active'.

The amino acid composition of protein BTI-CMe may be important in determining its haze stability. There are six amino acid substitutions between the BTI-CMe3.1 and BTI-CMe1 variants listed on the NCBI database. From the sequence data obtained in this study there was one further predicted amino acid substitution (Chapter 5, Figure 6). Potentially of interest to BTI-CMe's haze forming potential is a proline-alanine substitution at position 135 in the sequences of BTI-CMe1and BTI-CMe3.1. A proline residue is present in the BTI-CMe3.1 (SE –ve) variant, however, this is replaced with an alanine residue in the BTI-CMe1 (SE +ve) variant (Chapter 5, Figure 7). This amino acid change could provide another binding site for polyphenols to interact with the BTI-CMe protein. In this case, the proline substitution is in the SE –ve/BTI-CMe variant (3.1) that has been shown to improve beer haze stability. Substitution of this proline residue could disrupt the confirmation of the SE –ve protein thus reducing its ability to form haze.

Critical to BTI-CMe's haze forming potential could be the stage of the brewing process in which the SE/BTI-CMe protein contributes to haze formation. Is it before or after packaging? The BTI-CMe protein has been shown to be a heat stable protein (Mikola and Suolinna, 1969), therefore it is likely to survive the brewing process relatively intact to be present in the finished beer to be involved in haze formation. This indicates that the survival of the BTI-CMe protein may be akin to that of protein Z (Dahl *et al.,* 1996; Hejgaard, 1977; Lundgard and Svensson, 1989). The BTI-CMe protein can be detected with the SE antiserum in beer brewed from SE +ve varieties at the same molecular weight (~12000), right through the brewing process (Chapter 3, Figures 5 and 10) as well as in commercial brews (Chapter 4, Figures 7 and 8), indicating that the SE

+ve/BTI-CMe protein can survive the brewing process to be present and available for haze formation.

In this thesis, the removal of the SE protein from beer brewed from SE +ve varieties, filtered using a new set of filter sheets for each beer, was related to the level of haze formed in the beer after force testing (5 days at 55°C, 1 day at 0°C). From the brewing trials completed in this study, the 50 L SE +ve brews (Stirling, Schooner, Franklin, Gairdner, Grimmett and Lindwall) completed at Lion Nathan (Chapter 3, Figure 1; Appendix B, Figure 2; Evans *et al.*, 2003), contained the SE protein after filtration and were less haze stable than the 100 L Scarlett brews completed at VTT Biotechnology that did not contain the SE protein after filtration (Chapter 3, Figure 11). Commercial beer brewed from Scarlett (SE +ve) malt was also found to form less haze when it did not contain the SE protein after filtration (Chapter 4, Figures 3 and 4). As stated previously, the hypothesis linking the SE protein and haze potential is based on those beers that were filtered using a new set of filter sheets for each beer. However, in the 300 L Ballarat University pilot brews completed in which the filtration order is unknown, the Stirling (SE +ve) brews did not contain the SE protein after filtration but their stability was not substantially improved (Chapter 3, Figures 4 and 5). One of these Stirling brews had similar haze stability to the SE -ve brews (Harrington) while the other brews had reduced haze stability as expected (Chapter 3, Figure 4). At this point this anomaly cannot be adequately explained but may be a result of the larger filter used and the random selection of the keg for haze stability testing or unexplained experimental error. On balance, the evidence presented in this thesis supports the hypothesis that the immunological absence of the SE protein is associated with improved haze stability.

6.2.2 Mechanisms for the role of the SE/BTI-CMe protein in haze formation

The proline/glutamine content of BTI-CMe, along with its proline composition and conformation may be important in haze formation. The BTI-CMe protein differs from the previously suggested concepts of what a haze active protein should be because of its low level of proline. BTI-CMe is not rich in proline (8.3% mol/mol) or glutamine (5.8% mol/mol) (Appendix C - Table 1). This is surprising as haze active proteins are conventionally thought to contain high levels of proline to facilitate the interaction with polyphenols to form storage hazes (Bamforth, 1999a; Siebert, 1999). However, the observation by Outtrup *et al.,* 1987, that the haze activity is not simply determined by the amount of proline present but also its distribution within the protein, may explain this inconsistency. Although the X-ray crystallography structure of BTI-CMe has not been determined, the configuration of the proline residues in BTI-CMe variants may influence its haze forming potential.

The haze potential of the SE +ve (BTI-CMe1) variant may well be modified and improved by the proteases active during malting and mashing. This would allow for the proline residues to be presented in a conformation that facilitates polyphenol binding to form haze. Certainly after malting a second lower molecular weight, SE antisera binding band, is evident in malt and in some cases it survives into the finished beer (Chapter 2, Figure 1).

An alternative role for BTI-CMe in influencing haze stability, is that the BTI-CMe1 (SE +ve) variants limit the proteolytic degradation of the haze active protein components (hordeins), and in doing so allows for higher levels of haze active proteins to be carried through into the finished beer. It has been demonstrated that another of the serine

proteinase inhibitors, a bifunctional barley α-amylase/subtilisin inhibitor (BASI), may influence the rate of starch degradation during the early stages of mashing, but its inhibitory activity is lost at normal mashing temperatures (Munck *et al.,* 1985). It is unlikely that BTI-CMe would have a similar effect in controlling the rate of protein degradation as it has not been shown to be active against the endogenous proteinases from green malt (Mikola and Suolinna, 1969). To date no serine proteases have been shown to be inhibited by BTI-CMe. Zhang and Jones (1995), have also concluded that serine protease enzymes do not solubilise storage proteins and thus may have little effect on the level of soluble proteins in the final mash and hordeins have also been shown to be resistant to digestion by trypsin (Kapp and Bamforth, 2002). It is therefore unlikely that BTI-CMe impacts on the haze potential of beer by modulating the hydrolysis of the haze active fragments of hordeins.

The diversity and activity of trypsin inhibitors is of interest to plant breeders for the improvement of grain quality and the protection of the grain against micro-organisms and pests. However, in this study BTI-CMe was being investigated for its ability to influence the colloidal or haze stability of beer. In Chapter 3 (section 3.3.1), SE +ve varieties (BTI-CMe1 variant), were identified as less haze stable compared to those identified as SE -ve varieties (BTI-CMe3.1 variant). The level of trypsin inhibitor activity (TIA) has been identified in BTI-CMe3 variants to be the highest amongst all the variants tested and to be significantly higher (mean BTI content U/g), than BTI-CMe1 variants that have an intermediate level of activity (Ladogina, 1997; Moralejo *et al.,* 1993). It follows that the level of TIA of BTI-CMe variants may in some way contribute to their haze forming potential. At this stage, a plausible explanation as to how this would affect haze formation is not available.

With the 2-D gels of SE +ve and SE –ve varieties (Chapter 5, Figure 2), there appears to be less of the SE protein spot in the appropriate position as detected by Coomassie blue staining in the SE –ve variety compared to the SE +ve variety. The detection of the SE +ve protein by the SE antiserum, but not the SE –ve protein could also indicate a lower level of the SE –ve protein present in the grain. This may indicate that the SE antiserum is detecting the relative amount of BTI-CMe present in the grain, and that those varieties identified as SE –ve (Chapter 2, section 2.3.1.1, Table 1) have lower levels of BTI-CMe, therefore not detectable with the SE antiserum. The evidence for this theory of a lower level of BTI-CMe in some barley varieties is not conclusive, an ELISA assay using BTI-CMe specific antibodies for the quantitation of the BTI-CMe protein in malt and beer is required.

Assuming that there is less SE –ve/BTI-CMe3 protein than SE +ve/BTI-CMe1, the lower relative level could result in greater proteolytic degradation of haze active proteins thus improved haze stability in varieties containing the SE –ve/BTI-CMe3 type. Lower levels of BTI-CMe in the grain could also mean potentially less 'haze active' material being available to the proteases to hydrolyse and thus less haze forming protein present in the finished beer. It is possible that the SE -ve (BTI-CMe3/3.1) variant may not be as effective a protease inhibitor compared to the SE +ve (BTI-CMe1) variant. Again, the amount of BTI-CMe that is present within the grain in determining haze stability is of importance. It is possible that the relative abundance of the BTI-CMe protein in SE -ve varieties is considerably less than in SE +ve varieties. Again for these hypotheses to be valid, a target protease for BTI-CMe that impacts on the hydrolysis of haze proteins needs to be identified.

6.3 Future Directions

There are a number of factors that may be contributing to why the haze forming potential is lower in varieties classified as SE –ve compared to SE +ve varieties. It could be that the amount of BTI-CMe in SE –ve varieties is less than in SE +ve varieties. Expression studies could be completed to examine the level of the inhibitor expressed within the grain in an SE +ve variety as compared to an SE –ve variety. It may well be determined that the relative abundance of BTI-CMe in the mature grain is directly related to the haze forming potential of a variety. Diaz *et al.* (1995) suggests that the promoter of the *Itr* gene confers different tissue specific expression. Examination of variation in the promoter region between SE +ve and SE –ve varieties may also help to explain the pattern of expression within the grain in an SE +ve variety as compared to an SE –ve variety.

The amount of BTI-CMe present within a barley variety would be most usefully determined by using quantitative ELISA's, with antibodies designed to the SE +ve and SE -ve BTI-CMe proteins. The raising of antibodies from BTI-CMe proteins derived from a bacterial vector system was unsuccessful in this study due to the poor immunogenicity of the purified recombinant BTI-CMe proteins. Alternatively, instead of using an antibody system based on barley genes cloned into bacteria, the protein purified and then used for immunisation, synthetic peptides could be produced to the C-terminal region in which amino acid differences were observed between BTI-CMe1 (SE +ve) and BTI-CMe3/3.1 (SE –ve) variants (Chapter 5, Figure 6). From these synthetic peptides, specific BTI-CMe antibodies could be raised. In the future, successful raising of polyclonal antibodies to recombinant BTI-CMe proteins will allow the development of quantitative (ELISA) assays to ascertain the haze potential of beer (irrespective of the

malt variety brewed with), enabling the monitoring of haze potential through the brewing process that will give brewers greater control and optimisation of stabilisation and filtration regimes during brewing.

The level of TIA within the grain could also be important in determining haze forming potential. This could be investigated by measuring of the level of TIA in those varieties that have been shown to have improved haze stability (i.e. Harrington, Barke, Saana) and those varieties that have been shown to have decreased stability (i.e. Gairdner, Stirling, Scarlett) (Chapter 3, section 3.3.1). This experiment could be completed using a similar method to that described by Ladogina (1997), in which the inhibitor activity (U) is defined by the amount of trypsin that can be inhibited by a variety over a set time period. If the level of TIA was higher in those varieties displaying improved stability, this could also help in explaining the mechanism by which BTI-CMe contributes to haze formation.

In this study, a polymorphism for HA proteins was identified and surveyed by immunoblot throughout the brewing process, in barley, malt, beer and haze (Chapter 2, Figure 1). The identification of single-nucleotide polymorphisms (SNPs) between SE +ve and SE −ve varieties will facilitate the development of DNA based markers to validate BTI-CMe as the HA gene and facilitate MAS for the SE trait to improve the quality of malting barley.

In this study, pilot-brewing trials demonstrated that beer brewed from SE −ve malt varieties formed less haze during haze force testing trials (5 days at 55°C, 1 day at 0°C), than beer produced from SE +ve malt varieties (Chapter 3, Figures 4 and 7; and in

Evans *et al.*, 2003). Pilot brewing trials could be extended to involve stabilising agents such as silica (protein), PVPP (polyphenol) and protease treatments (protein) applied to assess haze stability in beer brewed from SE +ve and SE -ve malts. It would be of particular interest to examine the effect of proteases on the BTI-CMe protein and its ability to form haze. A paper by Lopez *et al.* (2004), describes the use of Brewers Clarex™ a proline-specific protease produced from *Aspergillus niger* which only cleaves proline residues resulting in improved haze stability in beer. This protease could be very useful in examining this question.

This thesis showed that the absence of the SE protein in combination with the level of total protein present in filtered beer was related to its haze forming potential (Chapter 4, Figures 3, 4, 5 and 6). The protein:polyphenol ratio has been demonstrated to be very important in the haze forming potential of beer (Siebert *et al.*, 1996b). It would be of interest to examine the interaction between BTI-CMe and polyphenols in haze formation. This would help in understanding the mechanism by which BTI-CMe contributes to haze formation and whether this interaction in haze formation is a critical as the presence or absence of the SE/BTI-CMe protein. Potential experiments to determine the interaction between the polyphenolic components and BTI-CMe, could include the use of purified BTI-CMe recombinant protein in model haze formation systems to follow the kinetics of haze formation. In addition, the application of proteases to the haze particles formed could yield "protected" peptides that indicate the sites of interaction with polyphenols. Recombinant BTI-CMe protein constructs could be expressed *in vitro* in *E. coli* cultures to produce sufficient purified protein to be able to add into both beers brewed from SE +ve or SE -ve malt varieties in an attempt to examine which of the BTI-CMe proteins or it's fragments is most haze promoting.

6.4 Conclusions

The role that the SE/BTI-CMe protein plays in beer haze formation represents an intriguing problem, the solution of which will significantly advance the understanding of protein/polyphenol interactions in haze formation. Currently, few commercial malting varieties used by brewers throughout the world contain the desirable SE -ve property. Selection for the SE-ve/BTI-CMe trait in breeding programs would ensure that future malting barley varieties contain this desirable property. By improving the quality of new malting barley varieties along with the optimisation of stabilisation and filtration regimes during brewing, the cost of beer stabilisation could be reduced.

Appendices

Appendix A: A revised pedigree chart for Australian barley varieties (Sparrow *et al.*, 2000).

188
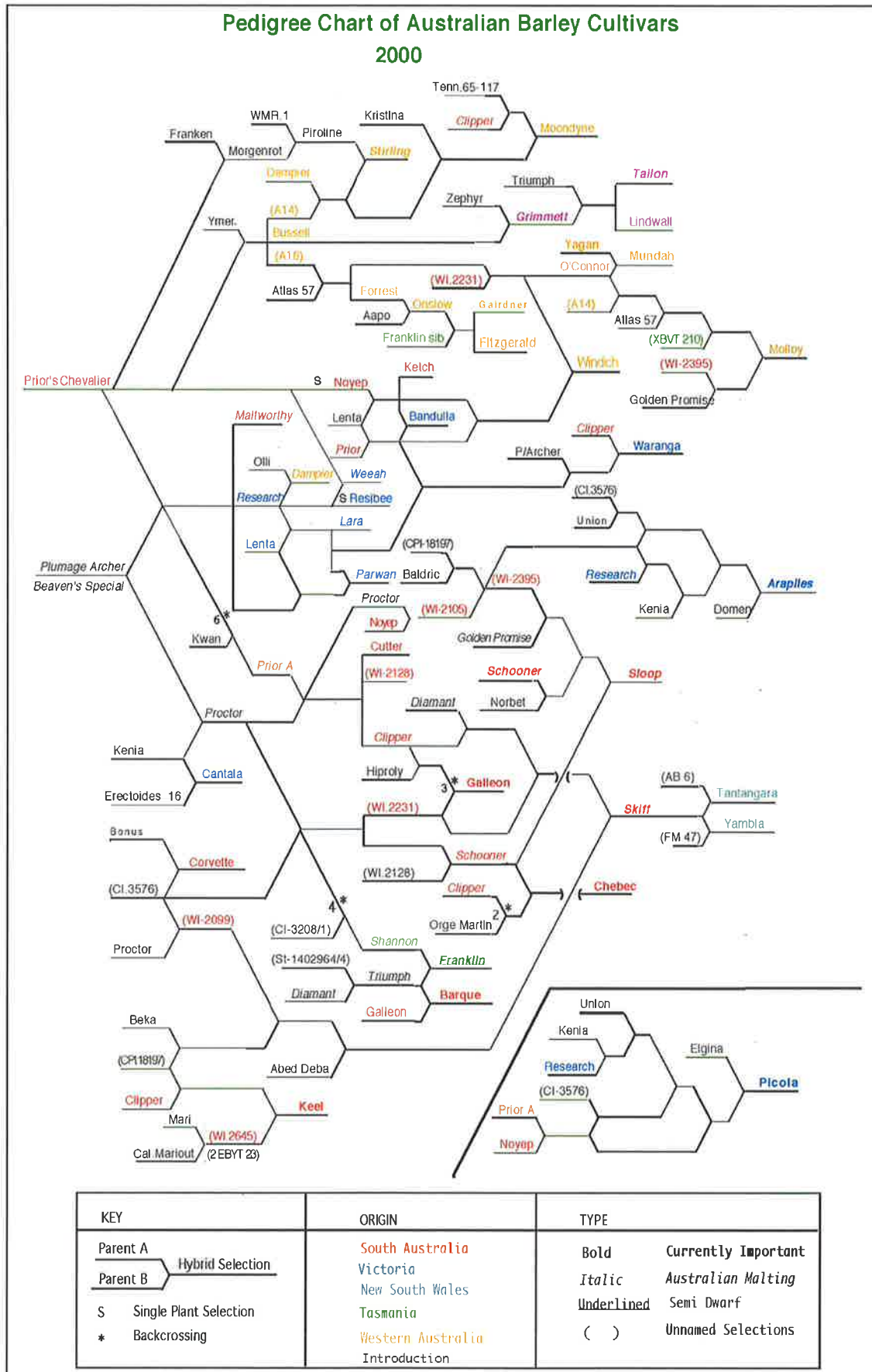
Appendix B – Additional brewing data

Table 1: Malt specifications for the SSB trials (Experiment 1 - Chapter 3).

Variety	Source	Malt Mois. (%)	Malt Prot. (%)	Kolbach Index (%)	Soluble Prot. (%)	DP[a] (°WK)[b]	Sacch. rate	HWE (% EBC)	Visc. (cP)	Fri. (%)
Unicorn	Kirin (WA)[1]	4.00	11.5	41.0	4.71	267	0-10	82.7	1.55	84
Harrington	Kirin (WA)[1]	4.50	10.1	46.0	4.64	238	0-10	82.3	1.53	85
Stirling	JWM (WA)[2]	4.60	9.5	46.1	4.40	245	10-15	82.4	1.56	85
Schooner	BBM (Vic)[3]	4.50	10.7	47.1	5.05	220	10-15	80.6	1.50	84
Franklin	IMC (Vic)[4]	5.20	10.3	44.0	4.55	271	10-15	82.5	1.53	83
Gairdner	JWM (SA)[5]	5.20	10.2	37.4	3.80	275	10-15	81.9	1.56	81

[1] Kirin Australia, Western Australia; [2] Joe White Maltings, Western Australia; [3] Barrett Burston Malting Company, Victoria; [4] International Malting Company, Victoria; [5] Joe White Maltings, South Australia. Malt specifications for the Grimmett and Lindwall malts used in experiment 1 were not available. [a]DP = Diastatic power, [b]WK = Windisch-Kolbach

Table 2: Malt specifications for the 300 L pilot brewing trials – University of Ballarat (Experiment 2 – Chapter 3).

Variety	Site/ Origin	Mois. (%)	Fine. Ext. (%db)	Ext - S.P	Col. EBC	DP °WK	DP/ T.P.	T.P. (%db)	S.P. (%db)	KI (%)	Coarse. Ext. (%db)	Visc. (cP)	AAL (%)	β-Gase (U/kg)	a-Amy (D.U.)	Wort β-G (mg/l)	F.A.N (mg/100ml)	Clarity	Friability
Unicorn	Kirin (WA)[1]	6.5	81.2	76.2	3.4	321	25.9	12.4	5.04	40.6	79.1	1.68	79.2	483	35.6	72.5	21.5	1	82.6
Stirling	JWM (WA)[2]	4.5	82.2	77.3	4.1	279	28.3	9.9	4.81	48.8	80.5	1.69	75.5	530	46.9	315	20.1	1	81.5
Harrington	Kirin (WA)[1]	5.5	80.1	75.2	3.4	313	28.0	11.2	4.88	43.7	79.1	1.55	79.8	583	47.3	47.5	22.9	1.0	85.1

[1] Kirin Australia, Western Australia; [2] Joe White Maltings, Western Australia. T.P. = Total protein, S.P. = Soluble protein, KI = Kolbach Index, DP = Diastatic power, WK = Windisch-Kolbach.

Table 3: Malt specifications for the 100 L (Experiment 3 – Chapter 3) and 10 L (Chapter 4) pilot brewing trials – VTT Biotechnology.

Variety	Source	Moisture (%)	Extract (Fine) (%)	DP[a] (°WK)[b]	Total Protein (%)	Kolbach Index (%)	Wort Viscosity (cP)
Barke	Polttimo[1]	4.0	81.4	320	10.9	35.1	1.44
Kustaa	Raisio[2]	4.3	79.5	240	12.0	36.0	1.48
Saana	Polttimo[1]	4.2	80.2	350	11.3	35.4	1.43
Scarlett	Polttimo[1]	4.1	82.1	280	11.1	36.4	1.45

[1] Polttimo Companies, Lahti, Finland [2] Raisio Malt, Raisio, Finland. [a]DP = Diastatic power [b]WK = Windisch-Kolbach

Table 4: Wort and Beer Analysis, SSB trials (Experiment 1 – Chapter 3, section 3.3.1.1).

Beer #	Variety	Source	Wort Analysis		Beer Analysis						
			Viscosity	Extract	Viscosity	Gravity	Protein	Bitterness	Alcohol	β-glucan	Polyphenols
			[mPa.s]	[°P]	[mPa.s]	[°P]	[mg/L]	[BU]	[% v/v]	[g/L]	[mg/L]
1	Unicorn	Kirin[1]	1.854	12.56	1.493	1.32	172	19.0	4.86	0.07	71.88
2	Harrington	Kirin[1]	1.84	12.43	1.489	1.21	179	17.6	4.95	0.22	82.04
3	Stirling	JW/WA[2]	2.04	12.33	1.642	1.42	171	17.9	4.76	0.43	71.88
4	Schooner	BBM[3]	1.761	12.14	1.486	1.95	171	17.3	4.72	0.23	78.13
5	Franklin	IMC[4]	1.751	12.06	1.471	1.42	178	18.0	4.95	0.18	64.85
6	Grimmett	JW/TM[5]	1.697	11.29	1.477	1.84	177	-	4.73	0.41	74.23
7	Lindwall	Admalt[6]	1.757	10.98	1.553	1.75	188	17.4	4.62	0.41	60.16
8	Gairdner	JW/SA[7]	1.749	11.40	1.509	1.72	166	17.1	4.86	0.41	63.29
9	Unicorn	Kirin[1]	1.881	12.82	1.513	1.08	171	16.0	5.13	0.10	67.98
10	Harrington	Kirin[1]	1.798	12.00	1.495	1.22	168	18.1	4.77	0.20	85.95
11	Stirling	JW/WA[2]	1.918	11.03	1.644	1.76	169	17.4	4.72	0.42	76.57
12	Schooner	BBM[3]	1.722	11.51	1.486	1.83	177	17.8	4.89	0.26	76.57
13	Franklin	IMC[4]	1.77	12.17	1.471	1.51	175	17.2	4.73	0.19	66.41
14	Grimmett	JW/TM[5]	1.687	11.13	1.470	1.73	184	16.3	4.7	0.39	75.01
15	Lindwall	Admalt[6]	1.768	11.16	1.541	1.69	178	16.9	4.76	0.41	60.16
16	Gairdner	JW/SA[7]	1.699	10.94	1.505	1.80	170	17.0	4.97	0.43	63.29
17	Unicorn	Kirin[1]	1.74	11.23	1.484	1.19	175	16.0	4.95	0.04	64.07
18	Harrington	Kirin[1]	1.796	11.79	1.461	1.94	173	17.6	4.79	0.14	75.76
19	Stirling	JW/WA[2]	1.952	11.40	1.599	1.63	170	14.7	4.56	0.27	66.41
20	Schooner	BBM[3]	1.759	11.53	1.497	1.78	175	16.7	4.76	0.33	72.13
21	Franklin	IMC[4]	1.726	11.24	1.496	1.24	175	15.4	4.67	0.07	64.85
22	Grimmett	JW/TM[5]	1.656	10.22	1.469	1.96	186	16.5	4.49	0.19	68.76
23	Lindwall	Admalt[6]	1.746	10.51	1.617	1.69	174	17.2	4.8	0.28	67.98
24	Gairdner	JW/SA[7]	1.675	9.94	1.515	1.81	168	17.1	4.77	0.39	57.04

[1] Kirin Australia, Western Australia; [2] Joe White Maltings, Western Australia; [3] Barrett Burston Malting Company, Victoria; [4] International Malting Company, Victoria; [5] Joe White Maltings, NSW; [6] Adelaide Malting Company, South Australia; [7] Joe White Maltings, South Australia.

Table 5a: Treatment 1 Haze Analysis, SSB trials (Experiment 1 - Chapter 3, section 3.3.1.1). Samples were filtered (0.45 μm), and pasteurised before force testing (5 day 55°C, 1 day at 0°C).

Beer #	Variety	Source	IPH[a]	IPH[a]	IPH[a]	IPH[a]	IPH[a]	IPH[a]	Average	Std Dev	Std Error
1,9,17	Unicorn	Kirin[1]	0.44	0.44	0.38	0.40	0.48	0.47	0.44	0.04	8.59
2,10,18	Harrington	Kirin[1]	0.40	0.41	0.47	0.48	0.44	0.43	0.44	0.03	7.30
3,11,19	Stirling	JW/WA[2]	0.33	0.34	0.40	0.40	0.39	0.41	0.38	0.03	9.08
4,12,20	Schooner	BBM[3]	0.35	0.34	0.32	0.35	0.36	0.34	0.34	0.01	4.35
5,13,21	Franklin	IMC[4]	0.35	0.38	0.36	0.39	0.38	0.41	0.38	0.02	5.93
6,14,22	Grimmett	JW/TM[5]	0.41	0.42	0.37	0.36	0.41	0.42	0.40	0.03	6.81
7,15,23	Lindwall	Admalt[6]	0.34	0.31	0.45	0.46	0.36	0.39	0.38	0.06	15.48
8,16,24	Gairdner	JW/SA[7]	0.43	0.41	0.44	0.45	0.40	0.42	0.43	0.02	4.32

Beer #	Variety	Source	5PH[b]	5PH[b]	5PH[b]	5PH[b]	5PH[b]	5PH[b]	Average	Std Dev	Std Error
1,9,17	Unicorn	Kirin[1]	7.01	5.02	2.12	2.75	2.72	1.80	3.57	2.03	56.81
2,10,18	Harrington	Kirin[1]	4.41	6.17	6.46	6.75	5.05	4.25	5.52	1.08	19.67
3,11,19	Stirling	JW/WA[2]	7.83	5.56	5.04	1.70	7.52	5.15	5.47	2.20	40.31
4,12,20	Schooner	BBM[3]	6.85	6.15	4.86	5.88	3.34	3.94	5.17	1.36	26.30
5,13,21	Franklin	IMC[4]	6.46	6.48	3.63	5.56	3.27	3.70	4.85	1.49	30.67
6,14,22	Grimmett	JW/TM[5]	3.95	4.18	5.71	4.12	4.85	4.00	4.47	0.69	15.44
7,15,23	Lindwall	Admalt[6]	5.06	4.01	5.50	5.55	4.36	4.01	4.75	0.71	15.03
8,16,24	Gairdner	JW/SA[7]	6.74	5.04	3.44	4.10	3.39	4.40	4.52	1.25	27.70

Beer #	Variety	Source	ICH[c]	ICH[c]	ICH[c]	ICH[c]	ICH[c]	ICH[c]	Average	Std Dev	Std Error
1,9,17	Unicorn	Kirin[1]	1.88	1.21	1.19	1.72	1.12	1.26	1.40	0.32	22.89
2,10,18	Harrington	Kirin[1]	1.45	1.66	1.69	2.03	1.78	1.54	1.69	0.20	11.96
3,11,19	Stirling	JW/WA[2]	1.76	1.42	1.84	1.82	1.50	1.90	1.71	0.20	11.59
4,12,20	Schooner	BBM[3]	1.77	1.55	1.73	1.94	1.45	1.91	1.73	0.19	11.26
5,13,21	Franklin	IMC[4]	1.84	1.67	1.53	1.99	1.59	1.49	1.69	0.19	11.52
6,14,22	Grimmett	JW/TM[5]	2.03	1.95	1.67	2.14	2.08	1.88	1.96	0.17	8.61
7,15,23	Lindwall	Admalt[6]	1.46	1.34	1.59	1.63	1.42	1.44	1.48	0.11	7.39
8,16,24	Gairdner	JW/SA[7]	1.85	1.77	1.98	2.01	1.83	2.05	1.92	0.11	5.91

Beer #	Variety	Source	5CH[d]	5CH[d]	5CH[d]	5CH[d]	5CH[d]	5CH[d]	Average	Std Dev	Std Error
1,9,17	Unicorn	Kirin[1]	9.90	4.79	5.22	8.36	5.70	5.67	6.61	2.04	30.90
2,10,18	Harrington	Kirin[1]	9.70	9.57	10.10	11.20	9.46	8.96	9.83	0.77	7.78
3,11,19	Stirling	JW/WA[2]	10.40	9.79	9.49	9.89	7.19	8.46	9.20	1.18	12.80
4,12,20	Schooner	BBM[3]	9.73	7.93	7.86	10.24	10.30	7.67	8.96	1.26	14.09
5,13,21	Franklin	IMC[4]	10.00	9.29	6.25	10.80	8.23	6.72	8.55	1.81	21.22
6,14,22	Grimmett	JW/TM[5]	7.15	8.67	8.22	7.67	8.18	7.72	7.94	0.53	6.70
7,15,23	Lindwall	Admalt[6]	8.49	9.88	7.67	8.32	8.36	6.94	8.28	0.98	11.81
8,16,24	Gairdner	JW/SA[7]	11.60	7.42	7.96	9.18	8.96	7.24	8.73	1.61	18.49

[a]IPH = Initial permanent haze, [b]5PH = 5 day permanent haze, [c]ICH = Initial chill haze, [d]5CH = 5 day chill haze. Standard error is presented as a %.
[1] Kirin Australia, Western Australia; [2]Joe White Maltings, Western Australia; [3] Barrett Burston Malting Company, Victoria; [4] International Malting Company, Victoria; [5]Joe White Maltings, NSW; [6]Adelaide Malting Company, South Australia; [7] Joe White Maltings, South Australia.

Table 5b: Treatment 2 Haze Analysis, SSB trials (Experiment 1 - Chapter 3, section 3.3.1.1). Samples were pasteurised before force testing (5 day 55°C, 1 day at 0°C).

Beer #	Variety	Source	IPH[a]	IPH[a]	IPH[a]	IPH[a]	Average	Std Dev	Std Error
1,17	Unicorn	Kirin[1]	0.51	0.53	0.53	0.50	0.52	0.01	2.73
2,18	Harrington	Kirin[1]	0.48	0.52	0.50	0.46	0.49	0.02	5.08
3,19	Stirling	JW/WA[2]	0.45	0.50	0.49	0.50	0.49	0.02	4.50
4,20	Schooner	BBM[3]	0.47	0.45	0.50	0.42	0.46	0.03	7.11
5,21	Franklin	IMC[4]	0.45	0.48	0.47	0.46	0.46	0.02	3.28
6,22	Grimmett	JW/TM[5]	0.44	0.49	0.46	0.45	0.46	0.02	4.29
7,23	Lindwall	Admalt[6]	0.39	0.49	0.44	0.41	0.43	0.04	9.82
8,24	Gairdner	JW/SA[7]	0.49	0.46	0.49	0.42	0.46	0.03	7.33

Beer #	Variety	Source	5PH[b]	5PH[b]	5PH[b]	5PH[b]	Average	Std Dev	Std Error
1,17	Unicorn	Kirin[1]	3.13	4.81	3.50	4.26	3.93	0.75	19.22
2,18	Harrington	Kirin[1]	7.35	5.27	6.23	5.24	6.02	1.00	16.56
3,19	Stirling	JW/WA[2]	4.07	5.23	4.50	5.01	4.70	0.52	11.08
4,20	Schooner	BBM[3]	5.80	3.85	5.20	4.75	4.90	0.82	16.77
5,21	Franklin	IMC[4]	6.40	2.44	3.25	4.67	4.19	1.74	41.48
6,22	Grimmett	JW/TM[5]	6.19	4.45	5.12	4.23	5.00	0.88	17.62
7,23	Lindwall	Admalt[6]	5.81	7.73	6.23	5.74	6.38	0.93	14.54
8,24	Gairdner	JW/SA[7]	2.73	4.93	2.95	3.56	3.54	0.99	27.93

Beer #	Variety	Source	ICH[c]	ICH[c]	ICH[c]	ICH[c]	Average	Std Dev	Std Error
1,17	Unicorn	Kirin[1]	1.95	1.56	1.76	1.82	1.77	0.16	9.16
2,18	Harrington	Kirin[1]	1.69	1.85	1.92	1.78	1.81	0.10	5.43
3,19	Stirling	JW/WA[2]	1.95	2.35	2.02	1.85	2.04	0.22	10.60
4,20	Schooner	BBM[3]	2.23	2.04	2.01	2.15	2.11	0.10	4.81
5,21	Franklin	IMC[4]	2.45	1.78	1.92	2.03	2.05	0.29	14.12
6,22	Grimmett	JW/TM[5]	1.89	2.09	2.05	1.97	2.00	0.09	4.43
7,23	Lindwall	Admalt[6]	1.95	2.22	2.25	2.06	2.12	0.14	6.64
8,24	Gairdner	JW/SA[7]	2.42	2.17	2.15	2.56	2.33	0.20	8.56

Beer #	Variety	Source	5CH[d]	5CH[d]	5CH[d]	5CH[d]	Average	Std Dev	Std Error
1,17	Unicorn	Kirin[1]	6.69	7.37	6.21	7.23	6.88	0.53	7.73
2,18	Harrington	Kirin[1]	11.70	10.10	10.45	10.56	10.70	0.69	6.48
3,19	Stirling	JW/WA[2]	6.73	5.99	7.11	6.34	6.54	0.48	7.40
4,20	Schooner	BBM[3]	10.50	6.37	10.21	6.73	8.45	2.20	26.09
5,21	Franklin	IMC[4]	10.80	9.56	10.34	9.31	10.00	0.69	6.89
6,22	Grimmett	JW/TM[5]	11.00	7.65	11.54	7.84	9.51	2.05	21.55
7,23	Lindwall	Admalt[6]	9.40	10.30	9.23	9.98	9.73	0.50	5.13
8,24	Gairdner	JW/SA[7]	7.69	9.94	7.34	10.19	8.79	1.48	16.87

[a]IPH = Initial permanent haze, [b]5PH = 5 day permanent haze, [c]ICH = Initial chill haze, [d]5CH = 5 day chill haze. Standard error is presented as a %.
[1] Kirin Australia, Western Australia; [2]Joe White Maltings, Western Australia; [3] Barrett Burston Malting Company, Victoria; [4] International Malting Company, Victoria; [5]Joe White Maltings, NSW; [6]Adelaide Malting Company, South Australia; [7] Joe White Maltings, South Australia.

Table 5c: Treatment 3 Haze Analysis, SSB trials (Experiment 1 - Chapter 3, section 3.3.1.1). Samples were filtered (0.45 μm), pasteurised then stored in the dark at 20°C before haze measurements were recorded.

Beer #	Variety	Source	IPH[a]	IPH[a]	IPH[a]	IPH[a]	IPH[a]	IPH[a]	Average	Std Dev	Std Error
1,9,17	Unicorn	Kirin[1]	0.45	0.36	0.42	0.36	0.38	0.43	0.40	0.04	9.45
2,10,18	Harrington	Kirin[1]	0.36	0.49	0.37	0.39	0.46	0.38	0.41	0.05	13.36
3,11,19	Stirling	JW/WA[2]	0.30	0.39	0.43	0.35	0.41	0.41	0.38	0.05	12.69
4,12,20	Schooner	BBM[3]	0.29	0.38	0.34	0.31	0.40	0.34	0.34	0.04	12.68
5,13,21	Franklin	IMC[4]	0.34	0.39	0.35	0.30	0.38	0.37	0.36	0.03	8.75
6,14,22	Grimmett	JW/TM[5]	0.30	0.32	0.43	0.31	0.31	0.45	0.35	0.07	18.99
7,15,23	Lindwall	Admalt[6]	0.31	0.45	0.32	0.32	0.46	0.34	0.37	0.07	19.22
8,16,24	Gairdner	JW/SA[7]	0.36	0.38	0.34	0.37	0.40	0.32	0.36	0.03	7.06

Beer #	Variety	Source	ICH[c]	ICH[c]	ICH[c]	ICH[c]	ICH[c]	ICH[c]	Average	Std Dev	Std Error
1,9,17	Unicorn	Kirin[1]	1.21	1.36	1.56	1.75	1.90	2.02	1.63	0.31	19.24
2,10,18	Harrington	Kirin[1]	1.22	1.43	1.59	1.76	1.92	2.03	1.66	0.31	18.41
3,11,19	Stirling	JW/WA[2]	1.27	1.43	1.61	1.76	1.93	2.05	1.68	0.30	17.73
4,12,20	Schooner	BBM[3]	1.30	1.44	1.61	1.79	1.94	2.15	1.71	0.32	18.62
5,13,21	Franklin	IMC[4]	1.32	1.47	1.68	1.81	1.94	2.15	1.73	0.30	17.65
6,14,22	Grimmett	JW/TM[5]	1.34	1.50	1.68	1.82	1.95	2.17	1.74	0.30	17.33
7,15,23	Lindwall	Admalt[6]	1.34	1.53	1.68	1.86	1.98	2.23	1.77	0.32	18.12
8,16,24	Gairdner	JW/SA[7]	1.35	1.55	1.72	1.89	1.98	2.27	1.79	0.33	18.20

[a]IPH = Initial permanent haze, [c]ICH = Initial chill haze. Standard error is presented as a %.
[1] Kirin Australia, Western Australia; [2]Joe White Maltings, Western Australia; [3] Barrett Burston Malting Company, Victoria; [4] International Malting Company, Victoria; [5]Joe White Maltings, NSW; [6]Adelaide Malting Company, South Australia; [7] Joe White Maltings, South Australia.

Table 6a and 6b: Wort and Beer Analysis, 300 L micro brewing trials – University of Ballarat (Experiment 2 - Chapter 3, section 3.3.1.2).

6a			Wort Analysis		Beer Analysis* - SCABA						Beer Analysis* - SCALA		
			Specific Gravity	pH	Alcohol	Original Extract	Apparent Extract	Real Extract	Apparent Fermentability	Energy	Bitterness	pH	Colour
Trial #	Variety	Source			[% v/v]	[°P]	[°P]	[°P]	[%]	[kJ/L]	[BU]		[°EBC]
1	Unicorn	Kirin[1]	1.043	5.47	5.03	11.70	-	-	-	-	25.50	4.32	-
1	Harrington	Kirin[1]	1.040	5.50	4.92	12.19	-	-	-	-	27.00	4.39	-
1	Stirling	JW/WA[2]	1.043	5.65	5.01	12.22	-	-	-	-	26.00	4.41	-
2	Unicorn	Kirin[1]	1.044	5.60	4.99	11.34	1.93	3.72	82.90	1704	26.50	4.29	7.0
2	Harrington	Kirin[1]	1.043	5.35	4.59	10.89	2.22	3.87	79.60	1636	28.50	4.22	8.6
2	Stirling	JW/WA[2]	1.048	-	4.91	12.59	3.45	5.19	72.50	1912	24.00	4.39	8.8
3	Stirling	JW/WA[2]	1.045	5.52	5.02	12.10	2.70	4.49	77.7	1828	27.00	4.44	10.7
3	Harrington	Kirin[1]	1.047	5.54	5.49	13.35	3.16	5.10	76.3	2030	26.00	4.43	9.3
3	Stirling	JW/WA[2]	1.045	5.30	5.09	12.50	2.99	4.80	76.0	1894	26.00	4.46	9.0

6b		Beer Analysis* - Volatile Flavour Components								
Trial #		1	1	1	2	2	2	3	3	3
Variety		Unicorn	Harrington	Stirling	Unicorn	Harrington	Stirling	Stirling	Harrington	Stirling
Source		Kirin[1]	Kirin[1]	JW/WA[2]	Kirin[1]	Kirin[1]	JW/WA[2]	JW/WA[2]	Kirin[1]	JW/WA[2]
Acetaldehyde	mg/L	-	-	-	4.45	5.27	4.24	6.35	5.46	3.85
Dimethyl Sulphide	μg/L	-	-	-	64.4	48.3	68.90	60.4	75.7	46.5
Ethyl Acetate	mg/L	-	-	-	32.6	28.8	29.20	31.8	40.5	23.6
n-Propanol	mg/L	-	-	-	19.6	17.4	16.80	19.5	20.9	0.1
iso-Butanol	mg/L	-	-	-	10.6	10.3	9.10	9.70	13.1	9.8
iso-Amyl Acetate	mg/L	-	-	-	2.18	1.74	1.43	1.71	2.55	1.21
iso-Amyl Alcohol	mg/L	-	-	-	54.2	38.1	40.30	44.2	53.6	38.3
Ethyl Hexanoate	mg/L	-	-	-	0.38	0.38	0.35	0.335	0.401	0.247
Ethyl Octanoate	mg/L	-	-	-	0.799	0.657	0.83	0.534	0.911	0.349
Total Fusel Alcohol	mg/L	-	-	-	84.45	65.81	66.17	73.42	-	-
Ester Index		-	-	-	1.28	1.06	0.89	1.02	-	-
Ester Ratio		-	-	-	5.74	4.58	4.09	5.11	-	-
Diacetyl	mg/L	-	-	-	0.044	0.066	0.03	0.049	0.019	0.040
Pentanedione	mg/L	-	-	-	0.015	0.024	0.01	0.023	0.009	0.020
Total VDK	mg/L	-	-	-	0.059	0.0895	0.04	0.0715	-	-

[1] Kirin Australia, Western Australia; [2] Joe White Maltings, Western Australia. * Brewing Analysis was completed by the Technical Department – Central Laboratory, Tooheys Brewery (Lion Nathan, Sydney, Australia).

Table 7: Wort Analysis, 100 L pilot brewing trials – VTT Biotechnology (Experiment 3 - Chapter 3, section 3.3.1.3).

Variety	Extract [°P]	Colour (EBC) 25 mm Cuvette	pH	Soluble Nitrogen (mg/L)	Fatty Acids[a] (mg/g)	F.A.N (mg/L)
Barke	12.0	7.0	5.37	1181	3.5	190
Barke	11.7	7.5	5.36	1158	3.3	178
Saana	12.1	11.5	5.31	1236	2.7	208
Saana	11.7	11.0	5.31	1235	3.3	200
Scarlett	12.1	10.0	5.32	1321	4.7	227
Scarlett	12.4	10.5	5.32	1325	4.1	228
Kustaa	12.2	10	5.30	1345	4.0	233
Kustaa	12.1	10.5	5.28	1299	4.6	224

[a]Fatty acid values equal to the sum of the total acids measured: palmitic acid, stearic acid, oleic acid, linolic acid and linoleic acid. F.A.N = Free Amino Nitrogen.

Table 8: Beer Analysis, 100 L pilot brewing trials – VTT Biotechnology (Experiment 3 - Chapter 3, section 3.3.1.3).

Variety	SG	Alcohol	pH	Colour	PT Value	PT Value	Chill Haze[a]	Permanent Haze[a]	Polyphenols	Total Prot.
					P40	T125				
		[%v/v]		[EBC]	[mg/100 mL]	[mg/100 mL]	[EBC FU]	[EBC FU]	[mg/L]	[mg/L]
Barke	1.00766	5.46	4.54	5.0	19.4	38.4	4.28	0.80	52	240
Barke	1.00772	5.41	4.49	5.5	18.4	11.0	10.25	0.85	73	400
Saana	1.00803	5.53	4.48	7.5	23.8	11.0	16.22	2.45	84	496
Saana	1.00077	5.36	4.52	7.0	26.8	13.8	2.95	0.37	62	347
Scarlett	1.00878	5.46	4.51	7.0	21.3	10.9	12.92	0.75	71	465
Scarlett	1.00850	5.67	4.49	6.5	20.5	10.0	16,23	0.54	102	478
Kustaa	1.01018	5.30	4.60	7.0	20.9	22.8	2.59	0.38	68	361
Kustaa	1.01054	5.39	4.56	7.5	18.5	10.6	9.88	0.55	93	470

[a]Chill and permanent haze values equal to mean of triplicate samples. PT = Protein/tannin.

Table 9: Wort Analysis, 10 L pilot brewing trials – VTT Biotechnology (Experiment 3 - Chapter 3, section 3.3.1.3).

Variety	Extract [°P]	Colour (EBC) 25 mm Cuvette	pH	Soluble Nitrogen (mg/L)	Fatty Acids[a] (mg/g)	F.A.N (mg/L)
Scarlett Control	12.1	9.0	5.61	1140	8.61	148
Scarlett Control	12.0	9.0	5.64	1137	8.57	155
Scarlett Nitrogen	12.1	7.0	5.64	1145	-	150
Scarlett Nitrogen	12.0	7.0	5.64	1132	6.14	150
Scarlett Oxygen	12.1	14.5	5.55	1196	11.05	153

[a]Fatty acid values equal to the sum of the total acids measured: palmitic acid, stearic acid, oleic acid, linolic acid and linoleic acid. F.A.N = Free Amino Nitrogen.

Table 10: Beer Analysis, 10 L pilot brewing trials – VTT Biotechnology (Experiment 3 - Chapter 3, section 3.3.1.3).

Variety	SG	Alcohol [%v/v]	pH	Colour [EBC]	PT Value P40 [mg/100 mL]	PT Value T125 [mg/100 mL]	Chill Haze[a] [EBC FU]	Permanent Haze[a] [EBC FU]	Polyphenols [mg/L]	Total Prot. [mg/L]
Scarlett Control	1.00759	5.51	4.25	7.0	37.2	13.1	6.34	1.0	100	237
Scarlett Control	1.00733	5.46	4.21	7.5	31.3	11.3	16.24	1.92	107	390
Scarlett Nitrogen	1.00723	5.46	4.28	5.5	37.9	11.1	8.32	2.25	99	220
Scarlett Nitrogen	1.00733	5.47	4.29	6.0	35.2	9.5	16.03	1.47	114	334
Scarlett Oxygen	1.00759	5.65	4.26	8.5	24.3	13.7	16.49	4.59	75	425

[a]Chill and permanent haze values equal to mean of triplicate samples. PT = Protein/tannin.

Table 11: Wort and Beer Analysis including V_{max}, from small scale brewing trials used to assess the micro-filtration of SE +ve and SE –ve malts.

Beer #	Variety	Source	Wort Analysis		Beer Analysis						
			Viscosity [mPa.s]	Extract [°P]	Viscosity [mPa.s]	Gravity [°P]	Vmax [mL]	Vmax [mL]	Av Vmax [mL]	St Dev	Standard Error [%]
1	Unicorn[B]	Kirin	1.63	12.01	1.373	1.73	**685.73***	**685.81***	**685.77***	0.057	0.008
2	Harrington[B]	Kirin	1.545	11.57	1.340	1.59	486.69	484.57	485.63	1.499	0.309
3	Stirling[B]	JW/WA	1.631	12.33	1.372	2.43	501.09	516.26	508.67	10.726	2.109
4	Unicorn[B]	Kirin	1.63	11.98	1.385	1.64	273.66	275.83	274.74	1.535	0.559
5	Harrington[B]	Kirin	1.588	12.21	1.336	1.47	496.64	505.04	500.84	5.941	1.186
6	Stirling[B]	JW/WA	1.684	13.02	1.368	1.87	541.83	529.48	535.65	8.731	1.630
7	Unicorn[B]	Kirin	1.703	12.89	1.389	1.49	291.15	287.13	289.14	2.844	0.984
8	Harrington[B]	Kirin	1.531	11.35	1.347	1.33	462.66	456.89	459.77	4.079	0.887
9	Stirling[B]	JW/WA	1.685	13.03	1.375	2.10	536.49	554.04	545.26	12.415	2.277
10	Unicorn[T]	Kirin	1.747	13.26	1.383	1.59	332.24	339.98	336.11	5.470	1.627
11	Harrington[T]	Kirin	1.662	12.81	1.355	1.57	359.89	371.30	365.59	8.064	2.206
12	Stirling[T]	JW/WA	1.657	12.66	1.383	1.80	315.54	323.29	319.41	5.484	1.717
13	Unicorn[T]	Kirin	1.651	12.41	1.419	1.44	370.45	390.43	380.44	14.124	3.713
14	Harrington[T]	Kirin	1.618	12.48	1.344	1.40	383.94	365.96	374.95	12.711	3.390
15	Stirling[T]	JW/WA	1.602	11.79	1.419	1.89	**119.43***	**117.07***	**118.25***	1.670	1.412
16	Unicorn[T]	Kirin	1.612	12.31	1.350	1.89	377.73	381.30	379.51	2.526	0.666
17	Harrington[T]	Kirin	1.572	11.89	1.339	1.50	364.65	349.07	356.86	11.018	3.087
18	Stirling[T]	JW/WA	1.622	12.41	1.387	1.38	323.39	322.63	323.01	0.536	0.166
Standard	WE Draught	SAB	-	-	-	-	257.41	227.82	242.61	20.924	8.624

[B]Malt used in the 300 L Ballarat trials (Experiment 2), [T]Malt used in the SSB trials (Experiment 1) and the 50 L Lion Nathan trials.
*Samples in **bold** were outliers and were not included in the data presented in Figures 9a and 9b (Chapter 4 – section 4.3.3).

Figure 1a: Filtration temperature profiles recorded using a PC logger 2100 (INTAB, Stenkullen, Sweden) for beers 1 (-1°C), 2 (2°C) and 3 (5°C).
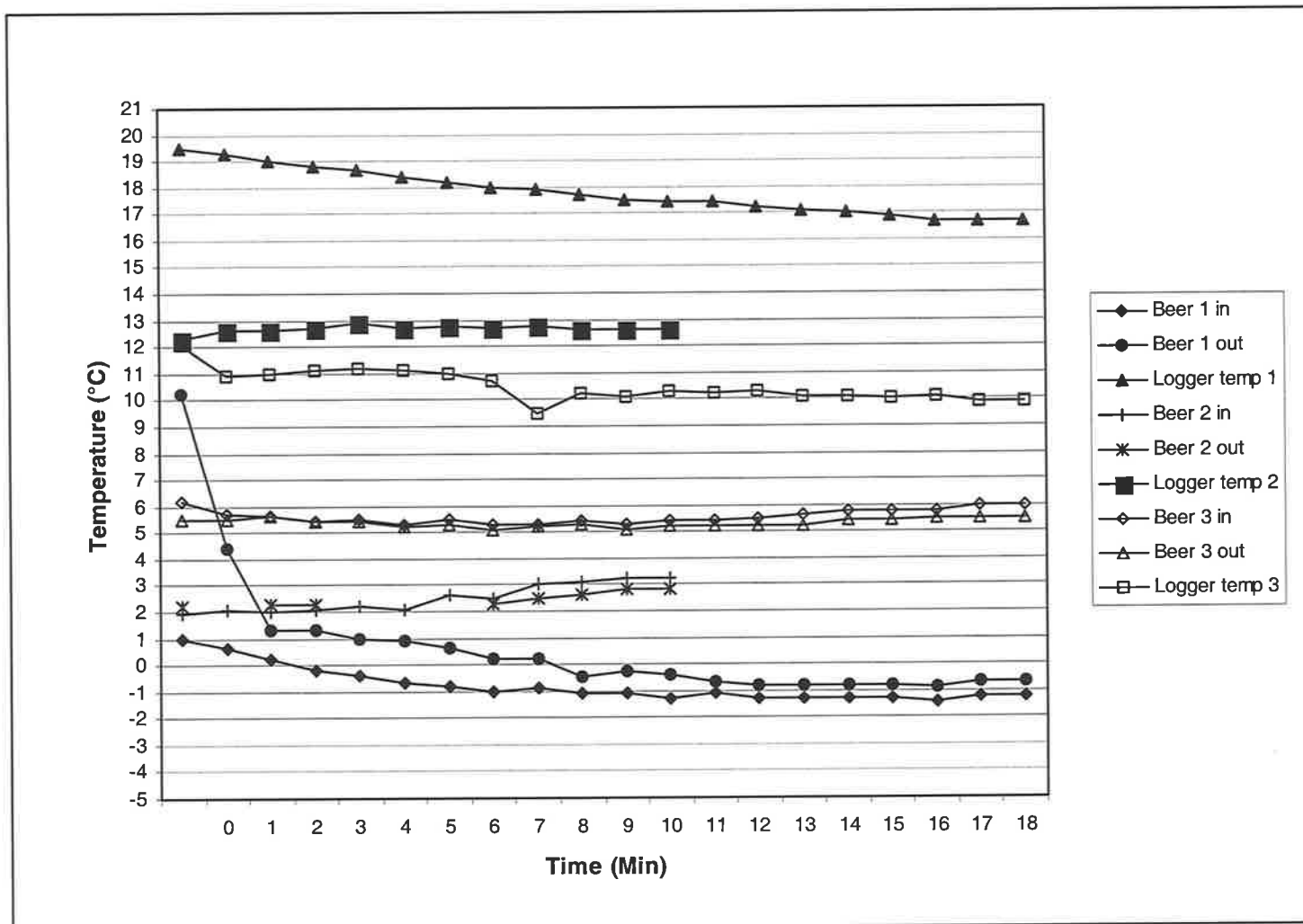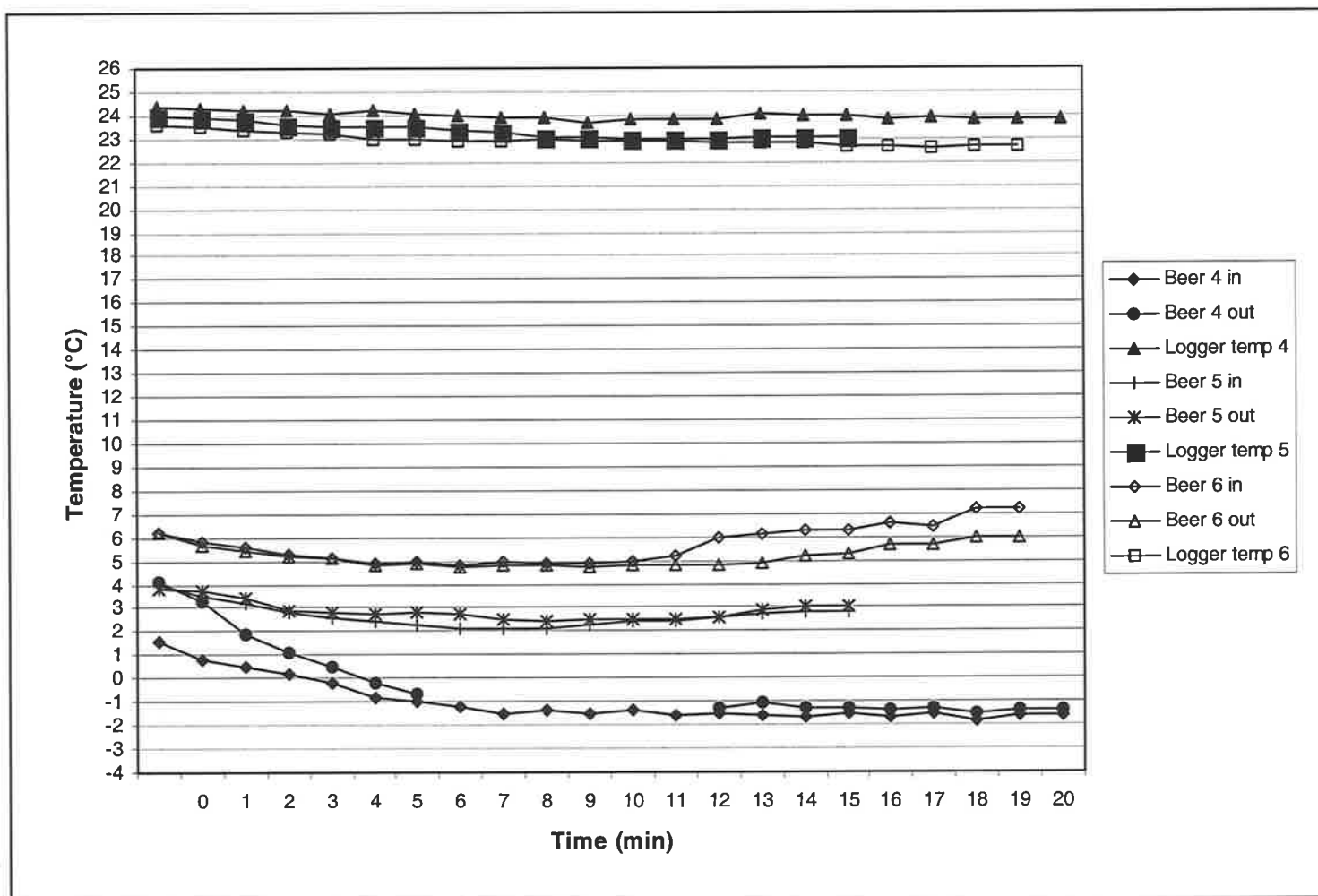
Figure 1b: Filtration temperature profiles recorded using a PC logger 2100 (INTAB, Stenkullen, Sweden) for beers 4 (-1°C), 5 (2°C) and 6 (5°C).
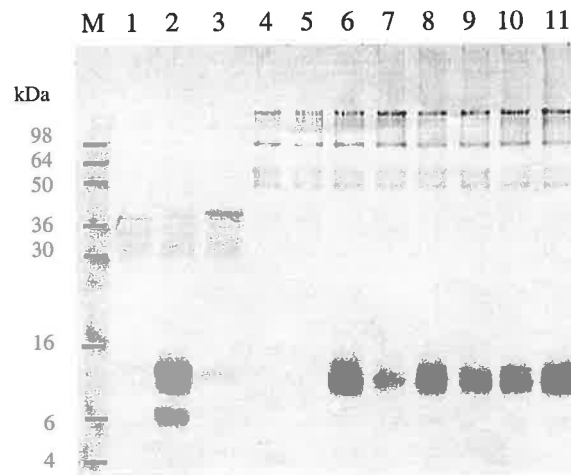
Figure 2: SDS-PAGE immunoblot analysis using the anti SE serum (1/1000) dilution of beer produced in the 50 L Lion Nathan trials. Total protein extracts from Unicorn (SE −ve), Stirling (SE +ve) and Harrington (SE −ve) malts (lanes 1-3), and 5-day haze force tested beers (lanes 4-11). Lane 4 = Unicorn (SE −ve), lane 5 = Harrington (SE −ve), lane 6 = Schooner (SE +ve), lane 7 = Stirling (SE +ve), lane 8 = Gairdner (SE +ve), lane 9 = Franklin (SE +ve), lane 10 = Grimmett (SE +ve) and lane 11 = Lindwall (SE +ve).
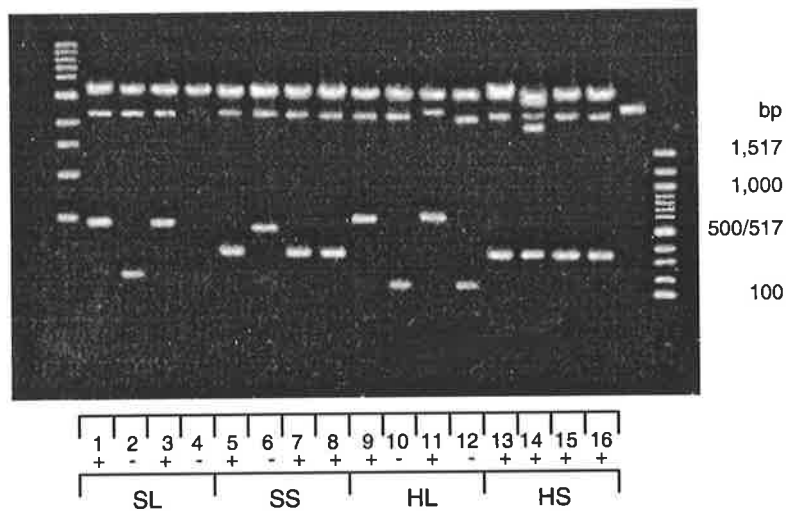
Figure 1: Agarose gel electrophoresis of the BTI-CMe plasmid DNA purified from

E.coli. SL = Sloop Long (full length protein), SS = Sloop Short (truncated protein). HL

= Harrington Long (full length protein), and HS = Harrington Short (truncated protein).

Table 1: Amino acid composition of BTI-CMe. The sequence of IAAE_HORVU (P01086) was analysed using the ExPASy – ProtParam Tool (Swiss-Prot/TrEMBL) (http://www.expasy.org/sprot). Computation based on the sequence of the mature protein.

Number of amino acids: 120, Molecular weight: 13258.0, Theoretical pI: 6.95

Amino Acid Composition:

Ala (A)	11	9.2%
Arg (R)	9	7.5%
Asn (N)	4	3.3%
Asp (D)	5	4.2%
Cys (C)	10	8.3%
Gln (Q)	7	5.8%
Glu (E)	6	5.0%
Gly (G)	9	7.5%
His (H)	3	2.5%
Ile (I)	5	4.2%
Leu (L)	9	7.5%
Lys (K)	2	1.7%
Met (M)	2	1.7%
Phe (F)	3	2.5%
Pro (P)	10	8.3%
Ser (S)	7	5.8%
Thr (T)	6	5.0%
Trp (W)	1	0.8%
Tyr (Y)	6	5.0%
Val (V)	5	4.2%

Appendix D

Buffers, Media and Solutions

All buffers, media and solutions were prepared under sterile conditions using nano-pure H_2O (Milli-Q® Ultrapure Water Purification Systems – Millipore, NSW, Australia) and autoclaved where appropriate.

SDS-PAGE and Immunoblotting

SE Extraction/Sample Buffer: 5 M urea, 4% (w/v) sodium dodecyl sulphate (SDS), Tris buffer pH 8.0. 6.05 g of Tris, 40 g SDS and 303 g urea was dissolved in H_2O and made up to a final volume of 1 L.

5x SDS-PAGE sample buffer: 0.225 M Tris-Cl, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT or 60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM β-mercapto-ethanol, 0.1 % bromophenol blue, pH 6.8.

2x SDS-PAGE sample buffer: 0.09 M Tris-Cl, pH 6.8; 20% glycerol; 2% SDS; 0.02% bromophenol blue; 0.1 M dithiothreitol (DTT).

Running gel buffer: 1.5 M Tris-HCl, pH 8.8. 36.3g of Tris was dissolved in H_2O and made up to a final volume of 200 mL. The pH was adjusted to 8.8 with HCl (4.4084 mL concentrated HCl).

Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8. 3.0 g of Tris was dissolved in H_2O and made up to a final volume of 50 mL. The pH was adjusted to 6.8 with HCl.

15% Running Gel: For 4 gels 10.5 mL acrylamide stock solution (Bio-Rad) (30% (w/v) acrylamide, 0.8% bis-acrylamide) was mixed with 5.24 mL running gel buffer and 4.90 mL H_2O. The solution was sonicated (Branson Ultrasonic Cleaner,

Danbury, CT, USA) then 210 μL of 10% (w/v) SDS, 100 μL 10% (w/v) ammonium persulphate and 10 μL TEMED (N,N,N,N'-Tetra-Methyl-Ethylenediamine) was added and the gel poured. H_2O was placed over the top of the gel during polymerisation.

15% Stacking Gel: For 4 gels, 1.59 mL acrylamide stock solution (30% (w/v) acrylamide, 0.8% bis-acrylamide) was mixed with 2.00 mL stacking gel buffer and 4.35 mL H_2O. The solution was sonicated, then 80 μL of 10% (w/v) SDS, 40 μL 10% (w/v) ammonium persulphate and 7.5 μL TEMED was added and the gel poured.

Protein gel electrophoresis buffer: (25 mM Tris-HCl, 190 mM glycine, 0.1% SDS, pH 8.3). For 4 L (1X); 12.0 g Tris, 57.05 g glycine, 4.0 g SDS and H_2O to 4 L.

Protein transfer buffer: (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, 0.05% SDS, pH 8.1-8.3). For 4 L (1X); 12.1 g Tris, 57.6 g glycine, 800 mL methanol, 0.2 g SDS, 3200 mL H_2O. The buffer was stored at $4^{\circ}C$.

1% (w/v) BSA/PBS: 5 g of Bovine Serum Albumin (BSA) in PBS to 500 mL.

PBS (phosphate buffered saline, pH 7.5) (10X solution): (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 4.3 mM Na_2HPO_4). 80 g NaCl, 2 g KCl, 2 g KH_2PO_4, 7.64 g $Na_2HPO_4.2H_2O$ or 11.5 g $Na_2HPO_4.7H_2O$, H_2O to 1 L. pH adjusted to 7.5.

Tween PBS (TPBS) (per litre): (Tween 20 - Polyoxyethylenesorbitan monolaurate (0.05%) - phosphate buffered saline, pH 7.5). 100 mL (10x PBS), 0.5 mL Tween-20, 900 mL H_2O.

TBS (tris buffered saline) (10X solution): (0.15 M NaCl, 10 mM Tris-HCl) - 87.66 g NaCl and 12.11 g Tris-HCl in 1 L of H_2O. pH adjusted to 7.5.

Horseradish Peroxidase (HRP) colour development substrate: 30 mg of 4-chloro-naphthol (Biorad #170-6534, HRP colour development reagent) was added to 10 mL of methanol on ice. 40 mL of TBS and 30 μL of H_2O_2 (30%) were then added.

Rehydration Solution with IPG Buffer: 8 M urea, 2% (w/v) (3[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid) (CHAPS), 0.5% (v/v) IPG buffer 3-10, bromophenol blue and 7 mg DTT per 2.5 mL aliquot of rehydration solution.

SDS Equilibration Buffer: 50 mM Tris-Cl pH 8.8, 6 M urea, 30% (v/v) glycerol, bromophenol blue, 2% (w/v) SDS.

Agarose sealing solution: Protein gel electrophoresis buffer with 0.5% agarose.

Colloidal coomassie (G250, modified Neuhoff stain) (per litre): (0.05% Coomassie blue G-250, 12% (w/v) ammonium sulphate, 2.25% (w/v) phosphoric acid, 25% (w/v) methanol). Ammonium sulphate (120 g) was dissolved first in H_2O, then phosphoric acid (19 mL of an 85% solution), and 250 mL methanol was added. 0.5 g Coomassie blue G-250 was dissolved/suspended in 20 mL of H_2O, then added to the ammonium sulphate/phosphoric acid/methanol solution and made up to 1 L with H_2O.

Colloidal coomassie de-staining solution: 1% (w/v) acetic acid solution.

Coomassie gel stain solution (per litre): (0.025% Coomassie blue R-250, 40% methanol, 7% acetic acid). 0.25 g of Coomassie blue R-250, 400 mL of methanol, 70 mL of glacial acetic acid, and 530 mL H_2O.

Coomassie de-staining solution I (per litre): (40% methanol, 7% acetic acid). 400 mL of methanol, 70 mL of glacial acetic acid, 530 mL H_2O.

Coomassie de-staining solution II (per litre): (7% acetic acid, 5% methanol). 70 mL of acetic acid, 50 mL of methanol, 880 mL of H$_2$O.

Cloning of BTI-CMe

DNA extraction buffer: (1% Sarkosyl, 100 mM Tris-HCl, 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) and 2% PVPP, adjust to pH 8.5 with concentrated HCl).

Phenol/chloroform/iso-amyl alcohol (25:24:1): Redistilled phenol was saturated with 0.5 M Tris-HCl, pH 8.0, then mixed with chloroform and iso-amyl alcohol.

10X Polymerase chain reaction (PCR) buffer: 500 mM KCl, 200 mM Tris-HCl, 25 mM MgCl$_2$, 1 mg/mL BSA, pH 8.4.

10X Tris-Acetate-EDTA (TAE) buffer: 400 mM Tris-HCl, 10 mM Na$_2$EDTA, pH 8.0.

1X Tris buffered EDTA (TE) buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

R40: 40 µL/mL RNaseA in TE Buffer

Purification of BTI-CMe

Lysis Buffer: (50 mM NaH$_2$PO$_4$, 0.3 M NaCl, 1% Triton, 5 mM imidazole, pH 8.0)

Wash Buffer 1: (50 mM NaH$_2$PO$_4$, 0.3 M NaCl, 5 mM imidazole, pH 8.0)

Wash Buffer 2: (50 mM NaH$_2$PO$_4$, 0.3 M NaCl, pH 6.0)

Wash Buffer 3: (0.1 M NaH$_2$PO$_4$, pH 6.0)

Elution Buffer: (0.1 M KH$_2$PO$_4$, 2 mM EDTA, pH 3.0)

Titration Buffer: (0.1 M NaH$_2$PO$_4$, 2 mM EDTA, pH 10.0)

Media

Luria-Bertani (LB) medium (per litre): 10 g of Bacto-Tryptone, 5 g of yeast extract, 10 g of NaCl, pH 7.0.

LB agar: LB medium containing 15 g of agar per litre.

Psi-broth: LB medium, 4 mM MgSO$_4$, 10 mM KCl.

SOB medium (100 mL): 2 g of Bacto-Tryptone, 0.5 g of Bacto-yeast extract, 1 mL of 1 M NaCl and 0.25 mL of 1 M KCl was added to 97 mL of H$_2$O, with stirring to dissolve. The solution was autoclaved and cooled to room temperature. One mL of 2 M Mg^{2+} stock (1 M MgCl$_2$·6H$_2$O/1 M MgSO$_4$·7H$_2$O) and 1 mL of 2 M filter-sterilised glucose were added. The solution was made to 100 mL with H$_2$O. The medium was filtered through a 0.2 μm filter unit. Final pH 7.0.

E.coli bacterial strains

XL1-Blue^r: Genotype: *SupE44 hsdR17 recA1 end A1 gyr A46 thi relA1 lac* F'[proAB$^+$, *lac*1^q *lac*Z^M15 Tn10 *(tet^r)*]; (Bullock *et al.*, 1987).

M15[pREP4]: Genotype: Nal^s, Str^s, Rif^s, Thi$^-$, Lac$^-$, Ara$^+$, Gal$^+$, Mtl$^-$, F$^-$, RecA$^+$, Uvr$^+$, Lon$^+$.

References

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