Potential of Isoelectric Focusing as a Screening Technique for Resistance to Black Point in Barley

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Introduction

Black point of barley (*Hordeum vulgare* L.) characterised by brown-black discolouration at the embryo end of the grain, is a major cause of downgrading malting quality barley to feed in Australia. *Alternaria alternata* and other fungi species such as *Bipolaris*, *Epicoccum*, *Fusarium*, *Cladosporium*, *Stemphylium*, and *Chaetomium* have been suggested to cause black point symptoms (Waldron 1934, Statler *et al.* 1975, and Southwell *et al.* 1980). More recent studies suggest no direct association between black point and fungal infection (Jacobs and Rabie 1987, Basson *et al.* 1990, Ellis *et al.* 1996 and Williamson 1997). Williamson (1997) was able to produce black point like symptoms, in vitro, by soaking mature wheat grains in catechol and placing them in hydrogen peroxide solutions. The solution turned pink-red and the grain showed some black staining at the germ end and along the crease. There was a clear distinction between the black stained area and the unstained portion of the grain. The pink-red colour developed in the hydrogen peroxide solution is an indication of peroxidase (E.C. 1.11.1.7) activity (Cochrane 1994b). Stress conditions or disruptions such as barley pre-germination, might bring the germ aleurone peroxidases to react with phenols under certain environmental conditions during grain filling and ripening (Cochrane 1994a). There is evidence available on the role of peroxidase in stress related processes such as wounding (Breda *et al.* 1993), salt stress (Botella *et al*. 1994) and disease resistance (Moerschbacher 1992). Hydrogen peroxide is produced in the plant under oxygen stress during photosynthesis (Matkovics *et al*. 1989). Catalase and peroxidase are oxygen scavengers (Droillard *et al*. 1987) and catalyse the reaction that degrade toxic hydrogen peroxide to water. Salicylic acid is a metabolite produced in response to stress. Catalase is inhibited by salicylic acid and other stress related factors, leaving peroxidase to catalyse the hydrogen peroxide reaction with a colour end product. Peroxidase also exhibits catalase activity as both enzymes follow the same path in oxidising Fe (III) to oxyferryl state Fe (IV) with reduction of hydrogen peroxide to water. However, the electron donor (co-substrate) used to reduce the Fe (IV) back to ferric ions is different for these enzymes. Peroxidase uses phenolic compounds, nitrite, cytochrome, but not hydrogen peroxide, whereas catalase uses another molecule of hydrogen peroxide as the reductant (Kruger *et al*. 1987). Further, Dixon and Webb (1966) consider that catalase can be regarded as a peroxidase with a different specificity.

Isoelectric focusing is an electrophoretic technique where separation is carried out in a pH gradient established between two electrodes and stabilised by carrier ampholytes. It offers good definition of protein bands and minimises the effects of further migration beyond the focus. Differences in peroxidase isoenzyme activity from black point resistant and susceptible wheat varieties were observed using the isoelectric focusing technique (Williamson 1997). The objective of this work was to see whether isoelectric focusing of peroxidase isoenzymes and water-soluble proteins is a useful tool for screening for resistance to black point in barley.

Materials and Methods

Sample extraction and electrophoresis

Barley grain of Kaputar, Schooner, WB 219, KTR/TLN 1-335, Kinu-Nijo 7, Grimmett, Lindwall, Bearpaw, Skiff, and Kinukei 20 from 1998 harvest was used. The wheat varieties used were Sunco, Cook, Sunlin and Tasman. The embryo end of three mature barley or wheat kernels of each variety were cut off, then placed in 30 μL of 20 % sucrose solution and shaken in an ultrasonic bath for 1 hour. Incubation was carried out overnight at room temperature.

Flat bed isoelectric focusing was carried out on 0.25 mm thick, 10 cm long, polyacrylamide gel containing 5% ampholyte (Ampholine 3.5 - 9.5). Constant voltage of 2000 V and a maximum power of 3W/cm were applied. The gel was pre-focused for 30 minutes and 10 μL of sample was loaded onto the gel.

Staining Techniques:

The polyacrylamide gels were stained for peroxidase isoenzymes and water-soluble proteins. Peroxidases were visualised by immersing the gels in alkaline buffered substrate solution (Liu *et al*. 1990), and in two different hydrogen donors (Catechol and *o*-dianisidine). Gels were submerged in catechol (1%) followed by hydrogen peroxide (0.03%) incubation (Williamson 1997). In other experiments, *o*-dianisidine (0.01M) and H_2O_2 (0.1M) were used as substrates (Laberge *et al.* 1973).

The barley water-soluble proteins were stained by Coomasie blue performed in four steps (fixing, equilibration, staining and de-staining). Fixing was done for 10 min using trichloroacetic acid (10%) and sulfosalysilic acid (5%) dissolved in water. Then the gel was immersed for 30 min in aqueous solution containing 25 % methanol and 5% acetic acid (v,v,v). The bands appeared after staining the gel with 0.025% Coomasie blue R-250, 1% acetic acid, and 40% methanol in water (w,v,v). The de-staining was performed with aqueous solution of 25 % methanol and acetic acid (v,v,v).

Results and Discussion

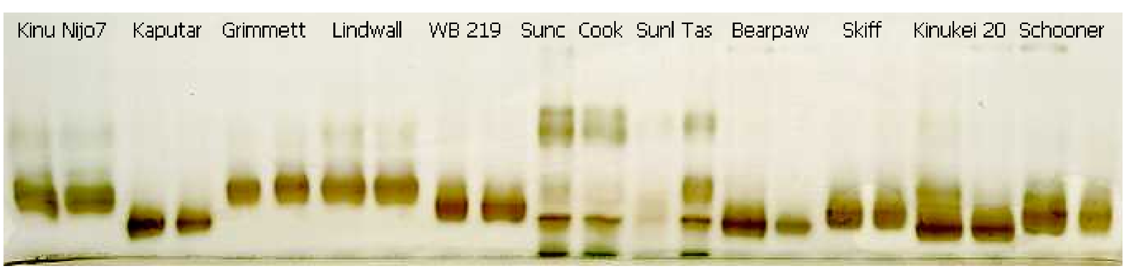

Figure 1. Isoelectric focused (pH 3.5-9.5) peroxidase isoenzymes of some Australian barley and wheat cultivars stained with alkaline buffered substrate solution (Liu *et al.* 1990). (anode (end), cathode (bottom)).

The alkaline buffer stain showed some differences between the peroxidase extract from barley and wheat varieties (Fig. 1). Barley samples contain one major group of peroxidase isoenzymes that have basic pI, and do not have the more basic isoenzyme seen in wheat. Barley also seems to contain more peroxidase than wheat as shown by the band intensity produced by the catechol reaction.

There is a varietal difference in the electrophoretic mobility of peroxidase isoenzymes present in barley. Varieties such as Kaputar, Kinukei 20, Bearpaw and Schooner contain peroxidase isoenzyme that focus at more alkaline pH compared to those present in Kinu-Nijo 7, Grimmett, Lindwall and WB 219.

Water-soluble protein and peroxidase extracts from WB 219, Schooner, Kaputar and TLN/KTR 1-335 were isoelectric focused and stained with catechol, *o*-dianisidine and Coomasie blue. Among these, WB 219 and Schooner contained the highest percentage of black point kernels whereas Kaputar and TLN/KTR 1-335 exhibited the least amount of black point grains from the 20 varieties screened from the 1998 harvest.

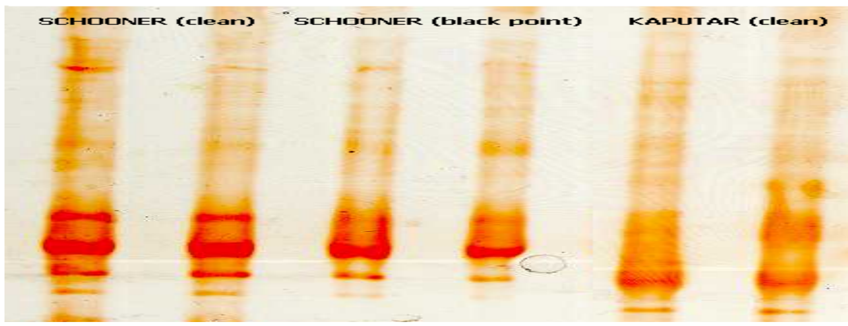

Figure 2. Isoelectric focused (pH 3.5-9.5) peroxidase isoenzymes of barley cultivars stained with *o*-dianisidine. (anode (top), cathode (bottom)).

The peroxidase isoenzyme patterns developed after incubation with *o*-dianisidine (Fig.2) shows one group of peroxidase isoenzyme missing (near the cathode end of the gel) from the extracts of Schooner kernels exhibiting black point symptoms. It also shows that Kaputar has less peroxidase activity compared to Schooner as seen by the stain intensity.

Extracts of barley husk of all varieties analysed showed very little peroxidase activity compared to that present in the extracts of mature barley kernels.

Better definition in the peroxidase isoenzyme pattern was obtained by using *o*-dianisidine instead of catechol as the hydrogen donor. The isoenzyme bands showed by the catechol reaction tend to diffuse rapidly, becoming difficult to distinguish.

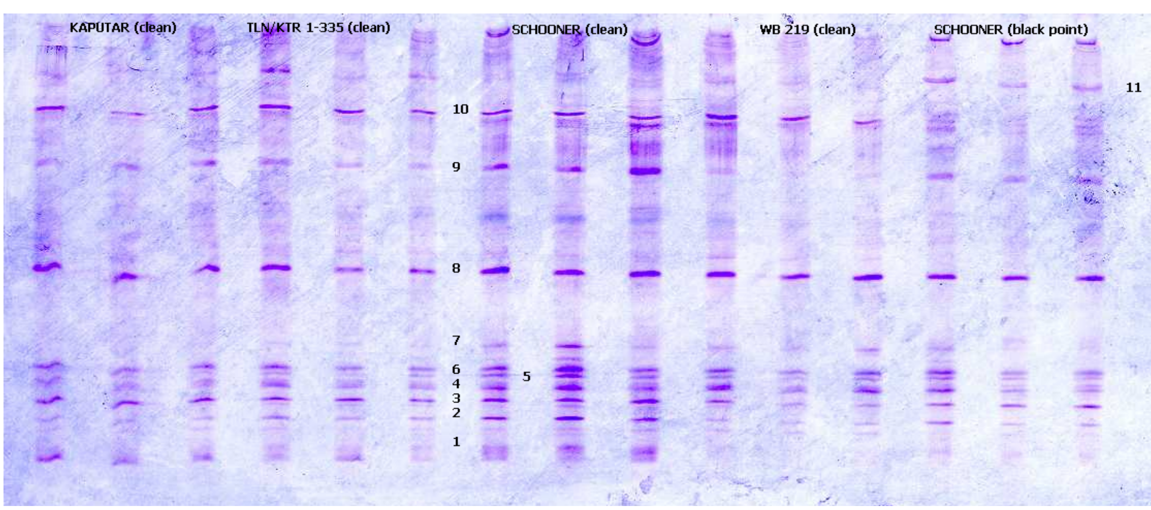

Figure 3. Isoelectric focused (pH 3.5-9.5) soluble proteins of some barley cultivars stained with Coomasie blue. (anode (top) and cathode (bottom)).

Water-soluble proteins (Fig. 3) extracted from some barley kernels were isoelectric focused and numbered from 1 to 11. Protein band 10 is present in all the clean kernels but not in the extracts from kernels with black point. Bands 5 and 7 are missing from Kaputar and TLN/KTR 1-335 while present in Schooner and WB 219 including the black point sample. Band 11 is only present in black point kernels, on the contrary, for the water protein 1, which is present in the clean sample from Schooner but missing in the black point sample. There is not appreciable difference in bands 3, 6 and 8. Protein 4 appears faded for Kaputar and TLN/KTR 1-335 while more intensely stained for WB 219 and Schooner.

Coomasie blue stained gels of barley husk do not show any water soluble protein present (results not shown).

Conclusions

The isoelectric focusing study showed some differences in the peroxidase isoenzyme pattern as well as in the water-soluble proteins among the different barley cultivars studied. Comparison of these enzymes and protein from barley kernels with or without black point symptoms also gave different banding patterns. However, accurate phenotypic data from all Australian varieties will be needed before any conclusions are reached.

The mechanism behind black point formation in wheat and barley may be quite different. In barley, unlike wheat, the husk tissue becomes discoloured. We have traced very little enzyme activity in this tissue of barley grain. Future research will focus on how precursors to black point move to this tissue.

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