1 Literature review

1.1 Introduction

Black point is a dark discolouration of the embryo end of otherwise healthy barley (*Hordeum vulgare* L.) grain (Figure 1-1). The discolouration occurs in the husk tissue (palea and lemma) and to a lesser extent in the germ aleurone tissue covering the embryo (Figure 1-2). Black point develops towards the end of grain development as the grain matures before it is harvested.

Figure 1-1 Black point affected grain (A) compared to healthy grain (B). On mature grain the discolouration can be readily observed in the husk tissue surrounding the embryo. The underlying germ aleurone tissue is also discoloured.

NOTE: This figure is included on page 2 in the print copy of the thesis held in the University of Adelaide Library.

Figure 1-2 Schematic longitudinal cross section of a mature barley grain showing the main tissue tissues of the grain. Black point symptoms are confined to the husk tissue and germ aleurone that surrounds the embryo (Source: http://www.crc.dk/flab/the.htm).

Black point also affects wheat grains in a similar way, the main difference being that mature wheat grains do not contain an outer husk tissue so the discolouration is concentrated in the germ aleurone tissue (Williamson, 1997).

Black point-affected grain is currently unacceptable to the malting industry for two main reasons. Firstly, the malting quality of black-pointed barley is perceived to be reduced, and secondly, discoloured grains are aesthetically unappealing and difficult to sell on the international market (*personal communication*, John Stuart, GrainCorp Limited, September 2005). As a consequence, malting barley can be downgraded to feed grade which can lower the value of the grain by up to $50 AUD per tonne.

Within Australia, black point occurs predominantly in Western Australia, South Australia, Victoria, northern New South Wales, and most severely in Queensland. Within South Australia and Victoria alone, it is estimated that in a severe black point

year approximately 200,000 tonnes of barley will be downgraded (Hadaway et al., 2005).

Given that the barley varieties most commonly grown are susceptible to black point, it is likely that black point will continue to be a problem for the barley industry. Therefore, there is a real need to develop black point resistant barley cultivars. To achieve this there needs to be a greater understanding of the causes of black point so that potential means of cultivar improvement might be identified. The use of new technologies such as proteomics and syntenic analysis may enable better understanding of black point formation. This literature review outlines the current scientific knowledge on black point and where further research is required to address knowledge gaps.

1.2 Possible causes of black point

Several factors have previously been identified as potential causes of black point including environmental conditions during grain development, fungal infections and most recently, enzymatic browning. Although the literature provides conflicting evidence, it is likely that a number of these factors are interacting to cause black point formation as discussed below.

1.2.1 Pre-harvest environmental conditions

Although often contradictory, environmental conditions such as rain, humidity and temperature have been reported to lead to black point formation (Waldron, 1934; Rees et al., 1984; Conner et al., 1992; Fernandez et al., 2000). Waldron (1934) noted that high temperature and low moisture conditions were associated with black point development, whereas Rees et al. (1984) observed that moist conditions during grain

development increased symptoms. Fernandez et al. (1994) supports the notion that moist conditions during grain development are involved but noted that conditions that delay ripening such as low temperatures and frost may also contribute.

It is likely that moist conditions during grain filling are involved but the timing and nature may be more important with intermittent precipitation during grain development increasing symptoms in comparison to one-off heavy rainfall events (Petr and Capouchova, 2001). More specifically, increased temperature and moisture between the grain development stages of milk and dough appear to be associated with an increase in the incidence of black point in wheat (Moschini et al., 2006).

Due to the variability observed in field-grown conditions, attempts have been made to reproduce black point symptoms under controlled environmental conditions. Higher levels of black point have been reported by growing plants in a plastic covered enclosure and applying overhead water misting to increase the humidity during grain fill (Sulman et al., 2001). Black point levels reported using this method still only reached a maximum level of 57% in highly susceptible varieties, compared to field grown levels of 47% for the same variety. It is worth noting that this study was conducted in Queensland where humidity levels are higher than in Southern Australia where black point levels that high are rarely observed. By artificially increasing the humidity, fungal infection unrelated to black point can also become a problem when using this method. Further research is therefore needed to determine precisely what environmental conditions are required to obtain consistently high levels of black point.

1.2.2 Fungal infection

Until recently, black point has been solely attributed to infection of the grain by

various fungi such as *Alternaria alternata, Bipolaris* spp., *Epicoccum* spp., *Fusarium graminearum*, *Cladosporium* spp., *Stemphylium* spp. and *Chaetomium* spp. (Waldron, 1934; Statler et al., 1975; Southwell et al., 1980) of which many are saprophytes. Throughout the literature there are mixed reports on the mechanism by which fungi cause symptoms. Bhowmink (1969) and Cromey and Mulholland (1988) hypothesised that in wheat, discolouration was due to a dense mycelial mat at the embryo end of the grain. However, these results are conflicting with an earlier study that found the beard end of the grain had far more fungal mycelium than the germ end (Hyde and Galleymore, 1951). Furthermore *Alternaria alternata* was found to grow over the entire grain and microscopic observations showed sites of infection were not associated with discoloured cells on black pointed wheat grains (Williamson, 1997).

In wheat, Koch's postulates (where an organism is isolated from symptomatic tissue and used to inoculate healthy tissue to reproduce symptoms before re-isolation of that same organism) have been used to confirm the involvement of *Fusarium proliferation* in Californian-grown wheat. Inoculation with *F. proliferation* produced symptoms identical to black point (Conner et al., 1996). Studies conducted outside North America and Canada however have not detected *F. proliferation* on black pointed grains (Jacobs and Rabie, 1987; Ellis et al., 1996; Moschini et al., 2006). Furthermore the fungal species that were isolated from the grain in these studies were present in comparable levels in both healthy and black pointed grains. The most common fungi isolated from black pointed grains grown in Australia is *Alternaria alternata* which was found not to induce black point when wheat heads were artificially inoculated with this fungus (Williamson, 1997).

Based on the literature it is unclear what role fungal infection has in black point. Any

link is further confounded by the fact that the same environmental conditions that induce black point are favourable for fungal growth. The fungal species proposed to be associated may also be simply present on senescing grain due to their saprophytic nature. Fungal contamination of black pointed grains may therefore be associated with symptoms but not be the causative agent of black point.

1.2.3 Enzymatic browning

The process of enzymatic browning has been well studied as a result of the large postharvest losses for many fruit and vegetables due to browning caused by wounding. In higher plants, phenolic compounds are oxidised by peroxidases and polyphenol oxidases into coloured end products (Kruger et al., 1987). Peroxidases differ to polyphenol oxidases in that they can oxidise numerous substrates, not just phenols. In addition, peroxidases require hydrogen peroxide to reduce the substrate (Halliwell and Gutteridge, 1989). Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) and is produced by plant cells in numerous ways (Mittler, 2002) and particularly during wounding (Smirnoff, 1993).

Recent studies have provided evidence that black point results from enzymatic oxidation of phenolic compounds as opposed to fungal infection. Black point symptoms have been reproduced in mature wheat grains *in vitro* (Williamson, 1997) by soaking grains in phenol and then transferring into a H_2O_2 solution. It was concluded from this experiment that firstly, the enzyme responsible for the oxidation must be produced by the grain and secondly, since H_2O_2 was required to produce the discolouration, the enzyme responsible is most likely a peroxidase. All varieties analysed developed symptoms with black point susceptible and tolerant varieties not being distinguishable.

It was noted that this experiment did not exclude the involvement of other oxidising enzymes in black point formation. Furthermore in barley, black pointed tissue was found to contain significantly higher levels of phenolic acids compared to that of healthy tissue (Michalowitz et al., 2001). The series of reactions that is proposed to lead to black point formation is described in Figure 1-3.

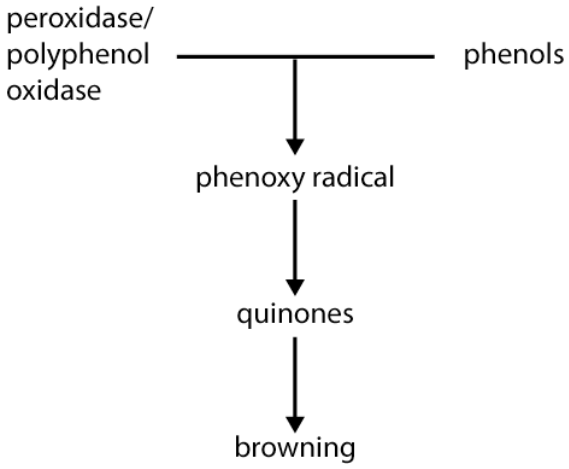

Figure 1-3 Proposed pathways involved in browning as adapted from Halliwell and Gutteridge (1989) and Pierpoint (1966). Phenolic compounds are oxidised by an enzyme such as a peroxidase or polyphenol oxidase to form the highly reactive phenoxy radical. This phenoxy radical can spontaneously form quinone. It is the quinone which can then self polymerise and also react with various amino acids and proteins resulting in discoloured end products.

While this process could result in the discolouration seen in black point-affected barley grains, it is yet to be determined precisely what events occur during black point formation *in vivo* that bring together the enzymes and substrates involved. Previous

studies have hinted at possible physiological mechanisms for how black point may arise during grain development. Cochrane (1994a) observed that with grains developing under certain environmental conditions, extension of the coleoptile occurs prior to grain maturity. The pericarp cells are not broken but the germ aleurone and testa cells are split and become very dark at the torn edge in response to wounding. This explanation is feasible as the high humidity conditions that cause black point are also known to induce pre-harvest sprouting (PHS). PHS (where the grain begins to germinate before it is harvested from the plant) may provide the wounding necessary to initiate black point.

Cochrane (1994b) also suggested that during grain filling, pericarp cells may be crushed, thus releasing phenols which may then be oxidised to form melanins. Black pointed grains have been found to be larger than healthy grains (Dimmock and Gooding, 2002). This increase in size may place increased stress on the grain, which could result in the crushing of cells, and initiate the enzymatic browning process to result in black point. Similar physiological events have been reported in pearl millet, where during normal grain development the embryo crushes the surrounding transfer cells and results in a dark discolouration visible on the outer surface of the grain (Fussell and Dwarte, 1980). It seems likely that enzymatic browning is involved in black point and is initiated by some form of wounding. Further research is required to understand what physiological events are leading to such wounding and importantly what enzymes are involved in the process.

For discolouration to occur in the embryo region of the barley grain, enzymes and substrates involved must therefore be produced either in the embryo region of the grain or transported there after being produced elsewhere. The following section discusses the production and supply of phenolic compounds, ROS, peroxidase and polyphenol

oxidase as well as their likely importance in black point development.

1.2.3.1 Phenols

Phenols are natural components of healthy grain. Ferulic and *p*-coumaric acids are the main phenolic compounds found in the cell walls of monocotyledonous plants (Regnier and Macheix, 1996). Within the barley grain, ferulic acid and *p*-coumaric acid are most concentrated in the outer tissues of the grain including the testa, pericarp, aleurone and husk tissue, with extremely low concentrations present within the endosperm tissue (Nordkvist et al., 1984). Ferulic acid acts to strengthen cell walls by cross-linking via covalent interactions between arabinoxylan chains (Holtekjolen et al., 2006). Ferulic acid and *p*-coumaric acid are produced from phenylalanine or tyrosine through a series of reactions, where the initial step is catalysed by the enzyme phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL) (Whitaker and Chang, 1996).

The supply of phenolic compounds to be oxidised may be a rate-limiting factor in black point formation and thus the activity of PAL and TAL may be influential. Interestingly PAL gene expression is up-regulated in wheat leaves exposed to cold stress (Gaudet et al., 2003) and PAL enzyme activity increases in response to aphid infestation of barley leaves (Chaman et al., 2003). It is still to be determined if PAL gene expression is up-regulated in response to environmental conditions which are known to increase black point formation. Recent experiments have identified that PAL mRNA (Genbank accession No. BQ134687) is highly expressed within the husk tissue of barley grains when compared to expression levels in the flag leaf (Abebe et al., 2004). It is unknown if this increased gene expression correlates with increased PAL

protein activity.

Comparisons between a black point susceptible and tolerant durum wheat variety has found that the susceptible variety contained considerably higher levels of ferulic acid (Regnier and Macheix, 1996). This study also observed higher levels of PAL activity and to lesser a extent TAL activity in the susceptible cultivar. Barley has also shown a 60-fold increase in ferulic and *p*-coumaric acid in the husks of black point-affected barley grain compared to healthy grain (Michalowitz et al., 2001), suggesting that PAL or TAL activity and gene expression may increase during black point formation. However, in contrast, the black point-affected tissue covering the embryo (testa and pericarp tissue) in wheat had reduced levels of ferulic and *p*-coumaric acid compared to healthy tissue. This may be due to husk tissue naturally being a rich source of phenolic compounds (Nordkvist et al., 1984). Further study is required to understand how the increased levels of phenolic compounds in black point affected tissue in barley are produced.

Phenolic compounds may play a secondary role in black point by regulating the occurrence of PHS. As highlighted in section 1.2.3 the initiation of mild levels of PHS could cause wounding that leads to enzymatic browning that results in black point. Generally phenolic compounds are thought to inhibit PHS, as is the case with vanillic acid that inhibits α-amylase activity *in vitro* and when applied exogenously to wheat grains increases dormancy (Sharma et al., 1986; Gatford et al., 2002). However, ferulic acid has been shown *in vitro* to reverse the inhibition of α-amylase by abscisic acid thus allowing germination to proceed (Sharma et al., 1986). Hence increased levels of ferulic acid within black pointed grains could lead to the initiation of PHS and then result in black point development.

Further investigation is required to determine what role individual phenolic acids have on links between PHS and black point formation and to understand the production and supply of phenolic compounds and substrates required for the enzymatic browning reaction to occur.

1.2.3.2 Generation and removal of ROS

ROS are unavoidable by-products of normal metabolic processes such as photosynthesis and respiration (Halliwell and Gutteridge, 1989). ROS include superoxide ($O_2^{\cdot -}$) and its protonated form, the perhydroxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$).

Since H_2O_2 is required for peroxidase activity, its production may be a rate-limiting factor in black point formation. H_2O_2 is produced in numerous ways by the cell. It can form spontaneously from the dismutation of $HO_2^{\cdot}/O_2^{\cdot -}$, although this reaction is relatively slow (Bielski et al., 1985). In contrast, when catalysed by the enzyme superoxide dismutase (SOD) it proceeds at an extremely rapid rate (Bielski et al., 1985). The role of SOD in the cell is to remove $HO_2^{\cdot}/O_2^{\cdot -}$ before it can react with H_2O_2 to form $OH^{\cdot}$, which is even more reactive causing damage to cellular components such as lipids, proteins, nucleic acids and carbohydrates (Halliwell and Gutteridge, 1989). There are three classes of SODs in plants which are based on the metal co-factor used: MnSOD, CuZnSOD and FeSOD (Bowler et al., 1994). Each class of SOD is located in different cellular compartments: MnSOD in the mitochondria and peroxisome; CuZnSOD in the chloroplast, peroxisome, and cytosol; and FeSOD in the chloroplast (Alscher et al., 2002). SOD may be a key enzyme involved in supplying the H_2O_2 substrate required by peroxidase enzymes for enzymatic browning to occur.

Stress is well known to increase the production of $HO_2^{\cdot}/O_2^{\cdot-}$ in plant cells. Pathogens, drought, salt, temperature, heavy metals and air pollutants all result in increased $HO_2^{\cdot}/O_2^{\cdot-}$ production from pathways such as photorespiration, the photosynthetic apparatus and from mitochondrial respiration (Mittler, 2002) as well as by enzymes located within the cell membrane such as NAD(P)H oxidase (Bolwell, 1999). $HO_2^{\cdot}/O_2^{\cdot-}$ produced within membrane-bound organelles cannot escape, as the lipid membranes are impermeable to the charged molecules (Takahashi and Asada, 1983). To prevent toxic levels of $HO_2^{\cdot}/O_2^{\cdot-}$ developing, SOD enzymes are located in all cellular compartments to catalyse $HO_2^{\cdot}/O_2^{\cdot-}$ dismutation to H_2O_2 which is then able to diffuse across lipid membranes (Bowler et al., 1994).

H_2O_2 is also produced by other mechanisms within the cell. Asthir et al., (2002) demonstrated that amine oxidases produce H_2O_2 in the chalazal cells of developing barley grains under non-stressed conditions. The H_2O_2 produced was thought to be involved in lignification of developing cell walls. Certain classes of barley germin and germin-like proteins (GLP) have also been shown to have SOD activity as well as H_2O_2 generating oxalate oxidase activity (Druka et al., 2002). Wu et al., (2000) identified a class of germins which are expressed only in the testa and pericarp tissue of the barley grain. These germins could be potentially involved in black point development by increasing the supply of H_2O_2 within these tissues to be used as a substrate in enzymatic browning reactions.

In the majority of barley varieties analysed by Hadaway (2002), SOD activity was higher in black pointed compared to non-black pointed barley grains of the same variety. In a separate study, no conclusive correlation between black point susceptibility and SOD gene expression during grain development was observed (March, 2003).

However one of the four FeSOD transcripts identified by northern analysis was expressed at higher levels in susceptible varieties during later stages of grain development. MnSOD was expressed at high levels in all varieties during grain development, whereas CuZnSOD expression was down regulated toward the end of development. It could not be determined from the two studies described above if the enzyme activity and gene expression observed was present in the tissue implicated in black point formation. However, if black point is associated with increased stress caused either by environmental conditions or wounding it would be expected that increased levels of SOD activity would be observed in the embryo region to combat this.

If H_2O_2 is involved in black point formation its removal may be an important mechanism in preventing black point development in tolerant varieties. Enzymes such as ascorbate oxidase, catalase and glutathione peroxidase are able to scavenge H_2O_2 (Mittler, 2002). Increased activity of these scavenging systems may be a tolerance mechanism employed by tolerant varieties.

Recently, a differential screening experiment has identified cDNA clones representing genes for GLP, catalase, and glutathione peroxidase which are highly expressed in the husk tissue of barley grain which suggest they could be involved in black point development (Abebe et al., 2004). Further research determining the activity and expression of each of these enzymes in relation to black point formation should divulge which ones, if any, are involved in symptom formation. Also determining the redox status of the grain during black point development should greatly assist in determining what particular ROS pathways are involved in providing H_2O_2 as a substrate for enzymatic browning to occur.

1.2.3.3 Peroxidases

Peroxidases are heme-containing enzymes that require H_2O_2 as an electron acceptor to oxidise a number of substrates, which include phenolic compounds (Yoshida et al., 2003). Since black point forms in the husk tissue and the germ aleurone tissue of the barley grain it seems logical that peroxidase enzymes would need to either be located in these tissues or be transported from an adjacent tissue.

Previous studies have identified peroxidase activity as well as specific isozymes in different tissue of the grain. However results presented by different research groups are often conflicting. This seems largely due to two factors: the phenolic hydrogen donor used to detect the peroxidase activity and the apparent varietal variation of peroxidase isozymes present in the grain.

Gel electrophoresis studies failed to detect significant levels of peroxidase isozymes in the husk, embryo, aleurone, testa/pericarp or scutellum tissue of barley kernels from the varieties Bonanza and Centennial harvested at 28 days post anthesis (dpa) (Laberge, 1975). Peroxidase activity was detected using 3-amino-9-carbazole as the hydrogen donor. However when *o*-dianisidine was used to detect activity, a significant level of a single isozyme was detected in the husk tissue from Centinnial alone. Following this, total peroxidase activity during grain development using *o*-dianisidine as the hydrogen donor was examined (LaBerge and Kruger, 1976). It was found that after 28 dpa, peroxidase activity in the husk and testa/pericarp tissue reduced to insignificant levels in both varieties. Activity in the embryo and scutellum remained extremely low late in development while activity in the aleurone significantly increased between 30 dpa and maturity.

Using 3-amino-9-carbazole as the hydrogen donor to identify peroxidase isozymes in mature grain of the barley cultivar Chariot, it was observed that there was no peroxidase isozymes present in husk tissue of this variety (Antrobus and Large, 1997). However, in contrast to LaBerge's (1975) findings, several isozymes and significant levels of activity were detected in the embryo and aleurone tissues. Whether this contradictory evidence is a result of cultivar differences, the development stage of the grain or experimental procedure is not known. The findings above suggest individual peroxidase enzymes are more substrate specific than once thought. If peroxidases are involved in black point formation then it is possibly a combination of a specific isozyme and phenolic compound that results in symptom development.

Interestingly, when black point symptoms were reproduced *in vitro* in wheat, symptom severity changed with different phenolic solutions (Williamson, 1997). When L-tyrosine was used only the tissue covering the embryo was discoloured, however, when replaced with catechol the entire embryo end and crease tissue discoloured. This suggests that different peroxidase isozymes are involved in developing symptoms within the different tissue types; at least in wheat.

Peroxidase isozymes present in mature whole grain samples from 16 barley varieties with ranging susceptibility to black point have been compared (Hadaway et al., 2003). Using guaiacol as the hydrogen donor to detect peroxidase activity it was generally observed (with the exception of the variety Keel) that susceptible varieties contained high pI isozymes that were absent in tolerant varieties. It was also observed that there was no difference between the peroxidase isozymes present between healthy and black point affected grain from the same variety. This indicates that peroxidase isozymes are not up-regulated in relation to black point development but does suggest certain

isozymes may be involved in black point susceptibility.

Several peroxidase genes have been identified in barley. Barley grain peroxidase 1 (*BP1*) is a major grain specific peroxidase that is predominately expressed in the endosperm tissue (Rasmussen et al., 1991). *Prx7* and *Prx8* have been shown in leaf tissue to be associated with abiotic stress (Kristensen et al., 1999). In relation to black point, *BP1* and *Prx7* were found to be differentially expressed between four varieties differing in black point susceptibility (March, 2003). *BP1* was expressed for longer during grain development in susceptible varieties, while *Prx7* was expressed at a consistently higher level in susceptible varieties towards the end of grain development. The expression of *Prx8* increased in all varieties towards the end of grain development regardless of susceptibility.

Whilst it appears that peroxidases are likely to be involved in black point formation based on the studies described above, further investigation is required to determine what tissue within the barley grain the enzymes in question originate from. It has also become apparent that other biochemical pathways that supply H_2O_2 and phenolic compounds are most likely required to trigger the peroxidase activity that results in black point development. In further research identifying peroxidases linked to black point it may also be useful to use detection techniques that are not influenced by substrate specificity. This would help to overcome some of the discrepancies described above.

1.2.3.4 Polyphenol oxidases

Polyphenol oxidases are well known to cause enzymatic browning in many fruits and vegetables and related enzymes exist in most plant material and fungi (Kruger et

al., 1987). There are three classes which are based on the enzyme's substrate specificity: tyrosinase, catechol oxidase and laccase which oxidise mono-phenols, *o*-diphenols and *p*-diphenols respectively. Oxygen is also required by the enzymes as a co-substrate to complete the oxidation reaction (Whitaker and Chang, 1996). The oxidised phenols form quinone, which is involved in the formation of brown end products.

It appears unlikely that catechol oxidases are directly involved in black point formation in barley as the two main phenolic substrates in the grain, ferulic acid and *p*-coumaric acid, are both inhibitors of this enzyme's activity (Whitaker and Chang, 1996). Furthermore, when whole-wheat grains are stained for PPO activity, the entire grain darkens (*personal communication,* Dr Daryl Mares, The University of Adelaide, 2003), not just the area where black point forms. This further suggests that PPO is not involved in the specific discolouration of the embryo region seen on black pointed grains. In contrast to this peroxidase activity has been shown to be concentrated to the embryo region and thus more likely to be involved in producing black point than PPO (Williamson, 1997). In addition, PPO has been associated with discoloration of the entire barley grain in susceptible varieties (*personal communication,* Christina Grime, University of Western Australia, Western Australia, September 2005).

PPO may however play a secondary role in black point formation. PPOs and peroxidases might act synergistically during enzymatic browning, because PPO may promote peroxidase activity by generating H_2O_2 from the oxidation of certain phenolic compounds (Richard-Forget and Gaulliard, 1997).

1.2.4 Determining which enzymes and/or genes are involved in black point formation

Based on studies described within this literature review, black point formation seems most likely to arise from an enzymatic browning reaction catalysed by peroxidases. However the studies on peroxidase involvement have highlighted that other biochemical pathways that control the supply of H_2O_2 and phenolic compounds are also likely to be involved. This suggests a broad range of genes may be contributing to black point formation as well as the level of susceptibility of an individual variety. Due to this, studies on the genetic and biochemical mechanism of black point lend themselves to broad-based research methodologies such as proteomics and map-based cloning approaches. Such techniques would allow identification of enzymes and/or genes involved in black point formation.

1.2.4.1 Direct identification using proteomics

Proteomics allows the analysis of proteins present in a tissue sample and the ability to compare between individual samples. The technique of two-dimensional gel electrophoresis is now commonly used to compare the proteomes of plant tissue (Jorrin et al., 2007). In wheat this technique has recently been used to compare the proteome of tissue from black pointed and healthy grain (Mak et al., 2006). This study identified many proteins that differed between healthy and black pointed samples, the most notable observation was that black pointed grains had a lower abundance of stress related proteins. From this the authors noted that black point might result from the grains inability to protect itself from stress. In support of the enzymatic browning model, this study also identified a partial sequence of a peroxidase-like protein that was

more abundant in black pointed grains, but this protein was not characterised further.

To date a similar study has not been completed in barley. Since there are differences in morphology between wheat and barley grains such a study is needed, as different proteins may be associated with black point between the two species.

1.2.4.2 Indirect identification through mapping of black point related genes

Within the literature there are reports of quantitative trait loci (QTL) being identified for both black point and kernel discolouration in barley. However, it is often not clearly stated if the two traits are the same. Black point refers to a darkening of the embryo end of the grain and is measured by visual assessment of a sample of grains (Sulman et al., 2001; Hadaway, 2002) while kernel discolouration (also referred to as weather damage) usually refers to melanization of the whole grain and can be measured using electronic colour detection systems (Li et al., 2003). However in a mapping study conducted by de la Penna et al., (1999) the two traits were both described as being a discolouration of the embryo region. Due to discrepancies in classification of these two traits, care needs to be taken when interpreting published research, as black point and kernel discoloration are not necessarily physiologically or biochemically related.

Within the double haploid mapping population Alexis x Sloop, a single putative QTL associated with tolerance to discolouration of the embryo end of the grain was identified on chromosome 2H (Hadaway, 2002). Li et al., (2003) identified QTL associated with total kernel discolouration tolerance within seven different mapping populations. These were located on chromosomes 2H, 3H, 4H, 5H, 6H and 7H. One of the QTL located on 2H was consistently detected in six of the seven populations.

In a study conducted in North America the mapping population Chevron x M69 was phenotyped by visually assessing the discolouration of the embryo end of the grain (de la Pena et al., 1999). In total, eleven QTL were identified for tolerance to discolouration on all chromosomes except 1H. Symptoms developed in this study were reported to be due to the inoculation of the barley heads with either *F. graminearum* or *B. sorokinana* compared to the Australian studies that were not artificially inoculated with fungi.

Mapping studies in wheat using double haploid populations derived from Sunco x Tasman and Cascades x AUS1408 identified QTL on chromosomes 2B and 2D respectively (Lehmensiek et al., 2004). The group 2 chromosomes in wheat are largely homologous with chromosome 2H in barley (Devos et al., 1993) and therefore similar genes may be controlling the two QTL. Further research would be required to determine if this is the case.

It appears that chromosome 2H is influential in black point formation, as it has been linked in all studies. Validation and alignment of these identified QTL is required to determine if the same genome regions are detected in all studies. Further research is then required to determine what genes underlie and control the identified QTL.

Identifying gene(s) underlying QTL is now commonly achieved by exploiting the synteny that exists between barley and the fully sequenced rice genome. By identifying syntenous regions in the rice genome, rice genes can be identified from sequence databases and then their presence in barley can be confirmed using gene mapping techniques (Varshney et al., 2006). This has been demonstrated recently in barley by using synteny with rice to characterise the genomic region that controls boron tolerance (Sutton et al., 2007). The results of this study has now enabled diagnostic marker

assisted selection to be used in breeding programs to identify lines that are boron tolerant instead of using resource intensive phenotypic screening methods. Using a similar approach to identify individual genes involved in black point, may also enable the implementation of diagnostic gene based markers in breeding programs to breed for black point tolerance.

1.3 Breeding barley cultivars which are resistant to black point

The evaluation of several Australian and international barley cultivars has identified that there is a wide range of susceptibility to black point (Sulman et al., 2001; Hadaway et al., 2003). Varieties range from the susceptible including Schooner, Keel and Sloop to more tolerant varieties including Alexis, Mundah and Harrington.

Black point tolerance has been found to segregate within mapping populations. This has been observed within the Arapiles x Franklin and Sloop x Alexis populations within Australia (Hadaway, 2002) and within the North American derived population Chevron x M69 (de la Pena et al., 1999). Establishing that black point formation is genetically controlled therefore indicates that it should be possible to breed for tolerance.

Currently, phenotypic assessment is the only method available to screen for black point susceptibility in breeding programs. High humidity growth tunnels have been developed and have been found to produce repeatable black point levels (Sulman et al., 2001). However, logistically it is not possible to screen the large number of lines present in the initial stages of a breeding program using such a system. This is due to both the unrealistic size of the tunnel that would be needed, and the time required to phenotype each individual line.

Because of the limitation imposed by phenotypic screening the need has arisen to

determine the genetic mechanism of black point formation. Knowledge of what genes are involved will allow the development of diagnostic markers that can be used to accurately screen lines within breeding programs. This approach will also eliminate the environmental influence, which has previously hindered phenotypic screening methods in breeding programs.

1.4 Conclusion and objectives

Black point results in substantial losses each year to the Australian grain industry through downgrading from malting quality to feed grade. Currently the prominent barley varieties grown within Australia are considered susceptible to black point. The need to breed increased tolerance into new varieties is therefore a pressing concern for the industry. Conventional breeding methods using phenotypic selection have shown limited success due to the large environmental influence on symptom formation. Because of this, research is now focussed on determining the genetic mechanism of black point so that marker-assisted selection can be implemented in breeding programs.

Research thus far has strongly indicated that black point arises from an enzymatic browning reaction within the grain. It has also been highlighted throughout this literature review that several biochemical pathways and individual enzymes may be implicated in this reaction.

Research to determine the genetic basis of black point has thus far focused on mapping QTL associated with the trait. To date, several QTL have been identified throughout the barley genome. All studies consistently detect QTL on chromosome 2H which indicates this region plays an important role in black point. For this QTL to be of more use to breeding programs the genes underlying it need to be identified so that

diagnostic gene-based markers can be developed.

This research project aimed to build on and complement the current research in determining the genetic basis of black point formation by firstly using a proteomics approach to identify individual proteins associated with black point development and secondly, exploit the synteny between barley and rice to identify genes underlying the black point associated QTL on chromosome 2H in barley. Identified proteins were then characterised in an attempt to determine what role they might play in black point while the QTL on 2H was further refined through fine-mapping.

2 Proteomic analysis of black pointed and healthy grain

2.1 Introduction

Research thus far has indicated that black point is the result of enzymatic oxidation of phenolic compounds within the grain as opposed to discolouration caused by fungal colonisation (Williamson, 1997). Several different classes of enzymes involved in the production and oxidisation of phenolic compounds are likely to be involved in black point development (refer to Chapter 1.2.3). Within barley, individual proteins associated with black point development have not yet been identified and as such this formed the major aim of the research presented in this chapter.

There are possibly many biochemical pathways involved in the discolouration reaction that result in black point. A broad-based experimental approach that is not reliant on a starting hypothesis would therefore be advantageous to identify proteins associated with black point. In recent years the method of 2-D electrophoresis has developed rapidly and has now been utilised to analyse the proteome from a large range of organisms (including barley) and tissues (Jorrin et al., 2007). The technique of 2-D gel electrophoresis involves two steps. In the first dimension protein samples are separated based on their charge (or isoelectric point) using an immobilised pH gradient (IPG) gel. In the second dimension, proteins are separated based on their molecular weight after denaturation using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

The barley grain proteome has been well characterised using 2-D gel

electrophoresis. Using this method in excess of 1000 barley grain proteins have been separated in a single experiment (Finnie et al., 2002). Changes in the barley grain proteome have subsequently been analysed throughout grain development and into germination (Finnie et al., 2002; Bak-Jensen et al., 2004; Østergaard et al., 2004). Recently 2-D gel electrophoresis was used to compare the proteome of healthy and black pointed wheat grains (Mak et al., 2006). This study analysed both the embryo and covering germ aleurone layer as well as the remaining endosperm and aleurone layer. This study found that there was a reduction in several stress-related proteins in black pointed grains compared to healthy grains. A similar study in barley would be of interest as in barley the husk tissue (which the majority of wheat varieties do not contain) is also discoloured. To date no such study comparing the proteome of healthy and black pointed barley grains has been reported.

Black pointed barley grains are not acceptable to the barley malting industry. This is largely due to the perception that black point results in reduced malting quality and may lead to microbial contamination within the brewery. However studies thus far indicate that black point has little or no effect on grain quality (Rees et al., 1984; Basson et al., 1990; Hadaway et al., 2005). Furthermore in wheat, the presence of fungal proteins within black pointed grains were not observed (Mak et al., 2006). The comparison of proteomes from healthy and black pointed barley grains would add further scientific knowledge to assist in managing this industry issue.

This study therefore aimed to examine the proteome of black pointed and healthy grain of barley. In particular, the husk proteome was examined because of the

unique symptoms of discoloration of this tissue in barley. The whole grain proteome was also examined to understand what changes occur in relation to black point development.

2.2 Materials and methods

2.2.1 Barley tissue samples

Barley grains used to optimise protein extraction and electrophoresis techniques were from *H. vulgare* (barley) plants (cultivar Sloop) grown in a glasshouse under natural light at the Waite Campus, Adelaide, South Australia. For comparison between black pointed and healthy grain barley was field grown during the 2004 season near Millicent, South Australia (latitude 37° 49′S longitude 140° 27′E) and mechanically harvested at maturity. Based on visual assessment, mature seeds were categorised into black pointed grain and clean grain samples. The cultivar Sloop is a mid- to early-maturing variety that is considered susceptible to black point.

2.2.2 Husk protein extraction

Using a scalpel the discoloured husk tissue from the embryo region of black pointed grain was removed and the corresponding tissue also removed from healthy grains. Collected husk tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. Ground tissue was stored at -80 ^{o}C prior to protein extraction using one of the methods described hereafter. All steps hereafter were performed at 4 ^{o}C unless otherwise stated.

To extract water-soluble proteins ground husk tissue (0.5 g) was resuspended in 2.5 mL of extraction buffer (5 mM Tris-HCl pH 8, 1 mM $CaCl_2$) in a 10 mL tube

and shaken on an orbital shaker (45 rpm) for 1 h. The supernatant was collected by centrifugation at 4000 g for 5 min. Proteins were precipitated by the addition of 4 volumes of acetone and placed at -20^{o}C for 5 h then centrifuged at 4000 *g* for 5 min to pellet the protein.

To extract ionically-bound cell wall proteins several methods were trialled. Ground husk material (0.5 g) was resuspended in 1 M KCl and shaken (45 rpm) for 1 h followed by centrifugation at 4000 *g* for 5 min. The supernatant was removed and proteins were sequentially re-extracted as described above from the husk tissue pellet using 1 M NaCl, 0.5 M $CaCl_2$, 50 mM ethylenediamine tetraacetic acid (EDTA), and 2 M LiCl. Proteins were precipitated from all collected supernatants by adding a 20% trichloroacetic acid (TCA) solution to achieve a final concentration of 15% and then placed on ice for 30 min. Proteins were collected by centrifugation at 12000 *g* for 10 min. Protein pellets were washed twice in 2 mL of cold 100% acetone and allowed to air dry.

Covalently-bound cell wall proteins were extracted by enzymatic degradation of the cell wall. Ground husk tissue (0.5 g) was resuspended in 1 mL of a cell wall degradation extraction buffer consisting of 50 mM sodium acetate pH 5, 2.5 % w/v pectinase (Sigma-Aldrich, Saint Louis, MO, USA), 0.65 % w/v cellulase (Sigma-Aldrich) and 5 % v/v protease inhibitor (Sigma-Aldrich, P-9599). Samples were shaken on an orbital shaker at 45 rpm for 12 h at 25^{o}C. Proteins were then precipitated with TCA as described above and collected by centrifugation at 12000 *g* for 10 min.

2.2.3 1-D gel electrophoresis of husk proteins

To compare the amount and quality of protein extracted from the different protein extraction methods 1-D gel electrophoresis was used. Protein pellets were resuspended in 1x lithium dodecyl sulfate (LDS) sample loading buffer (Invitrogen, Carlsbad, CA, USA) containing 50 mM dithiothreitol (DTT) and heated at 70^{o}C for 10 min then centrifuged at 12000 *g* for 5 min prior to gel loading to remove debris from the samples. To enable comparisons between protein extraction techniques, gel loadings were based on the initial husk tissue weight used for the extraction, rather than loading equal protein quantities. This enabled the protein extraction method which yielded the highest amount of proteins to be determined. Samples were electrophoresed on NuPAGE® 4-12% Bis-Tris gels with 4-morpholineethanesulfonic acid (MES) buffer using a XCell SureLock™ Mini cell gel tank (Invitrogen). Gels were electrophoresed at a constant 200 V for 35 min. Gels were then fixed for 15 min in 50 mL of gel fixative (30% ethanol, 10% acetic acid, 60% water). Proteins were visualised using a colloidal Coomassie brilliant blue (CBB) stain which was prepared by adding 15 mL methanol containing 0.005 g Coomassie G250 (Sigma-Aldrich) to 8.5 g ammonium sulphate dissolved in 29 mL water, to which 2 mL of phosphoric acid was added whilst stirring with a magnetic stirring bar. Gels were stained for 14 to 20 h with gentle shaking then washed in water for 30 min. Images were scanned and then cropped using Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA).

2.2.4 2-D gel electrophoresis of husk proteins

To separate and analyse individual husk proteins, 2-D gel electrophoresis was

used. Protein pellets were resuspended in rehydration buffer (8 M urea, 2% CHAPS, 50mM DTT, 0.2% ampholyte pH 3-10) for 3 h with shaking on an orbital shaker (45 rpm) then centrifuged for 10 min at 12000 *g* at room temperature. Protein concentration was determined using the *RC DC™* assay following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA) and using bovine serum albumin (BSA) as a standard. Using the program GenStat (6th Edition, Rothamsted Experimental Station, UK) a standard curve was generated based on the absorbance of the BSA standards. From this the concentration of the protein samples was calculated using the prediction function.

Protein samples (50 µg) were made up to a final volume of 125 µL with rehydration buffer. Protein samples were used to rehydrate 7 cm (IPG) dry strips pH 3-10 and then focussed using an Ettan™ IPGphor™ II IEF System as per the manufacturer's recommendations (GE Healthcare, Uppsala, Sweden). Focussing was performed at 20^{o}C for a total of 8164 Vh consisting of the following steps: 6 h of passive rehydration (0 V), 6 h of active rehydration (0 V), 300 V for 2 h, 30 min gradient to 1000 V, 80 min gradient to 5000 V then 5000 V for 3000 Vh. The second dimension was performed using NuPAGE® 4-12 % Bis-Tris ZOOM gels with MES buffer and an XCell SureLock™ Mini cell gel tank (Invitrogen). Gels were electrophoresed at a constant 200 V for 40 min. Proteins were visualised using colloidal CBB and scanned as described in section 2.2.3.

2.2.5 Amino acid composition of husk tissue

To determine the amino acid composition, acid hydrolysis of the husk tissue was performed essentially as described previously (Barkholt and Jensen, 1989). Husk

tissue was collected from the embryo end of the healthy grains (5 µg) and placed into a glass screw cap vial containing 0.1 mL hydrolysis solution (6M HCl, 0.05% phenol, and 5 µL of a 1% dithiodipropionic acid in 0.2 M NaOH) and incubated in an oven at 110°C for 18 h. Samples were then dried prior to being analysed using high performance liquid chromatography (HPLC).

HPLC analysis of hydrolysed husk proteins and amino acid standards was performed essentially as described previously (Ou et al., 1996). Firstly, Fmoc (9-fluorenylmethyl chloroformate) derivatisation of amino acids was performed as described previously (Haynes et al., 1991), prior to injection onto the chromatography column. A 10 µL volume of amino acid standard (500 pmol per amino acid in 250 mM borate buffer, pH 8.8) was mixed with 10 µL of Fmoc (4 mg mL^{-1}, w/v, in acetonitrile) and allowed to stand for 90 s. A 10 µL volume of cleavage reagent (made from a mixture of 340 µL of 0.85 M sodium hydroxide, 150 ml of 0.5 M hydroxylamine hydrochloride and 10 µL of 2- (methylthio) ethanol) was then added, the solution mixed, and allowed to stand for 3.5 min. The reaction was stopped by adding 10 mL of quenching reagent (20% glacial acetic acid, v/v, in acetonitrile). The mixing steps were conducted by the injection of 30 mL of air through the solution, and the entire derivatisation was carried out at room temperature. Seperation and detection of the derivatised amino acids was performed using reverse phase HPLC (Hewlett-Packard 1090 LC, Rockville, IL, USA). Amino acids were expressed as % mole of total amino acids present within the sample.

2.2.6 Whole grain protein extraction

Seed proteins were extracted as described previously (Finnie et al., 2002) with all steps carried out at 4°C unless otherwise stated. Barley seeds (4 g) were ground to flour with liquid nitrogen using a mortar and pestle. To isolate the proteins, the flour was shaken for 30 min at 100 rpm on a flat bed shaker in a solution containing 5 mM Tris HCl (pH 7.5) and 1mM $CaCl_2$ (20 mL). Insoluble materials were pelleted by centrifugation (4000 *g* for 30 min). The supernatant was then collected and stored in 2 mL aliquots at -20°C until required.

Protein aliquots (2 mL) were precipitated overnight at -20°C with four volumes of acetone and then collected by centrifugation at 10000 *g* for 5 min. Proteins were redissolved in a rehydration buffer (8M urea, 2% CHAPS, 0.5% v/v IPG buffer pH 3-10, 20mM DTT) with shaking on an orbital shaker (45 rpm) for 3 h. The protein concentration of the samples was determined as described in section 2.2.4.

2.2.7 2-D gel electrophoresis of whole grain proteins

Protein samples (500 µg) were made up to a final volume of 340 µL with rehydration buffer (8M urea, 2% CHAPS, 0.5% v/v IPG buffer pH 3-10, 20mM DTT) and used to rehydrate 18 cm IPG dry strips pH 3-10 which were then focussed using an Ettan™ IPGphor™ II IEF System as per the manufacturer's recommendations (GE Healthcare). Focussing was performed for a total of 42000 Vh at 20°C and consisted of the following steps: 6 h of passive rehydration (0 V), 6 h of active rehydration (30 V), followed by 2 h at 300 V, a gradient to 1000 V over 6 h, and then a gradient to 8000 V over 3 h, and finally 8000 V for 24000 Vh. IPG strips were then equilibrated in equilibration buffer (6 M urea, 2% SDS, 20%

glycerol and 50 mM Tris HCl, pH 8.8) containing 2% w/v DTT, for 20 min followed by equilibration buffer containing 2.5% iodoacetamide for a further 20 min. The second dimension was performed using a PROTEAN® II xi 2-D Cell (Bio-Rad) and hand-poured 10% Bis-Tris gels (pH 6.4) with MES running buffer (Invitrogen) as described previously (Graham et al., 2005). Gels were run at a constant of 200 V for 5.5 h. Proteins were stained as described in section 2.2.3. Two analytical replicates of three independent biological extracts were produced for both black pointed and healthy barley grain for image analysis.

2.2.8 Image analysis

Gels were scanned using an ImageScanner (GE Healthcare) operated by the software, Lab-Scan 3.00 (GE Healthcare). Intensity calibration was carried out using an intensity stepwedge prior to gel image capture. Image analysis was performed using the ImageMaster 2D Elite 4.01 software (GE Healthcare). Gels were compared manually as well as using the spot detection mode. Spots were matched, then the background was subtracted using the average on boundary method and spot normalisation performed using the total spot volume method. Standard error was calculated for the normalised values and a paired student *t*-test performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Spots showing greater than two fold difference with $P < 0.05$ were excised from the gel for mass spectrometry analysis.

2.2.9 Mass spectrometry (MS) of identified proteins

Protein identification was performed by the Adelaide Proteomics Centre (Adelaide, SA, Australia) using MS/MS analysis. Individual bands were excised,

destained and subjected to in-gel tryptic digestion essentially as described previously (Speicher et al., 2000).

Extracted tryptic peptides were desalted on a 1 × 5 mm 5 µm C18 pre-column (LC-Packings) before separation on a 0.075 × 100 mm 3 µm Atlantis dC18 NanoEase analytical column (Waters-Milford) with an acetonitrile gradient (5-70% v/v over 45 min containing 0.1% formic acid w/v) at a flow rate of 200 nL min^{-1} using a CapLC system (Waters-Milford). The eluted peptides were introduced into a Micromass Q-TOF2 mass spectrometer (Waters-Milford) through a NanoSpray source and ionised with 3 kV. A survey scan of the eluting material identified multiple charged species (+2, +3 and +4 charge states) and the most abundant were selected for fragmentation and MS/MS analysis. Once sufficient signal had been accumulated for each of the daughter-ion series, the system reverted to the survey scan mode to identify further multiple charged species of potential interest.

Following acquisition, MS/MS data were processed and analysed using ProteinLynx 2.0 software (Waters-Milford). *De-novo* sequence tags were determined from the fragmentation data and used to search the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) non-redundant protein database (October 2006 release) for potential protein identification matches using the program BLASTp. Where there was no protein match a tBLASTn was used to search against the translated DNA database (http://www.ncbi.nlm.nih.gov/). To determine theoretical Mw and pI values the compute pI/Mw tool was used (http://www.expasy.org/tools/pi_tool.html).

2.3 Results

2.3.1 Husk protein analysis

Initially in this study water-soluble proteins were extracted from the husk tissue. Separation of these proteins using 2-D gel electrophoresis resulted in the visualisation of two main protein spots (Figure 2-1). The main proteins identified had a mass of 70 kDa and 15 kDa respectively and a common pI of 4.5.

To extract proteins that were ionically-bound to the husk tissue cell walls, several different salt containing extraction methods were trialled. This approach required optimisation as it was found that salt was not soluble in acetone and thus was precipitated along with the proteins. The resulting high salt concentrations in the protein samples caused high currents and poor protein separation during electrophoresis. To overcome this 15% TCA was used to precipitate the proteins. However it was observed that small amounts of residual salt often remained in some samples.

Extraction of proteins with 1 M KCl resulted in greater protein yield from the same amount of husk tissue than that obtained using a water-soluble protein extraction buffer (Figure 2-2). There was a range of proteins extracted using 1 M KCl as indicated by a continuous smear throughout the lane. Two proteins of 25 kDa and 29 kDa were present in larger quantities compared to the other extracted proteins. Re-extraction of the same tissue with different extraction buffers (1 M NaCl, 0.5 M $CaCl_2$, 50 mM EDTA and 2 M LiCl) resulted in no additional proteins being extracted (Figure 2-2).

In an attempt to extract further proteins covalently bound to the cell wall, the husk tissue was degraded with the cell wall hydrolytic enzymes cellulase and pectinase. This protein extraction method resulted in the visualisation of a considerably larger quantity of protein (Figure 2-2). Comparisons made with a negative control lane consisting of just the extraction buffer showed that the proteins visualised in all lanes were products of cellulase and pectinase and therefore no husk proteins could be visualised using this method.

Since 1 M KCl was found to extract the most protein from the husk tissue, this protein extract was further analysed using 2-D gel electrophoresis to identify individual protein spots. Initial attempts to use the 1 M KCl extracts for IEF resulted in burning of the IPG strips due to high currents generated from residual salt as mentioned previously. The problem was improved significantly by adding an initial focussing step that consisted of 300 V for 2 h.

Separation of 1 M KCl extracted husk proteins using 2-D gel electrophoresis resulted in the detection of approximately 100 individual protein spots (Figure 2-3). These protein spots stained very faintly with coomassie indicating their low abundance. From the initial husk tissue using 1 M KCl it was calculated based on a Bradford assay that the extraction yield was 0.03% protein extracted / husk tissue (w/w). The low protein yield had two implications. Firstly due to limited tissue, large format gels that need larger quantities of protein but give superior separation could not be used. Secondly, the low protein yield did not allow for adequate gel replication to achieve statistically significant comparisons between gels. Further hampering this approach was the observation that samples were often affected by residual salt from the extraction buffer resulting in smearing of gels (Figure 2-3)

such that they could not be used for comparisons. Comparisons were made between black pointed and healthy husk tissue and differences were observed (Figure 2-3). Clear differences in protein abundance were visually observed for at least 4 protein spots. Reproducibility of these differences was affected by salt contaminated samples. The lack of black pointed husk tissue meant that these differences could not be statistically verified and therefore were not analysed further.

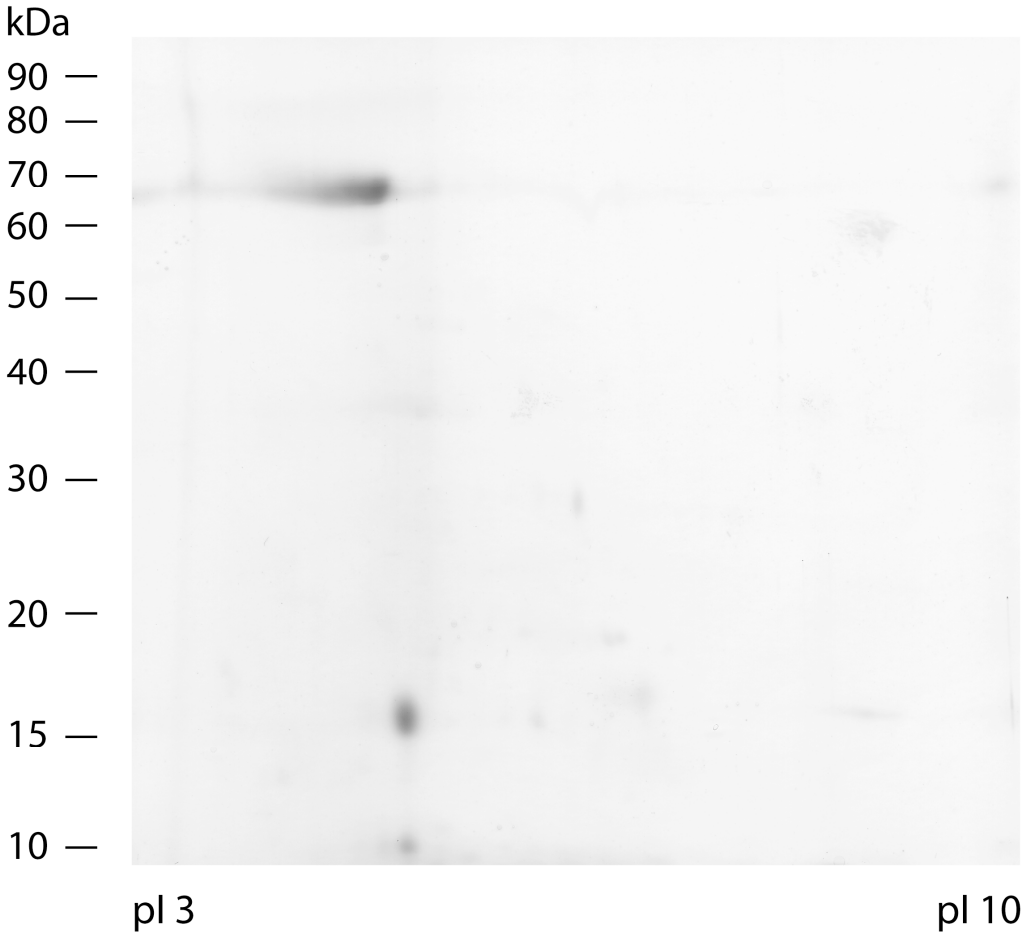

Figure 2-1 Representative 2-D gel electrophoresis of water-soluble husk proteins from healthy barley grain (n=3).

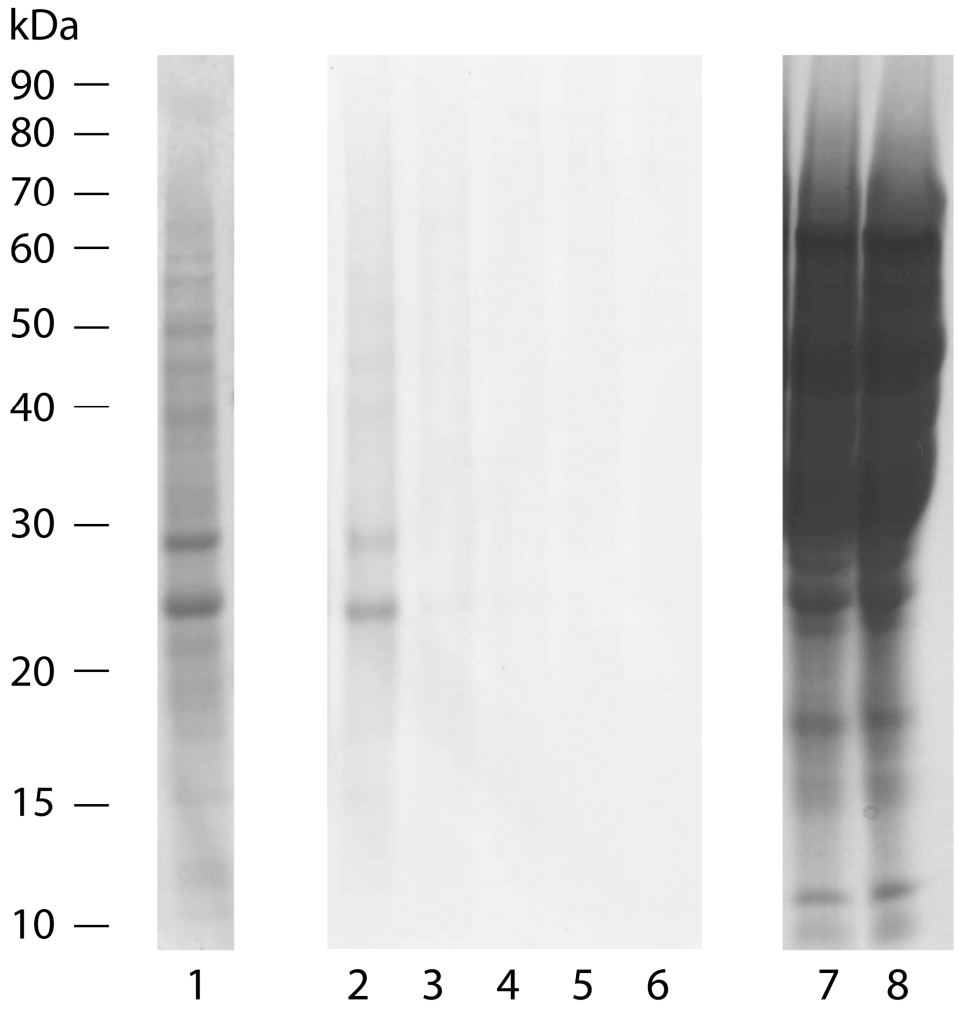

Figure 2-2 Extraction of cell wall proteins from barley husk proteins using different techniques. 1 M KCl was found to extract a range of proteins from husk tissue (lane 1). Sequential extraction with a range of salt extraction buffers did not extract any further proteins from husk tissue: 1 M KCl (lane 2), 1 M NaCl (lane 3), 0.5 M $CaCl_2$ (lane 4), 50 mM EDTA (lane 5), and 2 M LiCl (lane 6). Enzymatic degradation of husk tissue with cellulase and pectinase resulted in a high protein yield (lane 7) but a negative husk control (lane 8) showed the proteins present in lane 7 were products of cellulase and pectinase. Figure is representative of two replicates (n=2).

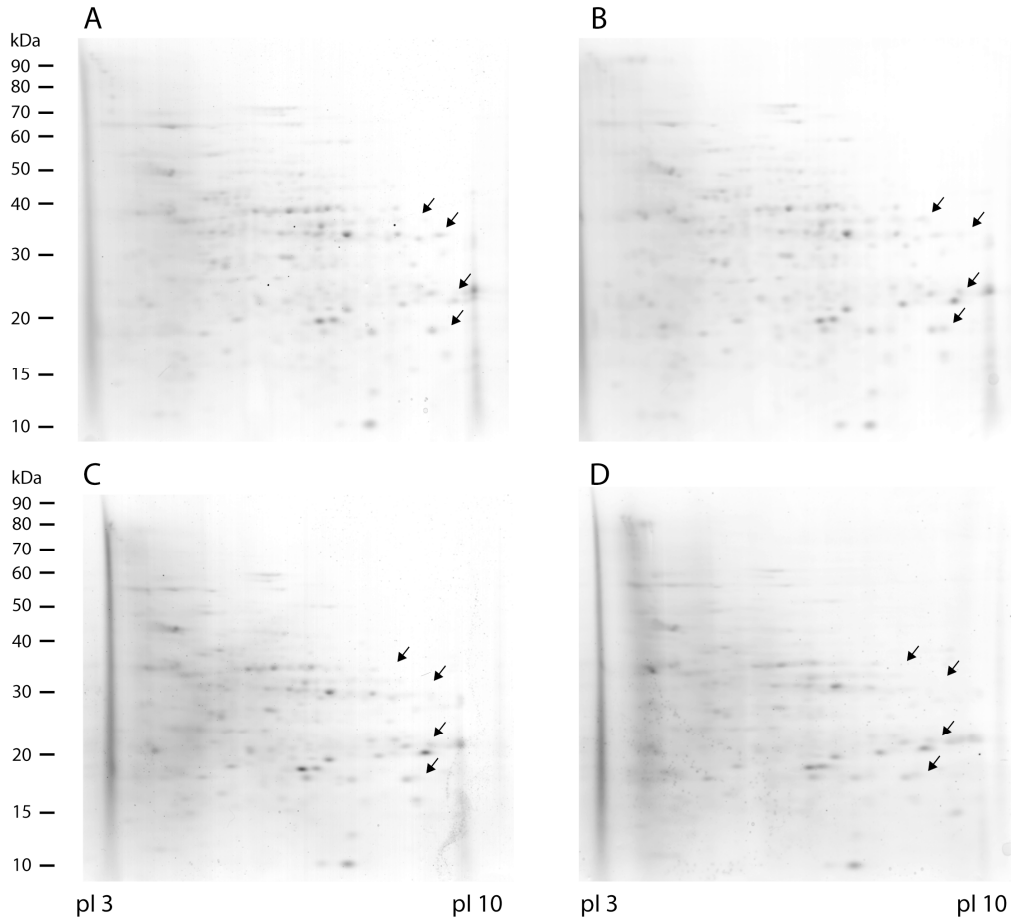

Figure 2-3 2-D gel electrophoresis of 1 M KCl extracted proteins from the husk of healthy and black pointed proteins. Comparisons were made between healthy (A) and black pointed (B) samples and protein spot differences were observed as shown with arrows. Reproducibility issues often arose in subsequent healthy (C) and black point (D) comparisons as residual salt contamination caused horizontal streaking (D).

2.3.2 Husk amino acid composition

Total amino acid composition and quantity was determined using acid hydrolysis of the husk tissue and analysed using HPLC. The most abundant amino acids present were proline, alanine and glycine while histidine and methionine were present at low levels (Figure 2-4). The total amino acid content of the husk tissue was calculated to be 0.7% w/w.

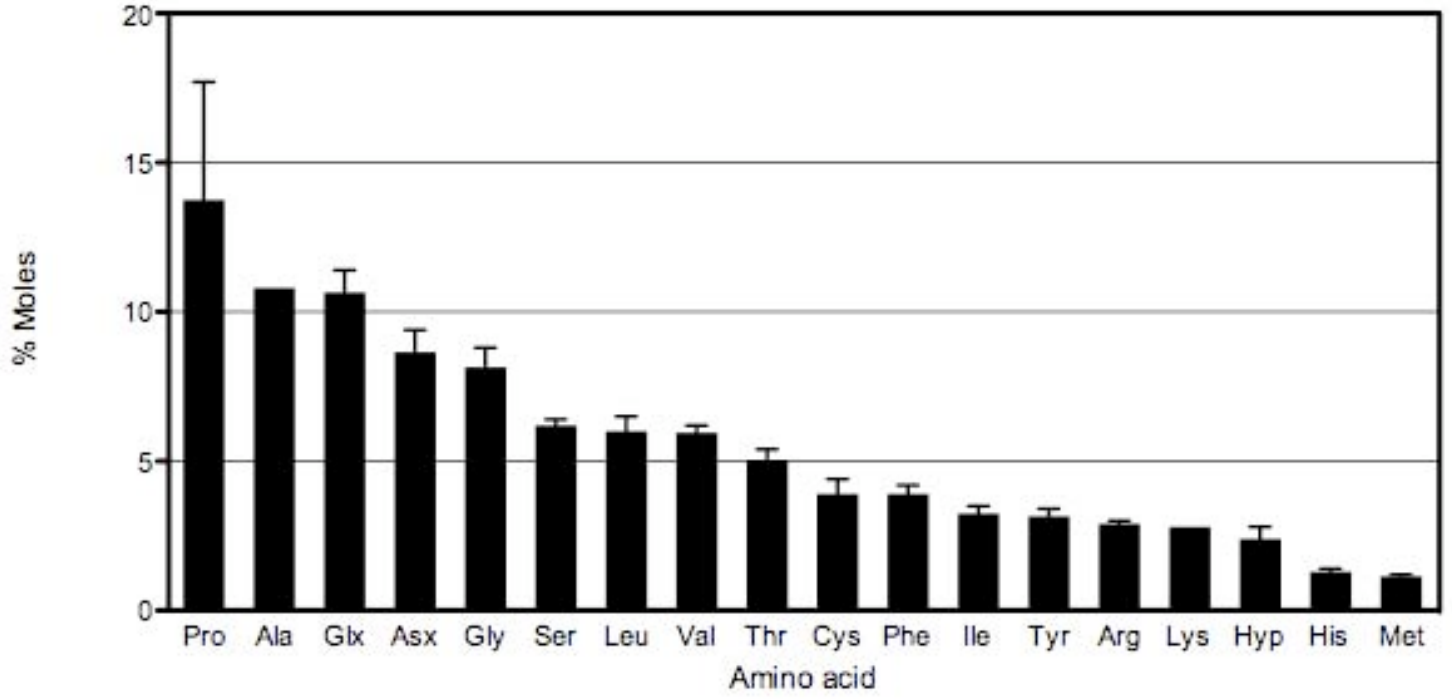

Figure 2-4 Amino acid composition of barley husk tissue. Husk tissue was acid hydrolysed and individual quantities of amino acids (% moles of total amino acids present within the sample) were determined using HPLC. Data is representative of two replicates Data shown are the mean ± SE of two biological replicates (n=2).

2.3.3 Whole grain protein analysis

Compared to husk tissue a significantly larger quantity of protein was extracted. The protein yield calculated based on a Bradford assay was 0.9% extracted protein / initial tissue (w/w) for whole grain in comparison to 0.03% protein extracted / husk tissue (w/w) (section 2.3.1).

Due to large quantities of protein, replicates were produced for black pointed and healthy grain (Figure 2-5). Coomassie Blue G-250 stained gel images were of high reproducibility between analytical and biological repetitions. In total, approximately 350 protein spots could be visualised and compared between healthy and black point sample replicates.

Comparison of black pointed and healthy gels identified three proteins that were more abundant in healthy grain and two additional proteins that were more abundant in black-pointed grain (Figure 2-6 A and B). In both cases, the difference in normalised volume was greater than two-fold between black pointed and healthy samples (Figure 2-6 C).

Each of the three proteins more abundant in healthy grain were approximately 70 kDa while their pIs were approximately 7.6, 7.9 and 8.1 respectively suggesting they were isoforms of the same protein. The two proteins more abundant in black pointed grain were 34 and 33 kDa respectively while they shared the same pI of 7.2.

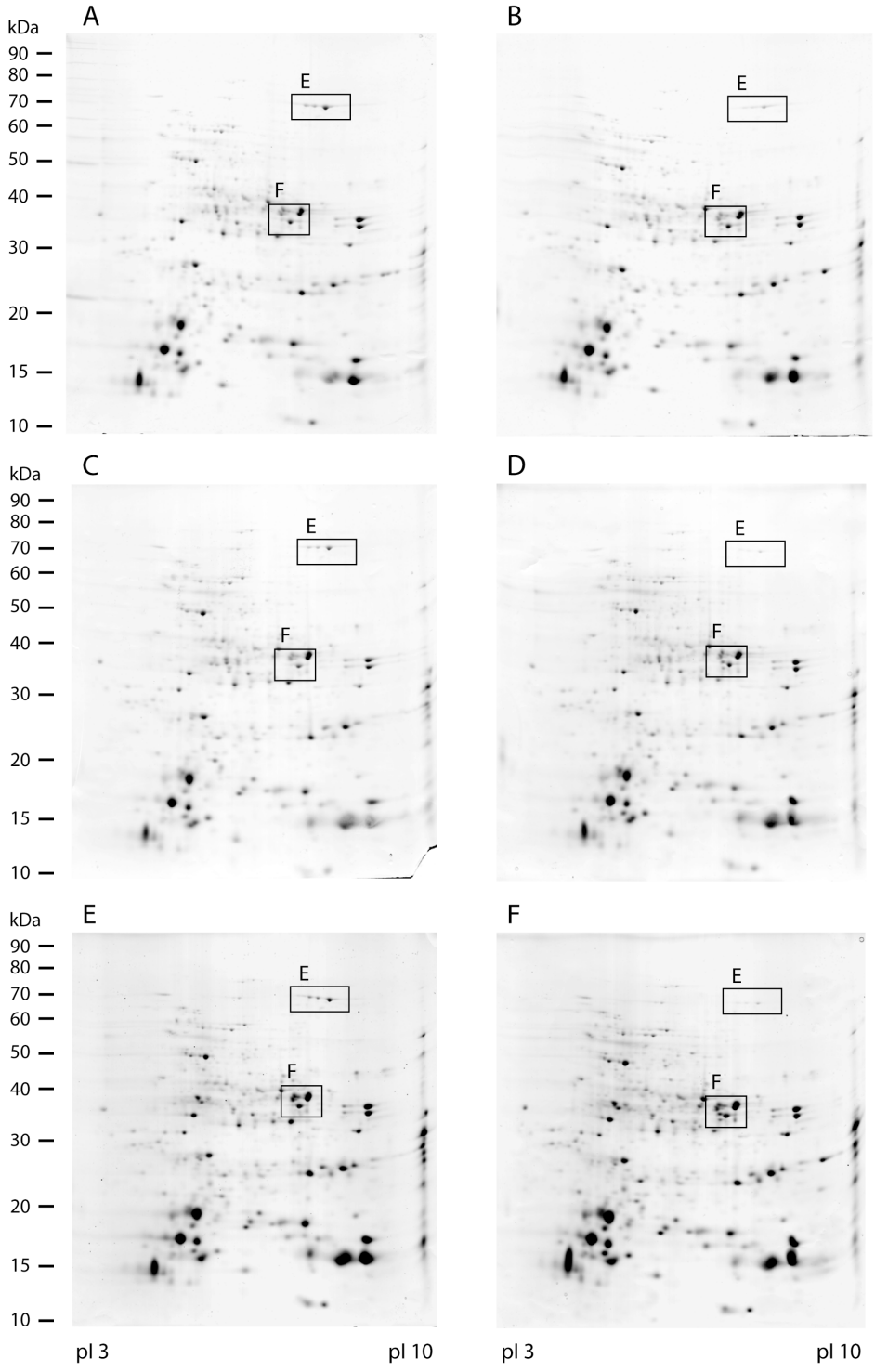

Figure 2-5 2-D gel electrophoresis of proteins extracted from the whole barley grain. Three replicates are shown of healthy (gels A, C and E) and black point (gels B, D and F) samples to indicate the high level of reproducibility. Boxed regions show areas with different protein abundance between healthy and black point samples.

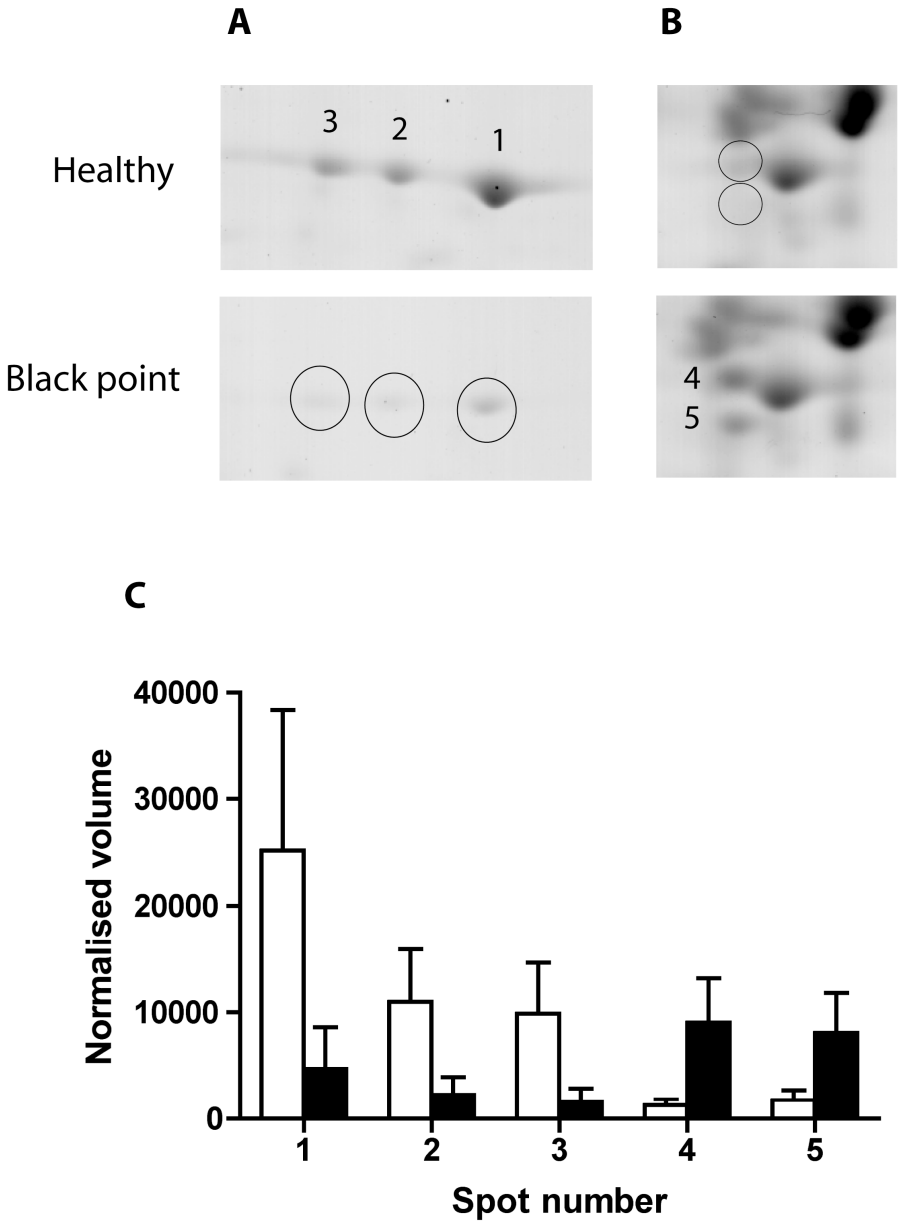

Figure 2-6 Identification of proteins associated with black point. Expanded boxed regions from Figure 2-5 highlight differences between healthy and black point samples. Three proteins were identified as being more abundant in healthy grain (A) and two proteins identified as more abundant in black-pointed grain (B). Quantification of the normalised spot volumes for healthy (white bars) and black pointed (black bars) samples confirmed this observation (C). Data shown are the mean ± SE of two gel replicates of three biological replicates (n=6).

2.3.4 Identification of whole grain proteins using MS/MS

For the three 70 kDa proteins, a total of four *de novo* peptide sequences (common to all) were obtained by MS/MS analysis (Figure 2-7). Using the tBLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/), two of the peptides identified matched to a translated 933 bp barley UniGene sequence Hv.10490. This UniGene sequence did not contain a stop codon. The longest partial open reading frame predicted from this sequence displayed 33% amino acid sequence similarity to a late embryogenesis abundant (LEA) domain containing protein from rice (XP_464846). The remaining two identified peptides were not present in the barley UniGene sequence Hv.10490 but showed sequence similarity to the rice LEA protein sequence (Table 2-1).

For the 34 and 33 kDa proteins, MS/MS identified six *de novo* peptides for each spot and database searching identified both spots as barley grain peroxidase 1 (BP1) with a 20.7 % coverage (Table 2-1).

2.3.5 Comparisons between the husk and whole grain proteome

Comparisons were made between the gel images from healthy husk tissue and healthy and black pointed whole grain protein extracts (Figure 2-8). There are some proteins that appear present in both tissues, in particular two proteins of approximately 5 kDa. The spots identified as being associated with either black pointed and healthy whole grain tissues were not detected in corresponding regions of the husk tissue gel.

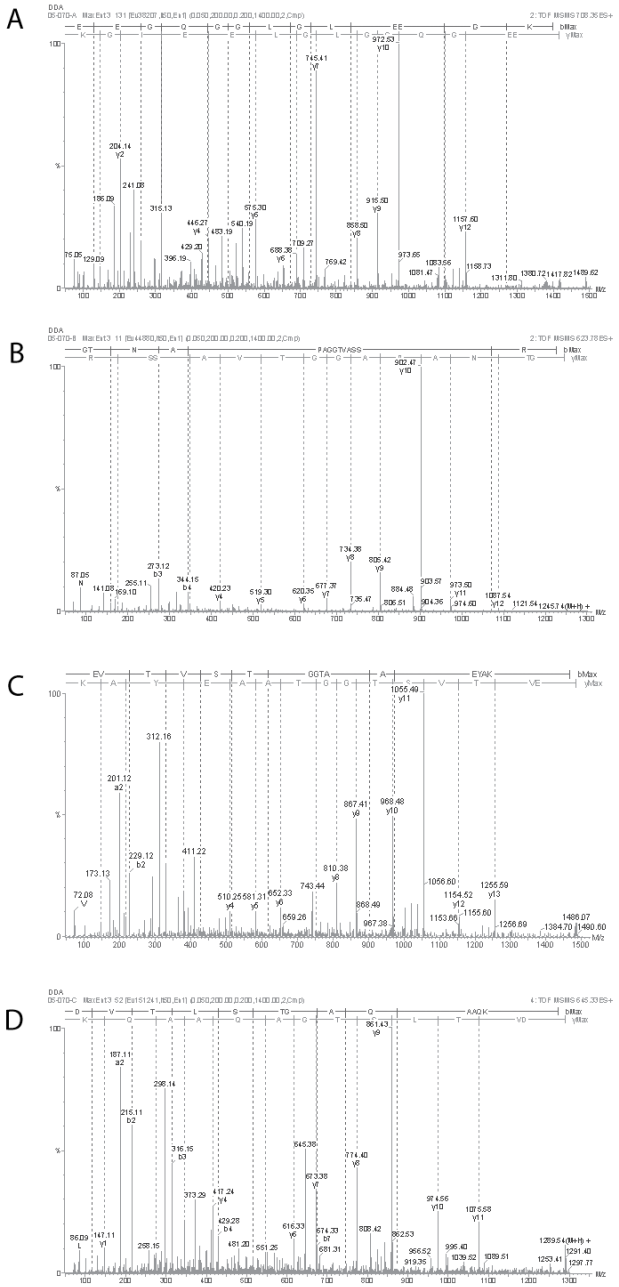

Figure 2-7 MS/MS analysis from spots 1, 2 and 3 from Figure 2-6. Each of the proteins identified were excised from the gel and digested with trypsin then subjected to MS/MS analysis. All three spots produced four common mass spectra in the +2 charge state, indicative of isoforms of the one protein. The spectra for the *de novo* sequences listed in Table 2-1 are shown for EEGQGGLGLEEIGK (A); GTNAPAGGTVASSR (B); EVTVSTGGTAAEYAK (C) and DVTLSTGAQAAQK (D).

Table 2-1 Identification of protein spots with different abundance between healthy and black pointed barley grains.

Spot	Protein identified	Accession	Identified peptides	Observed/ theoretical Mw (kDa) [b]	Observed/ theoretical pI[b]	Score[c]	E value[c]	Volume ratio BP:H[d]	% coverage[e]
1	Putative late embryogenesis abundant protein (partial sequence)	Hv.10490[a]	1. EEGQGGLGLEEIGK 2. GTNAPAGGTVASSR	75/unknown	7.6/unknown	n/a	n/a	1:5	10.0
2	Putative late embryogenesis abundant protein (partial sequence)	Hv.10490[a]	1. EEGQGGLGLEEIGK 2. GTNAPAGGTVASSR	75/unknown	7.9/unknown	n/a	n/a	1:4	10.0
3	Putative late embryogenesis abundant protein (partial sequence)	Hv.10490[a]	1. EEGQGGLGLEEIGK 2. GTNAPAGGTVASSR	75/unknown	8.1/unknown	n/a	n/a	1:6	10.8
4	Barley grain peroxidase BP1	GI:2624498	1. GAVVSCSDILALAAR 2. YYIDLVNR 3. DSVVVSGGPDYR 4. KDIGLAAGLLR 5. TPNVFDNK 6. DIGLAAGLLR	34/33	7.2/6.5	50.8	2e-05	7:1	20.7
5	Barley grain peroxidase BP1	GI:2624498	1. GAVVSCSDILALAAR 2. YYIDLVNR 3. DSVVVSGGPDYR 4. KDIGLAAGLLR 5. TPNVFDNK 6. DIGLAAGLLR	33/33	7.2/6.5	50.8	2e-05	4:1	20.7

[a] Barley UniGene sequences were obtained and a consensus sequence derived using Vector NTI Advanced™ 10.

[b] Observed Mw and pI were taken from the spot position on the gel and the theoretical Mw and pI determined using the calculate Mw/pI tool (http://www.expasy.org/tools/pi_tool.html).

[c] Score and *E*-value was determined from protein blast against the non-redundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/).

[d] The mean normalised spot volume (n=6) shown as a ratio between black pointed (BP) and healthy (H) grain samples.

[e] The percent coverage of the matched protein by the MS/MS derived *de novo* peptide sequences.

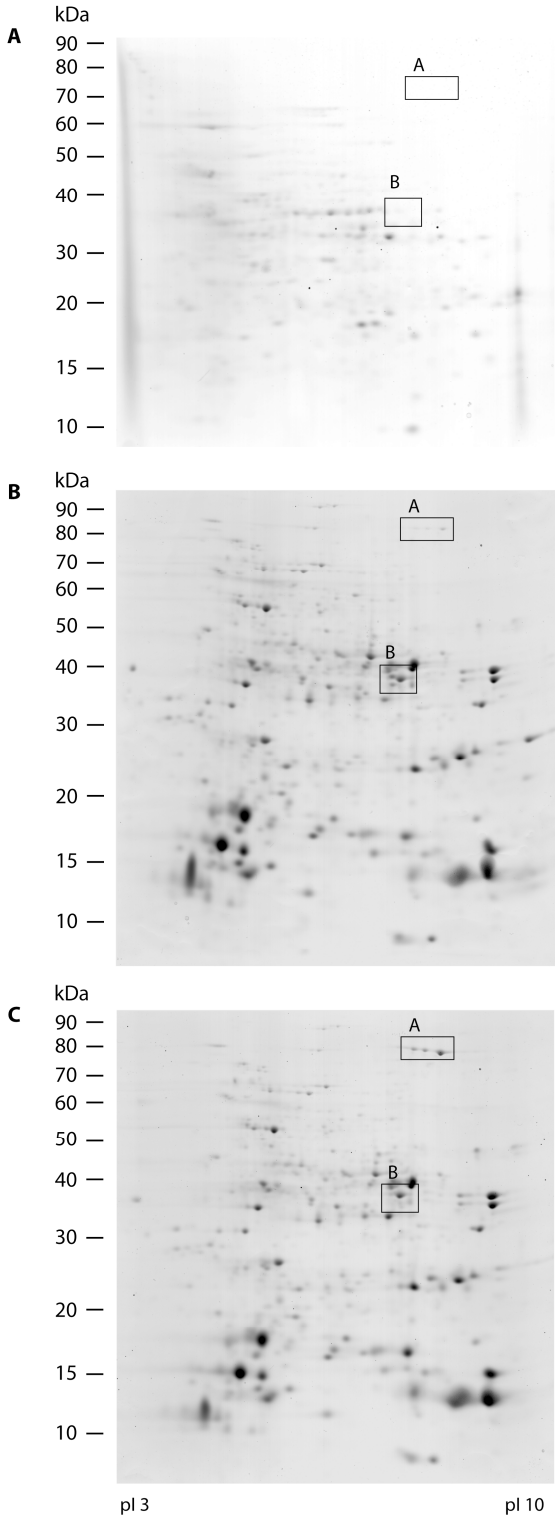

Figure 2-8 Comparison of the proteome of healthy husk tissue (A) compared to whole grain of healthy (B) and black pointed (C) whole grain. Boxed regions are from Figure 2-6 showing LEA (box A) and BP1 (box B), which are present in healthy and black pointed whole grains respectively, are not detected in the husk sample.

2.4 Discussion

The aim of this present study was to identify individual proteins associated with black point of barley grains. This was achieved by comparing the proteome of husk tissue and the whole grain tissue between healthy and black pointed grain samples.

Barley husk tissue from mature grain is a non-living tissue and primarily consists of cell wall material. Initially, the extraction of water-soluble proteins from husk tissue was attempted in this study. Using this method only two main proteins that were present at low abundance were able to be extracted. Considering mature husk tissue is primarily cell wall material this result was to be expected. Cell wall proteins are generally bound to the cell wall by ionic or covalent interactions and thus would not be extracted using a water-soluble protein extraction method (Carpita, 1996).

When 1 M KCl was used to extract proteins from the husk tissue a significant increase in protein yield was observed. High salt concentration extraction buffers containing NaCl, $CaCl_2$ or KCl are commonly used to extract cell wall proteins, with different salts able to extract different subsets of cell wall proteins (Robertson et al., 1997). Extraction buffers containing concentrated LiCl are also reported to extract cell wall-bound glycoproteins (Voigt and Frank, 2003) and tightly bound cell wall proteins (Melan and Cosgrove, 1988). EDTA is commonly used to extract arabinogalactan proteins (Immerzeel et al., 2006). The results from this study showed that after extraction with 1 M KCl no additional proteins could be extracted using any of the buffers described above. This suggests that the ionic strength of 1M KCl was strong enough to release the ionically bound cell wall proteins in the husk.

It also indicates there are low levels of arabinogalactan proteins contained in the husk tissue.

The protein yield obtained from husk tissue using 1M KCl was extremely low at 0.03% w/w. Upon acid hydrolysis of the husk tissue, the amino acid content of the husk tissue was found to be 0.7% w/w. Assuming that most amino acids were incorporated into proteins, it indicates that there is a considerable amount of protein remaining in the husk tissue after extraction with 1 M KCl. This protein is therefore most likely bound to the cell wall by covalent interactions and thus not extracted by salt containing extraction buffers. Previous studies have reported even higher levels of protein (5.9% w/w) within husk tissue, however these have been based on measuring total nitrogen content and using this value as an indicator of protein content (Parajo et al., 2004). From this it then appears there is a significant amount of free nitrogen present with husk tissue that is not incorporated into proteins.

Previous characterisation of barley husk proteins found the amino acids lysine, aspartic acid, serine, glycine, alanine and valine were most abundant (Pomeranz et al., 1976). This is in agreement with the findings presented here, with the exception of proline which was the most prominent amino acid detected.

Based on the amino acid content it can be predicted what type of cell wall bound proteins are contained within the husk tissue. Several classes of structural cell wall proteins have been identified in plants. These include glycine-rich proteins (GRPs) and proline-rich proteins (PRPs) (Showalter, 1993). The proposed function of these classes of proteins is to cross-link and strengthen the cell wall via covalent interactions between carbohydrate chains. The level of amino acids measured in the

husk tissue is consistent with the husk tissue containing high levels of these classes of protein. This further clarifies why little protein could be extracted from the husk tissue due to the fact these proteins are often covalently bound to the cell wall. There is also a further major class of cell wall proteins known as hydroxyproline-rich glycoproteins (HRGPs) (Carpita, 1996). Interestingly in this study levels of hydroxyproline were found to be low within the husk tissue compared to other amino acids, suggesting barley husk tissue contains limited amounts of HRGPs.

An attempt was made to hydrolyse the cell wall with cellulase and pectinase to release covalently bound proteins. This approach was unsuccessful as the cellulase and pectinase were present at high levels masking any husk proteins visible using gel electrophoresis. To overcome this problem a future approach could be to treat the husk tissue with a protease to cleave off peptides from the cell wall bound proteins. These peptides could then be characterised using a technique known as multi dimensional protein identification technology (MudPIT). MudPIT uses 2-D liquid chromatography to separate peptides based on size and charge and the peptides are then analysed using MS/MS to obtain sequence information (Link et al., 1999). The disadvantage of this technique is that it does not allow quantification of proteins as is possible with 2-D gel electrophoresis and thus would not allow comparisons of protein abundance between healthy and black pointed samples.

2-D gel electrophoresis of 1 M KCl-extracted proteins proved to be problematic in this study due to residual salt interfering with electrophoresis. Interfering salts during the IEF stage causes high currents that leads to heat build up and poor and inconsistent protein separation (Mao and Pawliszyn, 1999) making spot comparisons between gels difficult. The effect of salt was reduced by using TCA

rather than acetone to precipitate proteins. Further improvements were made incorporating an initial low voltage step to the IEF program (300V for 2 hours). The purpose of this additional IEF step was to remove salts early on from the gel prior to the higher voltage steps that focus the proteins. To fully optimise this method, further purification steps would be needed as problems were still encountered due to residual salt levels in some samples. Optimisation methods could include dialysis of the protein samples to remove the salt prior to electrophoresis although protein purification steps always result in loss of protein (Wang et al., 2005). Due to the already limited quantity of protein available in this study this approach was therefore not pursued.

The low amount of husk protein able to be extracted presented two main limitations to this study. Firstly, only small format gels (7 cm) could be used. These give less defined separation than larger format gels. Secondly, because the method could not be optimised to produce consistent results, not enough gel replicates could be produced to determine statistically significant differences between the proteome of black pointed and healthy samples.

For future experiments analysing husk tissue it may be advantageous to use more sensitive protein stains such as fluorescent dyes, and techniques including Difference In Gel Electrophoresis (DIGE) (GE Healthcare). DIGE is a technique that allows the labelling of up to three protein samples with different coloured Cy dyes, then all three samples can be separated on a single gel and protein spots compared. This method requires less replication to acquire statistically significant results as gel-to-gel variation is removed due to samples being run on the one gel (Tonge et al., 2001).

To extract proteins from the whole grain, a low salt and close to neutral pH extraction buffer was used. This extraction buffer is reported to suppress the extraction of the very abundant hordein proteins within the grain (Finnie et al., 2002). Overabundant proteins are problematic for 2-D gel analysis as they mask less abundant proteins that may be of greater interest thus difference in the abundance of these proteins cannot be measured.

Compared to the husk tissue, a significantly greater protein yield of 0.9% w/w was extracted from whole grains. The larger quantities of protein firstly allowed larger gels to be used which gave better protein separation (18 cm compared to 7 cm for the husk tissue) and secondly it allowed the comparison of biological and sample replicates for statistical analysis. By comparing the proteome of black pointed and healthy grain we identified a putative late embryogenesis abundant (LEA) protein that was less abundant in black pointed grain. The barley protein sequence was only a partial sequence derived from the barley EST database and thus appeared to be a novel barley protein. Only two of the four peptides identified by MS/MS matched to the barley EST sequence. Further research was therefore needed to obtain the full sequence of this barley LEA gene to determine if the remaining two identified peptides matched to it (see Chapter 3). The other protein identified was barley grain peroxidase 1 (BP1) which was more abundant in black pointed grains. BP1 is a well characterised protein with its full sequence and crystal structure having been resolved (Henriksen et al., 1998). However, its role in grain development is still largely unknown. This study has shown that LEA and BP1 are associated with black point however the role they have in black point development requires establishment.

In conclusion, the technique of 2-D gel electrophoresis is a powerful tool to study the barley proteome. This present study has shown that there are limitations in its application especially when dealing with limited amounts of protein samples and with interfering compounds such as salt. Further refinements to the optimisation procedure would most likely overcome these issues.