A *Botrytis cinerea* aspartic protease targets grape pathogenesis-related proteins and facilitates their removal from wine

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Abstract

Botrytis cinerea is a devastating plant pathogen whose broad host specificity allows it to infect many economically important crops, including grapes. Many enzymes produced by *B. cinerea* have been implicated in pathogenesis however a specific role for secreted aspartic proteases has not yet been ascertained. *B. cinerea* contains at least 14 genes encoding aspartic proteases (*Bcap1-14*), some of which are highly expressed during the early stages of pathogenesis. One of these proteases, BcAP8, represents 23% of the total secreted protein and 71% of secreted proteolytic activity. Strains in which these *Bcap* genes have been knocked out do not demonstrate an altered virulence phenotype. Nor are they more sensitive to grape pathogenesis-related (PR) proteins, a major defence response raise by the host plant. This is despite evidence that *B. cinerea* aspartic proteases can hydrolyse PR proteins.

In an effort to understand its physiological role, BcAP8 was expressed in *Pichia pastoris* and purified to homogeneity. It was produced as an inactive zymogen containing a glycosylated inhibitory prodomain. At pH 5 and below, this prodomain was removed through autocatalytic cleavage and the enzyme activated. Its maximal activity was at pH 3.5, correlating with the conditions of ripe fruit. Kinetic characterisation with a synthetic peptide substrate revealed that BcAP8 has a similar K_M and k_{cat} to several other fungal aspartic proteases, all of which differ to pepsin, a model gastric aspartic protease.

BcAP8 was demonstrated to be capable of hydrolysing grape chitinase and thaumatin-like (TL) protein, PR proteins which are inherently resistant to proteases including pepsin. This novel activity was investigated by digesting a purified grape TL-protein and determining the sites of cleavage. A substrate specificity was revealed for this enzyme which partially differs from that of pepsin. Whilst both enzymes cleave between hydrophobic residues, BcAP8 appears to have broader substrate specificity and be less prone to inhibition by unfavourable amino acid residues. This may in part explain its ability to digest the normally resistant PR protein of the host.

In order to ascertain the specific features of this specificity which allow BcAP8 to degrade proteolysis-resistant PR proteins, TL protein cleavage sites were mapped to a model three-dimensional structure of the protein and assessed for their likely

exposure to proteases. Accessible bonds which are cleaved by BcAP8 but not by pepsin are proposed as critical points for destabilising the tertiary structure of the TL protein, exposing further cleavage sites which allow its full degradation. This analysis suggests that it is the ability of BcAP8 to cleave bonds containing glutamine, proline, glycine and lysine which confers its ability to hydrolyse PR proteins.

Grape PR proteins persist through the winemaking process and are present in white wine. Despite their stability throughout fermentation, they are unstable in the wine and can aggregate to form an unappealing visible haze. In commercial wine production, these proteins are removed through the cation exchange activity of bentonite added to the wine. Bentonite removes PR proteins efficiently but can remove wine aroma compounds and retain some of the volume of treated wine, drawbacks which represent significant economic burdens. The novel ability of BcAP8 to hydrolyse these PR proteins presents an alternative method to remove these proteins before they form haze.

Previous studies have pasteurised grape juice to remove PR proteins, a process which can be improved by the addition of proteolytic enzymes. The potential of BcAP8 to enhance this method was investigated, revealing that BcAP8 is not sufficiently thermostable to survive pasteurisation, precipitating before it is able to have any further effect on PR proteins. Despite this, inhibition of aspartic protease activity during pasteurisation suggests the presence of a grape aspartic protease which remains active in this process and may represent another research target.

During fermentation of grape juice, BcAP8 is active against TL proteins and chitinases in a dose-dependent manner. Addition of BcAP8 to a concentration of 12.5 $mg.L^{-1}$ was sufficient to remove all PR proteins visible by SDS-PAGE with Coomassie staining, with dosages as low as 0.5 $mg.L^{-1}$ also significantly lowering the concentration of these proteins. Despite the removal of the intact forms of these proteins, wine stability tests demonstrated that their proteolysis products form more haze per mass of protein. However, on a positive note, bentonite appears to remove these proteolysis products more efficiently than it does intact PR proteins, suggesting that BcAP8 treatment during fermentation may reduce the quantity of bentonite required to stabilise wine, thus conferring potential economic and sensory gains to the final product.

Declaration

I, Nicholas Ian Warnock, certify that this thesis does not incorporate, without acknowledgment, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Nicholas Ian Warnock.

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Chapter 1: Introduction

White wine protein haze

Proteins present in white wine can denature and aggregate, forming a visible haze (Bayly and Berg, 1967, Hsu and Heatherbell, 1987a), a phenomenon triggered by increased temperatures often associated with wine transportation and storage. Although safe for consumption, haze-affected wine is unacceptable for sale for aesthetic reasons. Bentonite clay is used commercially to remove these proteins utilising its negative charge at wine pH to remove haze-forming proteins by cation exchange (Blade and Boulton, 1988). Small samples of wine treated with increasing concentrations of bentonite are subjected to stringent heat tests at 80ºC to induce haze formation, with the lowest dosage preventing haze formation used to treat the remaining wine. Despite the great efficacy with which this method stabilises wine, bentonite fining has several notable drawbacks.

Salazar and Achaerandio (2006) compared bentonite treated and untreated wine using controlled sensory analyses. Untreated wines were rated higher quality than bentonite fined wines, and it was hypothesised that this may be due to the loss of aroma compounds during fining. This was supported by the determination that bentonite fining reduced the level of eight wine aroma compounds in a dose-dependent manner (Lambri et al., 2010).

Another negative effect of bentonite fining arises from the inability to recover all treated wine from bentonite lees resulting from the treatment of wine in tanks. Based on observations that 3% of treated wine volume is unrecoverable, the cost to the Australian wine industry has been estimated at $A50 million per annum (Pocock et al., 2003). In total, the cost to the global wine industry associated with bentonite fining has been estimated to be US$1 billion per annum (Majewski, 2011).

For these reasons it is desirable to find alternative methods to remove haze-forming proteins from wine. Such improvements should offer greater specificity for haze forming protein removal with reduced effect on other wine components. Development of such methods requires a deeper understanding of the nature of these proteins, and the mechanism of haze formation.

Fundamental features of haze forming proteins

Early studies characterising wine protein haze established that it could be induced by heat. This heat-induced haze contained the same proteins as that found in non-heat induced, or naturally unstable wine (Koch and Sajak, 1959). Protein concentration in wine varies greatly (30-275 $mg.mL^{-1}$) however total protein does not correlate with wine stability due to the presence of proteins with differing thermal stability (Moretti and Berg, 1965, Bayly and Berg, 1967).

In order to better understand the nature of haze proteins, Hsu and Heatherbell (1987b) investigated their isoelectric point and molecular weight. The major proteins in wine ranged in pI from 4.1 to 5.8 and were between 20 and 30 kDa. A 28 kDa protein band separated into five proteins spanning the observed pI range. These predominant proteins were largely absent from bentonite-fined, protein-stable wines, whilst proteins larger than 55 kDa remained. This served to demonstrate that these larger proteins are less amenable to removal by bentonite, but also that they are heat stable and did not contribute to haze formation. Heat-induced haze was solubilised and analysed by gel electrophoresis, confirming that proteins of low molecular weight (12.6 and 20 to 30 kDa) and pI (4.1 to 5.8) contribute the most to haze formation.

Fractionation and thermal stability testing of wine proteins revealed that a 24 kDa wine protein generated the most haze per mass of protein (Waters et al., 1991). A 32 kDa protein also formed haze, though at only half the rate. The remaining fractions formed very little haze, although one of these contained a large amount of carbohydrate, which when added back to other haze forming fractions reduced haze formation: this will be discussed later. The conclusion of this study suggested that the 24 and 32 kDa proteins are predominantly responsible for haze formation.

Waters et al. (1992) then purified these same proteins to homogeneity and their haze potential was determined in ultrafiltered, protein-free wine (Waters et al., 1992). Both proteins formed haze in a linear, dose-dependent manner. In an attempt to remove these proteins through enzymatic hydrolysis, wine was incubated at 15ºC in the presence of a commercial protease preparation (Vinozym P). BSA was also added to these samples to verify the hydrolytic activity of the protease preparation, and proteolysis was monitored by SDS-PAGE. Whilst BSA was readily hydrolysed,

the wine proteins, including those of 24 and 32 kDa, were resistant. This result also served to confirm that wine does not contain inhibitors of Vinozym P. This resistance to proteolysis is an inherent property of grape PR proteins rather than a characteristic conferred by glycosylation or association with wine phenolic compounds (Waters et al., 1995).

In summary, the proteins responsible for haze formation are of low molecular weight and isoelectric point, and are resistant to proteolysis.

The origin of wine haze-forming proteins

Two potential sources of haze-forming proteins are apparent: grapes, and the microorganisms used in winemaking. These possibilities were investigated using antibodies raised against grape proteins, *Saccharomyces bayanus* (a winemaking yeast) and *Oenococcus oeni* (a lactic acid bacteria used in secondary fermentation) (Dambrouck et al., 2003). Wine proteins were probed with these antibodies but did not react with those raised to *O. oeni*, suggesting that this bacterium does not contribute proteins to wine. Several proteins reacted with *S. bayanus* antibodies, specifically those of 20, 38 and 80 to 100 kDa. The large majority of wine proteins reacted only with antibodies directed toward grape proteins, including those between 20 and 30 kDa. It was concluded that most wine proteins are derived from the grape, and furthermore, haze-forming proteins are among those.

Waters et al. (1996) directly identified wine haze proteins by N-terminal and peptide sequencing. Two 24 kDa proteins shared homology with the protein thaumatin and other plant thaumatin-like (TL) proteins. A 28 and 32 kDa protein shared homology with plant chitinases. Both of these protein classes are pathogenesis-related (PR) proteins. The identification of haze proteins as grape-derived chitinases and TL proteins, focussed research on their removal using a wealth of information already gathered about plant PR proteins.

Pathogenesis-related (PR) proteins

Pathogenesis-related (PR) proteins were first observed in tobacco by Van Loon and Van Kammen (1970), who demonstrated that upon infection by tobacco mosaic virus (TMV), *Nicotiana tabacum* var. “Samsun NN” expressed and accumulated four new

proteins. These proteins were host-derived, and their expression level corresponded to the density of viral lesions. Whilst their precise function was not ascertained at that time, it was hypothesised that they may reduce viral multiplication and spread. Infection-induced expression of these proteins was observed in uninfected parts of the plant. Furthermore, a correlation was found between this expression and the development of systemic acquired resistance, whereby subsequent pathogen attacks were rendered less severe (van Loon, 1975). As expression of PR proteins was triggered by infection, and was implicated in host resistance, it was concluded that expression of these proteins represents an active mechanism to prevent viral pathogenesis. Many of these proteins have been identified and grouped in seventeen families based on sequence homology (PR-1 to PR-17) (van Loon et al., 2006). Type IV chitinases found in wine haze (Robinson et al., 1997) are members of the PR-3 protein group, whereas TL proteins are PR-5.

As has been observed for wine haze-forming proteins, several properties broadly categorise PR proteins: they have a relatively low molecular weight, they are selectively extractable (and stable) at low pH, are resistant to proteolysis, and are predominantly localised in the extracellular space (van Loon, 1985). Many of these characteristics are linked, and are understandable given that PR proteins are uniquely adapted to the harsh environments in which they are found.

Expression of grape PR proteins is developmentally regulated

In the early stages of berry growth, most sugars transported into the fruit are metabolised. From véraison, the onset of ripening, sugars are converted to glucose and fructose for storage in the berry (Robinson and Davies, 2000), concurrent with increased PR protein expression.

Vitis vinifera thaumatin-like protein 1 (VVTL1), one of the grape TL proteins originally identified by Waters et al. (1996) is expressed only in the berry, not in flowers or leaves (Tattersall et al., 1997). *VvTL1* mRNA was detectable at very low levels from five weeks post-flowering and accumulated rapidly at 8 to 10 weeks post-flowering, coinciding with véraison. Transcript levels remained high up to 17 weeks after flowering. Western blot analysis of berry proteins confirmed that

VVTL1 was detectable 10 weeks post-flowering, accumulated substantially at 13 weeks then slowly increased until 17 weeks post-flowering.

Chitinase activity was not detected in grapes until véraison, at which point it increased rapidly in parallel with expression of two genes encoding acidic class IV chitinases, both of which continued throughout ripening. Two other genes encoding basic class I and III chitinases were expressed at insignificant levels in the berry. Seven acidic chitinase isoforms have been found in the developing grape which account for 95% of the chitinase activity (Derckel et al., 1996). Chitinase activity in the berry was ten-fold higher than in leaves. Furthermore, chitinase activity is strongly correlated with sugar content during grape ripening (Derckel et al., 1998).

High performance liquid chromatography has been used to quantify and identify soluble proteins in grape juice (Pocock et al., 2000). Although PR proteins represent the most abundant proteins in grapes, their concentration and the ratio between classes vary between cultivars. HPLC retention times of TL proteins was consistent between cultivars, whilst those for chitinases varied, indicating the existence of different isoforms.

Despite the accumulation of sugars and softening of berries during maturation, there is a link between increase grape maturity and increased disease resistance. Susceptibility to *Uncinula necator*, the fungus responsible for powdery mildew, decreases exponentially with increased maturity and beyond 7º Brix (equivalent to 7% (w/w) sucrose), grapes are resistant to new infection (Chellemi and Marois, 1992). Given PR proteins accumulate in parallel with sugars, we hypothesise that PR proteins play a role in this increased resistance at a time in which high sugar concentrations and berry softening could otherwise increase susceptibility to pathogens.

Function of grape PR proteins in disease resistance

Seeking to identify a direct relationship between fungal pathogenesis and PR protein expression, Jacobs et al. (1999) studied two chitinases and two TL proteins in grapes. These were not expressed prior to véraison, but were induced by infection with *U. necator*, and chitinase activity increased in proportion to infection severity. Two grape TL proteins are able to inhibit pathogenic fungi *in vitro*, restricting mycelial

growth and spore germination in *B. cinerea*, *U. necator* and *Phomopsis viticola*, the cause of 'dead-arm' (Monteiro et al., 2003). Analysis of *in vivo* interactions between TL proteins and *Elsinoe ampelia*, the cause of anthracnose, yielded similar results (Jayasankar et al., 2003). *Vitis vinifera* plants regenerated from embryonic cultures selected on fungal culture filtrates constitutively expressed a lipid transfer protein and two TL-proteins. These plants exhibited decreased symptoms during subsequent infection, attributed to inhibition of spore germination and germ tube growth.

PR proteins represent a strong defensive mechanism to prevent fungal infection in grapes. These proteins are absent at times when grapes are less susceptible, but expression is rapidly induced in response to fungal infection. It is likely that the accumulation of these proteins in the developing grape represents an insurance policy of sorts for the plant with the aim of securing the viability of the subsequent generation.

Physiological concentrations of grape chitinases are able to completely inhibit *B. cinerea* conidia germination *in vitro,* but grapes become more susceptible to *B. cinerea* infection as they mature (Derckel et al., 1998). The susceptibility of four different *V. vinifera* Pinot noir cultivars to *B. cinerea* was not determined by chitinase activity levels. This suggests that grape chitinases, which inhibit *B. cinerea* growth *in vitro,* are not effective *in vivo* and that *B. cinerea* may have developed means to negate the effect of chitinase activity.

Interactions between grape PR proteins and *Botrytis cinerea*

Supporting the hypothesis that *B. cinerea* can actively counteract the presence of chitinases, Marchal et al. (1998) observed that all grape proteins below 67 kDa were absent in grapes infected by this fungus, which itself contributed new proteins. These proteins appear to be hydrolysed by aspartic protease activity secreted by *B. cinerea* (Marchal et al., 2006). Proteins secreted by *B. cinerea* during fermentation of defined media were added to a model wine containing grape proteins. The concentration of almost all proteins below 50 kDa was significantly reduced by protease activity which was sensitive to the aspartic protease inhibitor, pepstatin. After two weeks at 30ºC, total protein content of the model wine had been reduced by 53%.

To investigate whether this effect was specific to *B. cinerea*, Girbau et al. (2004) compared its effects to those of *U. necator*. Juice was extracted from grapes infected by each of these pathogens, and protein content determined by HPLC. The main contributors to total protein were TL proteins and chitinases. *Uncinula necator* infection increased the TL protein concentration in proportion to the level of infection. These proteins survived the vinification process, increasing the haziness of the finished wine, again in proportion to the level of infection. In contrast, *B. cinerea* infection dramatically reduced the level of VVTL1, VVTL2 and chitinases in juice. It was suggested that the decreased PR protein content was likely due to proteolysis of PR proteins by *B. Cinerea*. In summary, it appears that the ability of *B. cinerea* to degrade grape PR proteins produced as a defence against fungal pathogens may confer its ability to infect grapes at maturity, in contrast to *U. necator* which lacks both of these abilities.

Regardless of its role in plant defence, this protease activity may represent a novel characteristic of this pathogen and a possible unique source of proteases with the potential to replace bentonite for protein stabilisation. It is this possibility that represents the major aim of this study.

B. cinerea aspartic proteases are implicated in pathogenesis

Movahedi and Heale (1990b) investigated protease activity secreted by *B. cinerea* during pathogenesis. Aspartic protease activity peaked after two days in *B. cinerea* infected carrot tissue but was not detected in uninfected tissue. An aspartic protease purified from the infected carrot caused cell death in carrot tissue and cell culture. It was argued that toxic compounds were released by proteolytic hydrolysis of plant cell wall proteins (Movahedi and Heale, 1990a). Treatment of *B. cinerea* spore suspensions with pepstatin (a specific aspartic protease inhibitor) did not affect germination, but did decrease subsequent disease symptoms by 40 to 100% without further application. Analysis of several *B. cinerea* isolates demonstrated that protease production invariably occurs in the early stages of pathogenesis, mostly preceding production of other enzymes whose expression was more variable. In addition, treatment of carrot discs with the purified aspartic protease prior to infection caused the release of plant-derived compounds which provided resistance against subsequent

infection, likely by acting as early indicators of infection and triggering defence responses in the plant.

When the environmental pH is between pH 3 and 4, *B. cinerea* secretes aspartic proteases which account for 95% of the secreted protease activity (Manteau et al., 2003). Ripe fruits range in pH from 3.3 to 4.4, with grapes being approximately pH 3.5. At pH 5 and above, *B. cinerea* secretes oxalic acid, effectively lowering the pH of its environment. Other potential virulence factors are also regulated in a pH dependent manner, including laccase and polygalacturonase activity, suggesting the existence of multiple sets of enzymes allowing broad host specificity. Modification of the pH closer to that required for aspartic protease expression and function supports a hypothesised role for these enzymes in pathogenesis.

B. cinerea contains at least 14 genes encoding aspartic proteases, designated *Bcap1-14*. Functional analysis of BcAP1-5 was performed utilising knockout mutants of these genes either individually or in pairs (ten Have et al., 2010), though none of these mutants displayed altered virulence or secreted aspartic protease activity. Analysis of the wild-type secreted protein profile indicated a predominant protein of 35 kDa representing 23% of the total secreted protein, identified as BcAP8. Transcript levels of *Bcap8* increased 1000- to 5000-fold after 12 hours in liquid culture, and reach similar levels when *B. cinerea* is grown on leaves. BcAP8 knockout mutants secreted 71% less aspartic protease activity but, as with other BcAP isoform knockouts, did not display an altered virulence phenotype. Despite previous evidence that BcAPs may play a role in hydrolysing PR proteins, BcAP knockout mutants studied so far do not exhibit increased sensitivity to grape PR TL proteins chitinases, which are able to inhibit wild-type growth *in vitro*. It seems likely that there is a high level of functional redundancy that exists within this aspartic protease family, five proteins of which are secreted. Only two of these secreted proteases (BcAP5 and BcAP8) have so far been investigated through individual gene knockout, and it is possible that removal of both of these, and others in the family, is required before any effect will become apparent.

Characterisation of the secreted proteome of *B. cinerea* after 16 hours growth in the presence of different host tissues determined that proteases comprise approximately 40% of the total secreted protein (Espino et al., 2010). In contrast to other studies, serine, glutamic and metallo-proteases were also detected. Despite this, BcAP8

always contributed at least 20% of the total secreted protein. Enzymes which hydrolyse pectin comprise approximately 18% when *B. cinerea* is grown in the presence of fruit extracts. These include the endopolygalacturonases BcPG1 and BcPG2 which are important factors required for full *B. cinerea* virulence (ten Have et al., 1998, Kars et al., 2005). This suggests that the 16 hour sampling point is appropriate for the study of virulence factors, again supporting a role of proteases in this process. Other classes of secreted proteins included glycosyltransferases, lipases, oxidoreductases, and cellulose- and other polysaccharide-degrading enzymes.

Application of *B. cinerea* aspartic proteases to protein haze prevention

As mentioned, heat unstable proteins in wine are grape pathogenesis proteins, specifically TL proteins and chitinases. These accumulate in the grape coincident with ripening and in response to fungal challenge, limiting the ability of pathogens to infect the developing berry. *B. cinerea* is able to infect grapes at full maturity, likely due to the production of aspartic proteases which can hydrolyse these defence proteins, dramatically reducing the protein content of juice. Due to the unique ability of *B. cinerea* aspartic protease activity to degrade these inherently resistant PR proteins, they may represent a novel and more specific means of removing haze proteins from wine. Whilst *B. cinerea* possesses a large number of genes encoding aspartic proteases, the BcAP8 enzyme accounts for approximately 70% of the secreted aspartic protease activity, representing an initial target for study.

In the early stages of the work which comprises this thesis, recombinant BcAP8 is produced in *Pichia pastoris.* BcAP8 and pepsin, a digestive enzyme which is a model aspartic protease, were added to grape juice prior to fermentation (Van Sluyter et al., 2013). Pepsin had no effect on soluble protein content, but BcAP8 significantly reduced the concentration of chitinases and TL proteins in the finished wine. Haze was assessed through a heat stability test at 55ºC, demonstrating that BcAP8 treatment reduced the instability of wine. This protease exhibited a unique ability to hydrolyse these otherwise proteolysis-resistant proteins in their native states, and has the potential to be a cheap and simple method of removing haze forming proteins from wine. Its application to winemaking, as well as characterisation of its biochemical properties forms the basis of this thesis. Application of exogenous

enzymes is widespread in the wine industry. These include pectinases, β-glucanases and β-glycosidases. For a detailed review, see Van Rensburg and Pretorius (2000).

The mechanism of wine protein haze formation

In order to develop more specific methods of haze prevention, knowledge about the mechanism of haze formation is crucial. There is a great deal of evidence demonstrating that it is a complex process involving many different wine compounds which can have implications for potential solutions.

Commercial thaumatin forms haze in wine, but not in a model wine solution of ethanol and tartaric acid (Pocock et al., 2007), suggesting that other factors present in wine are necessary for haze formation. The sulphate anion has been identified as essential for haze formation, and the turbidity of wine subjected to heat testing increases with increasing sulphate. Phenolic compounds and polysaccharides, whilst not essential, have been found associated with precipitated proteins in naturally formed wine haze (Esteruelas et al., 2009). Haze forming proteins demonstrate different responses to phenolic compounds, polysaccharides (Gazzola et al., 2012), sulphate concentration and ionic strength (Marangon et al., 2011b) in model wine, with these factors able to modulate the size and concentration of haze particles.

Individual wine proteins exhibit varying behaviour during heat stability tests, with possible implications for their roles in haze formation. Differential scanning calorimetry reveals that chitinases irreversibly unfold at 55ºC and aggregate upon cooling (Falconer et al., 2010). In contrast, TL proteins denature at higher temperatures of 55 to 62ºC, but return to their native conformation without aggregating. Whilst this implies that chitinases are responsible for haze formation, proteins studied in isolation do not necessarily mimic their behaviour in the presence of other wine proteins and compounds. Wine proteins in real wine compared to when in model wine have decreased melting temperatures (Marangon et al., 2011c). Falconer et al. (2010) suggest that the presence of TL proteins in haze may be due to interactions with denatured chitinases, a hypothesis supported by the demonstration that TL protein precipitation is dependent on chitinase concentration, although the TL proteins do not contribute to haze (Marangon et al., 2011c). Whether or not TL proteins participate directly in haze formation, current protein stabilisation methods

lack the specificity to selectively remove chitinases. Although TL proteins may not be implicated in the problem, they do affect the efficiency of the solution.

A crucial aspect of wine protein haze research is the determination of the quantity of haze in wine, measured as absorbance or turbidity. The standard industry heat test involves incubation at 80ºC for six hours, a temperature higher than is actually experienced during wine production and transportation. This may cause haze measurements to be falsely high and provide misleading information about the actual stability of the wine. Falconer et al. (2010) demonstrated that invertase, a wine protein which has not been found in natural protein hazes, unfolds at 81ºC. As Marangon et al. (2011c) demonstrated that the melting temperature of proteins in real wine is lower than that in model wine, invertase would likely be directly involved in haze formed in the 80ºC heat test. In addition, Dufrechou et al. (2010) demonstrated that different haze formation mechanisms prevail at different heating temperatures, affecting particle size and concentration. This suggests that haze formation in an 80ºC heat test is unlikely to form by the same process as natural haze. Despite this, longer term comparisons between heat test predictions and actual wine stability have demonstrated that a two hour, 80ºC heat test accurately predicts short to medium term stability of wine (Pocock and Waters, 2006). Marangon et al. (2011c) demonstrated that haze can be induced at 30ºC for 22 hours, the main component of which was chitinase. It may be that a heat test at these lower temperatures is more indicative of naturally formed haze, however in the absence of long term data regarding these alternative tests, an 80ºC test will remain the standard by which wine protein stability is assessed in research and in an industrial setting.

Defining the specific physical and chemical properties of wine in the context of the relative contribution and interaction of individual components to haze remains a challenge. The different composition of individual wines makes it difficult to determine the precise mechanism of haze formation in each case. Whilst this information has thus far provided information useful for guiding research into novel bentonite alternatives, the success of any replacement method will be determined by its ability to eliminate, or drastically reduce, the need for bentonite, rather than reduce the amount of haze produced in artificial tests.

Bentonite alternatives

The information which has been accumulated regarding the nature of haze proteins and the mechanisms which can affect their haze potential, has guided diverse research into bentonite alternatives.

Adsorbent materials

Zirconium dioxide has been investigated as an alternative adsorbent to bentonite. One study using 25 $g.L^{-1}$ of this product was able to completely stabilise two of three unstable wines tested, which previously required 0.9 and 1.6 $g.L^{-1}$ bentonite (Marangon et al., 2011a). The third wine demonstrated a bentonite requirement reduced from 1.7 to 0.2 $g.L^{-1}$. These decreases were achieved through the removal of large amounts of wine proteins in a non-specific manner. Zirconium dioxide could be regenerated for reuse following a simple procedure utilising common winery chemicals, with its efficiency showing no sign of decreasing after eleven rounds of regeneration. Sensory assessment of treated wines showed relatively few effects, with the reduction in acidity being the most noticeable. The main challenges for this method are the high dosages required (25 $g.L^{-1}$ wine) and the necessity for constant stirring over extended time periods (up to 100 hours). The latter has been addressed by adding zirconium dioxide into fermentations, utilising the natural agitation provided by this process (Lucchetta et al., 2013). Three juices were fermented with the same dosage of zirconium dioxide. Protein levels were reduced by 90% in two hours, a shorter time period than the previous study. In contrast to control wines which required bentonite, each treated wine was stable. Whilst this process shows great promise as an improved method of wine stabilisation with a decreased effect on sensory properties and reduced waste production, the high dosages of zirconium required for stabilisation currently prohibit its adoption as a commercial method.

Other adsorbents recently investigated include two polysaccharides, carrageenan and pectin (Marangon et al., 2012a) which are negatively charged at wine pH. Each was added to juice alone or in combination prior to fermentation. Protein content in the finished wine was reduced by between 58 and 72%, translating into reductions in bentonite requirement of between 38 and 76% respectively. Despite these significant reductions, each had several drawbacks. Pectin reduced total acidity and removed micronutrients which are used for yeast growth. Fermentations containing

carrageenan were susceptible to frothing which may lead to increased fermentation times.

In addition to alternative adsorbent materials, research is being conducted into improving the efficiency of bentonite. Lower fining rates would likely reduce the negative impacts of bentonite use, particularly in regard to wine volume losses. Nordestgaard et al. (2007) investigated the benefits of an in-line fining method over the standard batch processing method. This allowed removal of PR proteins within a three minute contact time with bentonite, and centrifugation of the treated wine increased the amount of wine recoverable from bentonite lees.

Alternative adsorbent materials share the ability of bentonite to remove haze proteins from wine, but come with issues of their own. The most promising, zirconium dioxide, currently requires high addition rates in order to stabilise wines. Whilst the rate of protein removal has been increased by applying this material during fermentation, the effect of this treatment on required dosage has not been investigated. Dosage rates must be reduced in order for zirconium to represent a viable alternative to bentonite.

Haze protective mannoproteins

Waters et al. (1991) fractionated wine proteins and assessed the thermal stability of each fraction. A carbohydrate-rich fraction was the most stable, and conferred this property on less stable fractions. A crude preparation of this fraction reduced the haze formed by wine proteins in a dose-dependent manner, down to 10% of the control at the highest level tested (Waters et al., 1993). This reduction in haze was not due to removal of wine proteins, but rather a reduction in the size of haze particles. A haze protective factor contained 4% protein and 96% carbohydrate, the latter of which was comprised predominantly of mannose with some glucose, suggesting it was derived from yeast cell walls. Further analysis of this haze protective factor confirmed its identity as a 420 kDa mannoprotein comprised of 30% protein and 70% sugars (Waters et al., 1994b). This protein was present at a low concentration in wine, representing only 0.007% of ethanol-precipitated polysaccharides, and was derived from yeast. Other glycoproteins have since been revealed to protect wine against haze formation, including arabinogalactan-proteins from wine and apple (Waters et al., 1994a), and invertase (Moine-Ledoux and

Dubourdieu, 1999). Brown et al. (2007) identified the *S. cerevisiae* gene encoding Hpf1p and another mannoprotein, Hpf2p, which also reduced haze. Deletion mutants for these genes showed reduced capacity for haze reduction compared to wild-type, whilst overexpression strains showed increased capacity. Haze protective factors remain soluble in wine when haze is formed, leading to the hypothesis that they compete with wine proteins for a wine component which is required for the formation of large haze particles (Dupin et al., 2000). The protection offered by Hpf2p can be attributed to its specific glycan structure, although it is not clear whether this interacts directly with wine components to prevent haze, or whether it provides structural support to the functional protein (Schmidt et al., 2009). The level of naturally occurring haze protective factors in wine is too low for commercial applications (Dupin et al., 2000) and difficulties have been experienced in their recombinant production (Schmidt et al., 2009). Despite this, new methods for harnessing their potential are being explored. Gonzalez-Ramos et al. (2009) generated yeast deletion mutants for cell wall biosynthesis genes and a regulatory gene. Fermentation performed with these modified yeasts increased the polysaccharide content of wines, and bentonite requirements for protein stability of these wines were decreased by up to 40%.

Pasteurisation and proteolysis

Pasteurisation of juice or wine has been proposed as an alternative method of wine stabilisation, utilising the same thermal instability of wine proteins which causes them to be a problem for winemakers. It was first investigated by Koch and Sajak (1959), who heated juice for two minutes at 75ºC. When this juice was fermented, it contained less protein than untreated juice and was more stable. Subsequent studies have combined the denaturing effect of pasteurisation with enzymatic hydrolysis. Pocock et al. (2003) pasteurised three wines at 90ºC for one minute, with or without the addition of pepsin or aspergillopepsin, a fungal aspartic protease. Pasteurisation alone reduced total protein between 15 and 52%. These reductions were enhanced by aspergillopepsin, with total protein reduced by between 50 to 56%, whilst the effect of pepsin was more varied. Heat alone reduced the bentonite requirement by between 27 and 50%, and aspergillopepsin and pepsin increased the effectiveness of this treatment, reducing bentonite requirement by between 43 to 71%. Pasteurisation was

demonstrated to have minimal effects on the sensory properties of the wines, despite the apparent harshness of the treatment.

A similar study treated two juices with aspergillopepsin and pasteurisation at 75ºC prior to fermentation (Marangon et al., 2012b). Pasteurisation alone reduced protein content in the finished wine by 16 and 41%, reducing the bentonite requirement by 19 and 27% respectively. Treatment of juice with aspergillopepsin alone caused reductions in total protein of 17 and 20%, corresponding to bentonite reductions of 32 and 7% respectively. A combined protease and heat treatment enhanced these effects, with total protein reductions of more than 80% and bentonite reductions of 96%. Pathogenesis-related proteins in their native state are resistant to proteolysis (Waters et al., 1995), but pasteurisation denatures these proteins allowing them to be hydrolysed by thermostable proteases. Degradation of native PR proteins in the absence of heat presents a challenge for these same proteases, dramatically reducing their effectiveness. These studies clearly demonstrate the great efficacy of pasteurisation, and its potential to be enhanced in combination with proteases. Despite this, adoption of this treatment in a winery setting would require significant investment in infrastructure to enable the heat treatment, presenting a barrier for smaller wineries.

The *B. cinerea* aspartic protease BcAP8 is able to hydrolyse grape PR proteins in their native state at winemaking temperatures (Van Sluyter et al., 2013), presenting a significant advantage over proteases which require pasteurisation to achieve their full benefits. Further investigation of the use of this enzyme for haze prevention forms the basis of this thesis.

Proteolytic enzymes

Proteolytic enzymes catalyse the cleavage of peptide bonds and can be broadly separated into two categories: exopeptidases that cleave bonds close to a peptide chain terminus, and endopeptidases, that cleave peptide bonds interior to a peptide. Proteases are also classed according to their catalytic mechanism, thus separated into aspartic, cysteine, serine, threonine, glutamic, and metallo-proteases (López-Otín and Bond, 2008). The differing functional groups confer distinct characteristics on each class of protease, including catalytic method, pH optimum and cofactor requirements, allowing them to participate in diverse physiological reactions. The focus of this review is aspartic proteases; for a comprehensive review of all protease classes refer to the work of Rao et al. (1998).

Aspartic proteases

Aspartic proteases (EC 3.4.23) are endopeptidases which utilise two catalytic aspartate residues to hydrolyse peptide bonds. They have been classified by sequence homology into sixteen families in the MEROPS peptidase database (Rawlings et al., 2012), with the A1 family containing pepsin A, a well-known digestive enzyme. Aspartic proteases are characteristically inhibited by pepstatin, a compound derived from *Actinomycetes* (Umezawa et al., 1970). Pepstatin binds to aspartic proteases in the same manner as do substrates (Figure 1 – red molecule), but contains the unusual amino acid statine which displaces the catalytic water molecule normally coordinated in the active site (Rich et al., 1985). The inhibitor molecule mimics a tetrahedral intermediate of the catalytic process. Pepstatin is an extremely effective and highly specific inhibitor of aspartic proteases.

Aspartic proteases are expressed as inactive zymogens containing an inhibitory prodomain of approximately 45 residues (Hartsuck et al., 1992), often preceded by a signal peptide to control secretion or localisation (Kageyama, 2002). Porcine pepsinogen (the zymogenic form of pepsin) contains a 44 residue N-terminal prodomain followed by a 326 residue mature protein. Two catalytic aspartic acid residues for this class of protease are located within the motif -Asp-Ser/Thr-Gly- (Rao et al., 1998).

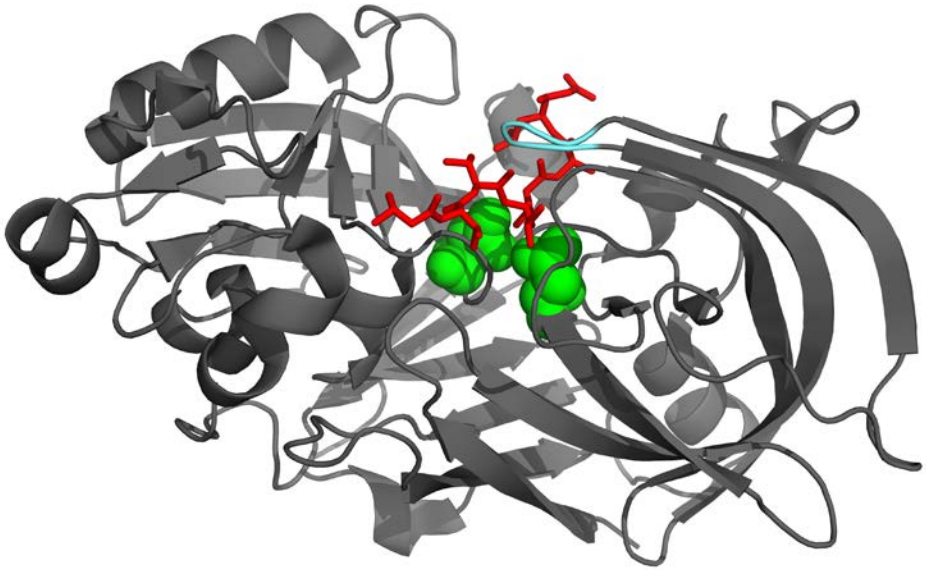

Figure 1: Three dimensional structure of *Irpex lacteus* aspartic proteinase

Irpex lacteus aspartic proteinase (grey) bound to the inhibitor, pepstatin (red). Catalytic aspartate residues are indicated as green spheres and the flexible flap region is shown in teal. PDB reference 1WKR (Fujimoto et al., 2004).

Approximately 70% of the aspartic protease peptide forms secondary structure, consisting predominantly of α-helix in the prodomain and β-sheet in the mature region (Hartsuck et al., 1992). These structural features prevent interaction between the two domains which could otherwise prevent removal of the prodomain during activation. Analysis of aspartic protease tertiary structure reveals the presence of two distinct lobes, each contributing one catalytic aspartic acid. These lobes demonstrate a great deal of structural homology, and are likely the result of gene duplication and fusion (Tang et al., 1978). Retroviral aspartic proteases are produced as single lobes, functioning as homodimers equivalent to the eukaryotic bilobal structure (Navia et al., 1989). The active site is located in a substrate binding cleft between the two lobes. Inactive zymogens contain a correctly formed active site which is inhibited by the prodomain (Figure 2A – red section) (Hartsuck et al., 1992). The lysine at position 36 of the prodomain occupies the position taken by a catalytic water molecule in the active structure. Two tyrosine residues, one of which is in the prodomain, form hydrogen bonds with the catalytic aspartic acid residues. In addition, substrate binding sites are distorted in zymogenic proteases, and the active site itself is filled by the prodomain and the first 13 residues of the mature enzyme. For an in-depth analysis of the inhibitory properties of the prodomain, see the work of Hartsuck et al. (1992).

Activation of aspartic proteases occurs at acidic pH, beginning with protonation of acidic residues in the mature part of the protein. This disrupts the electrostatic interactions which stabilise the prodomain in the active site (Richter et al., 1998). A conformational change occurs in which the scissile bond of the prodomain is placed within the active site (Glick et al., 1991). Cleavage of the destabilised prodomain occurs either in a single- or multi-step fashion, and can involve the formation of intermediate structures (Kageyama and Takahashi, 1983). Multi-step activation occurs when a portion of the prodomain is cleaved prior to removal of the remainder from the active protease. The precise cleavage sites are a function primarily of the substrate specificity of the enzyme. Activation can also proceed by intra- and inter-molecular mechanisms or a combination of both (Kageyama and Takahashi, 1987). Intra-molecular activation is achieved when a zymogen removes its own prodomain; inter-molecular activation involves the activity of another, active molecule. Upon activation, the prodomain dissociates from the active protease (Twining et al., 1981).

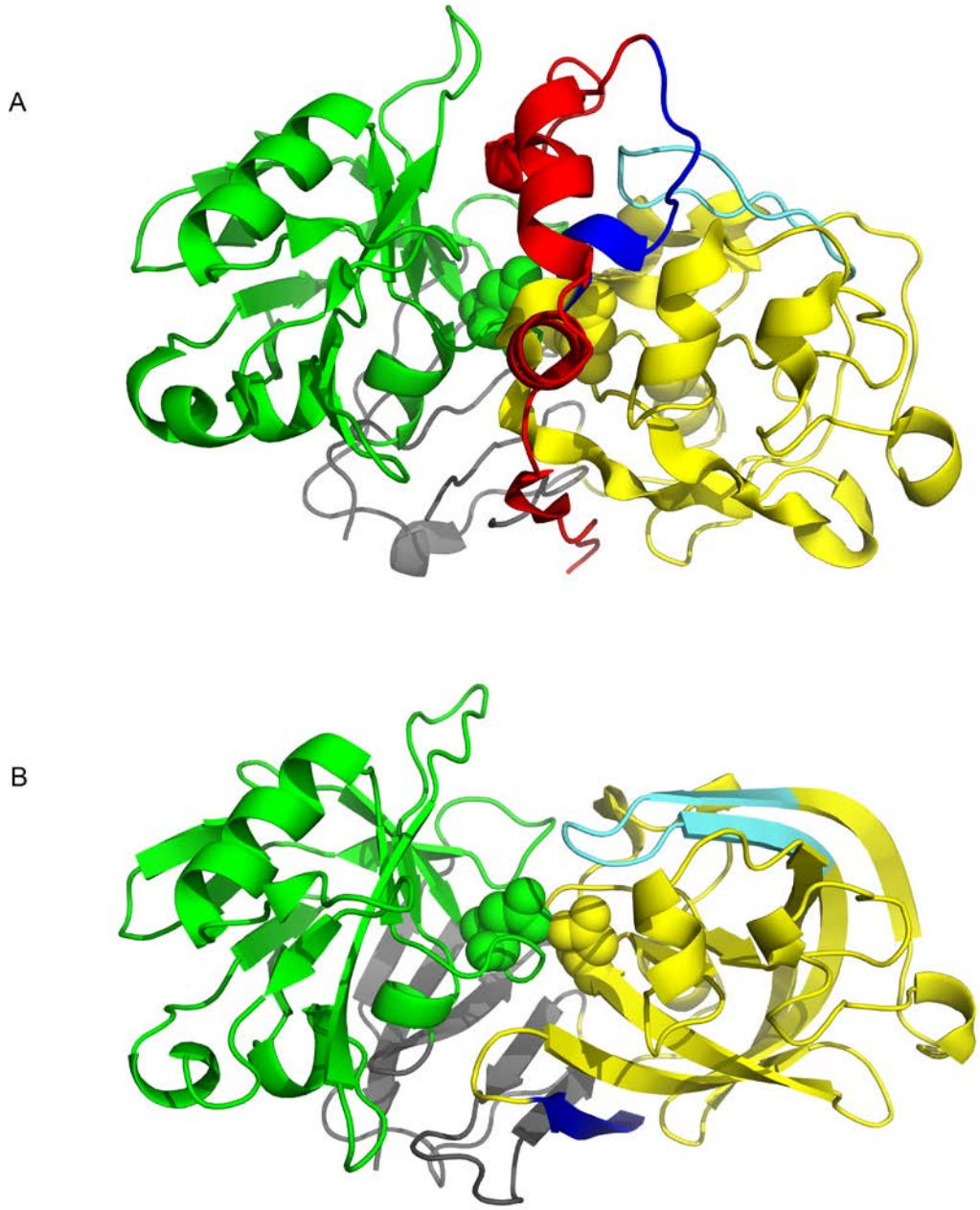

Figure 2: Representation of the tertiary structure of porcine pepsinogen (A) and pepsin (B), presented from similar observation points.

Different structural elements are represented: red, prodomain; blue, first nine residues of mature protein; yellow, N-terminal lobe; green, C-terminal lobe; teal, flexible flap region; grey, the remainder of the peptide chain. Each homologous lobe contributes one catalytic aspartate residue, represented as spherical structures at the centre of the molecule PDB reference for pepsinogen is 3PSG (Hartsuck et al., 1992), for pepsin is 5PEP (Cooper et al., 1990).

At this time, the first nine residues of the mature protease rearrange to replace the first nine residues of the prodomain in a β-sheet behind the active site, leaving the active site accessible for substrate binding (Figure 2B) (Hartsuck et al., 1992).

The active site of the enzyme contains seven subsites which are able to coordinate seven substrate residues (Powers et al., 1977). These subsites, designated S_4 to S_3' accommodate substrate residues P_4 to P_3', where the apostrophe indicates residues or subsites C-terminal to the scissile bond. The scissile bond of the substrate is therefore between P_1 and P_1' (Figure 3). The active site contains the two catalytic aspartic acid residues stabilised by a network of hydrogen bonds (Blundell et al., 1990). In addition, a water molecule is coordinated through hydrogen bonding with the aspartates. On entry of a substrate molecule into the active site, a flexible flap region (Trp-71 to Gly-83) closes down over the cleft (James et al., 1982). Hydrogen bonds are formed between residues in the flap and the peptide backbone of the substrate to correctly orientate the scissile bond of the substrate relative to the catalytic residues (James et al., 1982). Asp-32 protonates the carbonyl carbon of the scissile bond, which is then subjected to nucleophilic attack by a water molecule activated by Asp-215 (Veerapandian et al., 1992). A tetrahedral intermediate is formed before the substrate amine group is protonated, cleaving the peptide bond. The products of the reaction then exit the active site and another water molecule binds to the catalytic aspartates. A detailed model of this catalytic process is described by James et al. (1992).

Collation of data representing almost 7000 peptide bond cleavages was used to investigate the substrate specificity of pepsin, the archetypal aspartic protease (Powers et al., 1977). In general, pepsin cleaves preferentially between hydrophobic residues, with the P_1 residue exerting the most influence on bond specificity. Whilst other subsites played a smaller determining role, some amino acids alter the frequency of bond cleavage in either a positive or negative manner depending both on the amino acid and its location. Arginine at P_3 inhibits cleavage by pepsin even when a favourable amino acid occupies the P_1 position. Fungal aspartic proteases differ from pepsin in that they also favour the cleavage of bonds with Lysine in the P_1 position, demonstrated by the ability of aspergillopeptidase A to activate trypsinogen to trypsin through hydrolysis of a Lys-Ile bond (Abita et al., 1969). This altered substrate specificity is due to several sequence differences between pepsin

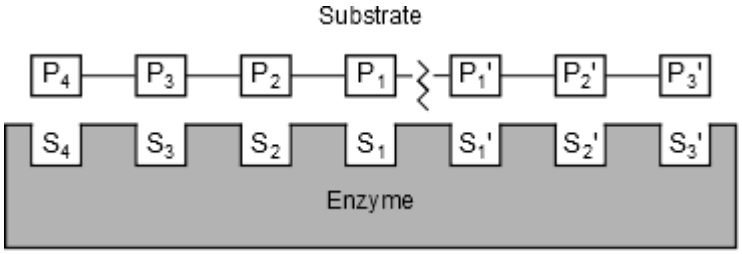

Figure 3: Schematic representation of subsites located within an aspartic protease active site.

P_4 – P_3' represent individual residues of a peptide substrate and S_4 – S_3' represent the enzyme subsites which bind those residues in the active site. The scissile bond is located between P_1 and P_1'.

and fungal aspartic proteases, specifically mutation of Thr-77 to Asp and insertion of a Serine residue between Gly-78 and Ser-79 (Shintani et al., 1997).

Aims and significance of this study

This work has several distinct aims: the first relates to improving our fundamental knowledge of BcAP8 activation and catalytic activity, whilst the second will continue to investigate factors affecting its use in the applied setting of wine production. The work presented here represents to the best of my knowledge, BcAP8 as the first aspartic protease from *B cinerea* to be expressed in a recombinant system.

The first results chapter of this thesis will detail the methods used to produce BcAP8 from *P. pastoris* and the subsequent purification and activation of the enzyme. The main aim of this section is to determine the kinetic parameters of BcAP8 which may be used to compare it to other enzymes, and to investigate its substrate specificity. The ability of BcAP8 to degrade grape PR proteins during fermentation has been described (Van Sluyter et al., 2013), and this ability will be further investigated using purified PR proteins. The substrate specificity of BcAP8 will be studied in a physiological context using a purified grape TL protein. Analysis of this data will reveal the differences in specificity between BcAP8 and pepsin which enable the former to hydrolyse PR proteins. These differences will be explored in an effort to identify a physiological role of BcAP8 in *B. cinerea* virulence, knowledge which has thus far proved elusive yet has far reaching implications.

The second results chapter of this thesis will expand on previously published research that demonstrates the ability of BcAP8 to hydrolyse protease resistant grape PR proteins in juice and small scale fermentation (Van Sluyter et al., 2013). This study, despite suggesting that BcAP8 is capable of reducing haze forming proteins in wine, only addresses one side of the problem. The aim of the study presented here is to take this application one step further and determine if BcAP8 can reduce or eliminate the need for bentonite in wine stabilisation. To achieve this, we explore different ways in which BcAP8 may be applied to the winemaking process, specifically as an additive during pasteurisation of juice, and during fermentation of larger volumes than have previously been used by Van Sluyter et al. (2013). In addition, this previous study tested the stability of BcAP8 treated wines using a heat test at 55ºC. It is possible that this test better simulates real haze, however it is not currently the standard industrial method. This work will investigate the effect of

BcAP8 treatment on haze formed during an 80ºC heat test in an attempt to satisfy winemakers, the ultimate target market for any bentonite replacement technology.

The data chapters of this thesis are both presented as manuscripts to aid their submission for publication in the near future. The rules governing the structure of theses at Flinders University can be found on page 30 of the Research Higher Degrees Student Information Manual, available at http://www.flinders.edu.au/fms/Officeof%20Graduate%20Research/documents/RHD Manual.pdf .

Chapter 2: A *Botrytis cinerea* aspartic protease utilises novel substrate specificity to degrade proteolysis resistant plant pathogenesis-related proteins.

This chapter represents a manuscript intended for publication. I have included the author list and affiliations and obtained feedback and recommendations from some of these people, however I am the sole author of this manuscript.

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Abstract

Botrytis cinerea is a fungal plant pathogen which can infect grapevine and many other commercially valuable crops. During the early stages of infection, it secretes aspartic proteases whose intended substrate is not known. BcAP8 is an aspartic protease which represents 70% of the secreted proteolytic activity of *B. cinerea.* Here we express the inactive zymogen of BcAP8 in *Pichia pastoris*. It is activated by autocatalytic pro-domain processing at pH 5 and below, with maximum activity at pH 3.5. A novel feature of this protease is its ability to hydrolyse grape pathogenesis related proteins which are resistant to proteolysis by the model aspartic protease, pepsin. This ability was confirmed and utilised to investigate differences in substrate specificity between BcAP8 and pepsin. BcAP8 preferentially cleaves peptide bonds containing hydrophobic or negatively charged residues, but its ability to hydrolyse a grape thaumatin-like protein appears linked to cleavage of bonds containing glutamine, lysine, proline and glycine. This supports the hypothesis that *B. cinerea* has developed aspartic proteases specifically adapted to remove the protective influence of plant-derived antifungal proteins.

Introduction

Botrytis cinerea is a fungal pathogen of over 200 plant species (Jarvis, 1977), including many which are commercially valuable (Elad et al., 2007). It gains broad host specificity through the secretion of a diverse arsenal of enzymes, including aspartic proteases, pectic lyase (Movahedi and Heale, 1990a), xylanase (Brito et al., 2006) and polygalacturonase (ten Have et al., 1998). These enzymes confer the ability to degrade many plant cell wall components.

Aspartic protease activity is present at different times during the disease cycle of *B. cinerea.* Activity is found in spores, and its inhibition prior to germination can reduce infection severity by 40 to 100%, without affecting germination rate (Movahedi and Heale, 1990a). In addition, investigation of eight *B. cinerea* isolates demonstrated that aspartic proteases are produced within six hours of infection, whilst other enzymes have later and more variable expression times. Although the specific role of these later expressed isoforms has not yet been determined, there is evidence to suggest they target plant defence proteins.

Botrytis cinerea secretes aspartic proteases across a narrow pH range (3-4) which coincides with that of ripe fruit (Manteau et al., 2003). As they mature, grapes become resistant to infection by *Uncinula necator*, the fungus which causes powdery mildew (Chellemi and Marois, 1992). Grapes accumulate antifungal pathogenesis related (PR) proteins in a developmentally regulated manner (Robinson et al., 1997, Tattersall et al., 1997) such that final concentrations in grapes reach levels sufficient to inhibit *B. cinerea in vitro* (Derckel et al., 1998). Despite this, *B. cinerea* is able to infect grapes at full maturity, and juice from infected grapes contains reduced levels of PR proteins (Girbau et al., 2004).

Fourteen genes potentially encoding aspartic proteases, *Bcap1-14*, have been identified in *B. cinerea* (ten Have et al., 2010). In an attempt to determine the physiological function of several of these genes, individual or pairwise knock out *B. cinerea* strains were generated, however susceptibility of these mutants to purified grape PR proteins was unchanged, suggesting redundancy between *BcAP* genes. Despite this lack of change, one knockout mutant secreted 70% less aspartic protease activity, attributed to the loss of BcAP8 which comprised 23% of total secreted protein in wildtype *B cinerea* strains.

Added to juice and fermentations, recombinant BcAP8 is able to degrade grape PR proteins, specifically chitinases and thaumatin-like (TL) proteins (Van Sluyter et al., 2013), despite the inherent resistance of these proteins to proteolysis (Waters et al., 1995). Pepsin, a model aspartic protease from the gut, is unable to degrade these proteins, suggesting that *B. cinerea* has developed aspartic proteases with novel substrate specificity in order to target these antifungal proteins.

In this study, a biochemical characterisation of BcAP8 produced in *Pichia pastoris* was performed. The ability of this protein to hydrolyse purified grape PR proteins *in vitro* was confirmed, and used as the basis for an investigation of substrate specificity towards physiologically relevant proteins.

Materials and methods

Preparation of BcAP8 expression construct

A BcAP8 expression vector was prepared as previously described (Van Sluyter et al., 2013) directing secretion of the recombinant protein through fusion with the *Saccharomyces cerevisiae* α-mating factor secretion signal peptide. A 6xhistidine tag was added to the native C-terminus by annealing two oligomers (HisF: 5'-GGCTCACCACCACCACCACCACTAGC-3' and HisR: 5'-GGCCGCTAGTGGTGGTGGTGGTGGTGAGCCGC-3') and ligating between SacII and NotI sites of the pPICZαC vector (Life Technologies, Australia). Oligomers were annealed at 20 ng.μL^{-1} each in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA by heating at 95ºC for 10 minutes then cooling to room temperature. The complete construct was electroporated into *E. coli* DH10B (Life Technologies, Australia). Plasmid DNA was linearised with *Pme*I and electroporated into *P. pastoris* according to the supplied protocol (Life Technologies). Transformed cells were selected on YPD agar containing either 100 or 600 µg.mL^{-1} Zeocin (Life Technologies).

Expression and purification of recombinant BcAP8

Extracellular expression of BcAP8 was achieved in *P. pastoris* by inoculating 5 mL of YPD medium with a single transformant and culturing overnight at 30ºC. This was transferred to 200 mL BMGY and grown for a further 72 hours at 30ºC and 250 rpm. Cells were harvested, resuspended in 200 mL BMMY, and grown for a further 72 hours, supplemented with 0.75% (v/v) methanol every 24 hours to induce *BcAP8* expression. Supernatant containing recombinant BcAP8 was obtained by centrifugation at 4,000 *g* for 10 minutes at 4ºC.

Purification and activation of BcAP8

The supernatant was filter sterilised, combined with 0.5 volumes 3x nickel binding buffer [60 mM sodium phosphate (pH 6), 300 mM NaCl, 15 mM imidazole] and applied to a nickel sepharose column at 1.5 mL.min^{-1} at 4ºC. The column was washed with 20 column volumes 1x nickel binding buffer, and bound protein eluted with 250 mM imidazole in the same buffer. Protein-containing fractions were pooled and dialysed against three changes of 20 mM sodium phosphate (pH 6) at 4ºC.

Purified BcAP8 was activated by combining with 0.5 volumes 3x activation buffer [600 mM disodium phosphate (pH 5), 300 mM citric acid] and incubating at 12ºC for 24 hours. Activated BcAP8 was washed in 1x activation buffer in a 30,000 MWCO Amicon Ultra-15 Centrifugal Filter (Millipore, USA) to remove cleaved prodomain peptides. Protein concentration at each stage of purification was determined by Bio-Rad Protein Assay Kit (Bio-Rad, USA).

Optimisation of BcAP8 activation

Nickel affinity purified BcAP8 was diluted 1:10 in activation buffers consisting of 0.1 M citric acid, 0.2 M Na_2HPO_4 at pH 1-7. Activation was performed at 12ºC. Samples were taken periodically in which activation was halted by the addition of SDS-PAGE sample buffer. Conversion of the BcAP8 zymogen to the mature form was determined by SDS-PAGE.

pH dependence of activity

Active purified BcAP8 was diluted to 1 µM in activation buffer (pH 5) then diluted to 10 nM in assay buffer consisting of 0.1 M citric acid, 0.2 M Na_2HPO_4 (pH 1 – 7). Activity assays contained 2.5 nM BcAP8 and 100 µM synthetic substrate Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu, where Phe(NO_2) is p-nitrophenylalanine (Biomatik, Canada) in each assay buffer. Cleavage of the substrate was monitored at 300 nm in a FluoStar Omega spectrophotometer (BMG Labtech, Germany) and activity expressed in relative terms as percentage of the highest activity.

Deglycosylation and N-terminal sequencing

Protein deglycosylation was performed using PNGase F (New England Biolabs, USA) according to the supplied denaturing protocol. Proteins separated by SDS-PAGE were transferred to PVDF membrane and stained with Coomassie Blue R-250. Protein bands were excised and analysis performed by the Australian Proteome Analysis Facility. Samples were subjected to 5 cycles of Edman N-terminal sequencing, using an Applied Biosystems 494 Procise Protein Sequencing system.

Kinetic analysis of BcAP8

Kinetic analysis of BcAP8 was performed in 100 mM sodium citrate, pH 3.5, at 25ºC, with reactions containing 5-160 µM synthetic substrate. An extinction

coefficient of 1480 $M^{-1}.cm^{-1}$ was used to determine reaction rates (Verissimo et al., 1996). Initial rates were determined in at least triplicate at each substrate concentration, and the analysis was conducted on three separate preparations of recombinant BcAP8.

Degradation of grape PR proteins by BcAP8

Grape thaumatin-like protein C (TL-C, accession 7406716) and chitinase A (accession 33329392) were purified according to Van Sluyter et al. (2009). Protein concentration was estimated by A_{280nm} using Abs 0.1% = 1.228 for TL-C and 1.472 for chitinase A, calculated using ProtParam (Gasteiger et al., 2005). Each PR protein at a concentration of 250 $mg.L^{-1}$ was incubated in 100 mM sodium citrate (pH 3.5) at 20ºC in the presence of pepsin or BcAP8 at 10 $mg.L^{-1}$ and PR protein degradation monitored by SDS-PAGE.

To investigate TL-C as an alternative BcAP8 substrate, BcAP8 activity assays were performed as described for kinetic assays using 20 µM of synthetic substrate in the presence of 20 µM TL-C.

Substrate specificity of BcAP8

Substrate specificity of BcAP8 was determined using reduced, alkylated TL-C as a substrate. TL-C was reduced in 10 mM DTT in 25 mM Tris (pH 8.8), 0.5% w/v SDS at 65ºC for 15 minutes and alkylated in 20 mM iodoacetamide for 60 minutes. Proteins were separated by SDS-PAGE, and Coomassie stained TL-protein bands excised. BcAP8 digestion of TL-C was performed by washing gel slices twice for 30 minutes in 100 mM sodium citrate (pH 3.5). Gel pieces were shrunk in acetonitrile and dried. BcAP8 in 100 mM sodium citrate (pH 3.5) was added at 1:20 and 1:40 BcAP8:TL-protein (w:w) and incubated for 16 hours at 25ºC. Samples were centrifuged and the supernatant analysed by LC-MS by Flinders Proteomics, South Australia.

Protein structure modelling

A model of the three dimensional structure of TL-C was generated using *Phyre* (Kelley and Sternberg, 2009). This model was based on the structure of VVTL1

(PDB 4L5H) (Marangon et al., 2013) as the two are 98% identical. An alignment of these proteins is shown in Supplementary Figure 1.

Results and discussion

Expression of recombinant BcAP8

Increasing selection can increase recombinant protein expression

P. pastoris transformants were selected on either 100 or 600 $\mu g.mL^{-1}$ Zeocin with the higher concentration used to select transformants containing multiple copies of the expression cassette, and therefore anticipated to express higher levels of recombinant protein (Cereghino and Cregg, 2000). Expression supernatants were analysed by SDS-PAGE and proteins of 48, 43 and 33 kDa were identified as recombinant BcAP8 by Western blot using an anti-6xhistidine antibody (data not shown). Densitometry revealed 3.2-fold higher expression in transformants selected at the higher level of Zeocin (SDS-PAGE image: Supplementary Figure 2). BcAP8 expression yielded approximately 470 $mg.L^{-1}$ of protein, consistent with Kars et al. (2005) who expressed five *B. cinerea* polygalacturonases in *P. pastoris* at between 5 and 1000 $mg.L^{-1}$ in unpurified supernatant. Attempts to produce BcAP1, 3 and 4 in the same way were unsuccessful, and BcAP5 appeared to be expressed in a hyper-glycosylated state which was not amenable to purification using the methods described here.

Active BcAP8 secreted by *B. cinerea* has previously been resolved at 35 kDa by SDS-PAGE (ten Have et al., 2010) which correlates well with the 33 kDa band observed in the current study. Whilst aspartic proteases are typically activated from zymogenic forms under acidic conditions (Dunn, 2002) the growth media used in this work was buffered at pH 6, preventing activation. The BcAP8 zymogen, including a 6xhistidine tag, is predicted to have a molecular weight of 39 kDa, smaller than either of the other two proteins present (48 and 43 KDa). N-terminal sequencing revealed that both of these proteins shared a common N-terminus which coincided with the N-terminus of the BcAP8 zymogen. Both proteins were also detected utilising the C-terminal histidine tag. This confirmed that both proteins are full length zymogenic BcAP8, suggesting post-translational modification may have occurred.

Recombinant BcAP8 is glycosylated

BcAP8 samples were deglycosylated with peptide-N-glycosidase, causing the 48 kDa protein to resolve at 43 kDa (Figure 1). The apparent molecular weight of the 43 kDa protein was unchanged, suggesting that the larger protein was an N-glycosylated form of the smaller. No size shift was evident when mature BcAP8 was subjected to the same treatment. An advantage of the *P. pastoris* expression system is its ability to perform post-translational modification of proteins, including the secretion of proteins via processing of signal peptides (Cereghino and Cregg, 2000), enabling the expression of relatively pure recombinant protein. *P. pastoris* has been shown to perform N-linked glycosylation of recombinant porcine pepsin (Yoshimasu et al., 2002). An N-glycosylation motif, –Asp-X-Thr/Ser-, where X is not proline. is generally necessary for glycosylation (Marshall, 1972), although is not in itself sufficient (Gavel and Heijne, 1990). This motif occurs twice in zymogenic BcAP8 (Figure 2), the latter of which is retained in the mature form predicted by ten Have et al. (2010). The 33 kDa protein that remained unchanged following peptide-N-glycosidase treatment suggests that this latter motif is not glycosylated. This is consistent with the observation that non-glycosylated motifs are more likely to be found towards the C-terminus of a protein (Gavel and Heijne, 1990). Regardless, the mature form of this heterologously produced BcAP8 (33 kDa) is the same size as active BcAP8 secreted from *B. cinerea* (ten Have et al., 2010), suggesting that no other modifications have been performed by *P. pastoris* and that this protein, once activated, is suitable for analysis of its enzymatic properties.

Purification of recombinant BcAP8

Despite the apparent purity of BcAP8 by SDS-PAGE, a purification strategy was developed to remove trace contaminant proteins, including any host-derived proteases, which may affect activity measurements. BcAP8 from 36 mL of *P. pastoris* supernatant was purified by nickel affinity chromatography. This sample was dialysed to remove imidazole, the protein activated at pH 5, and the cleaved prodomain removed by ultrafiltration to prevent it becoming a substrate. The purification strategy resulted in a 1.62-fold enrichment of activity, indicating the removal of contaminants not apparent by SDS-PAGE analysis, whilst retaining 81% of the total activity (Table 1). From 36 mL supernatant, 8.6 mg of highly pure

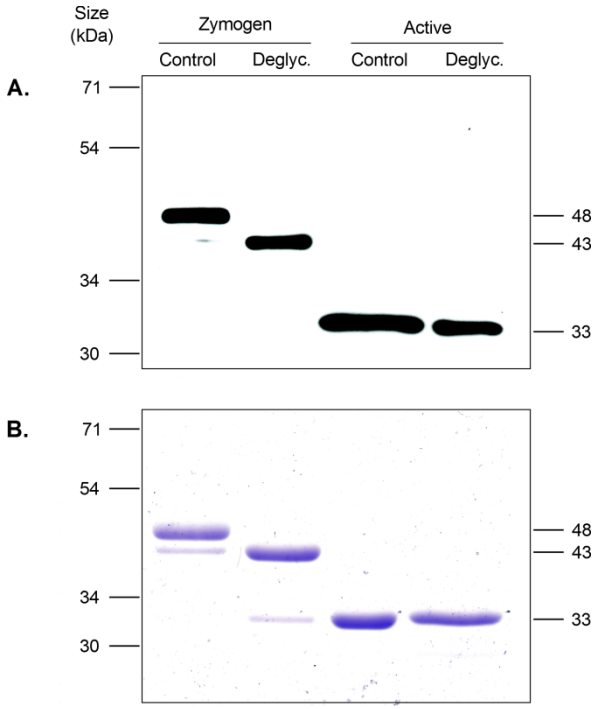

Figure 1: Deglycosylation of BcAP8.

BcAP8 in zymogenic and active forms was deglycosylated with PNGase F and analysed by SDS-PAGE followed by A) Immuno-blot probed with an anti-6x-His antibody (Rockland, USA), or B) Coomassie staining. The PNGase F enzyme is faintly visible in the deglycosylated zymogen lane at 33 kDa.

```
  1 TPIAQVKSAV LPLAKHSNVT SIKNIVDKGH ARLNKYNGIT TTDKRATAVS
 51 SGAVTNEDVS YVAPVVIGGA TWSLIVDTGS SNTWCGAQSS CEKTSTGVAS
101 GGTVSVSYGS GSFSGKEYTD TVSFGGLTVK AQSIGAATSA SGFSGVDGIL
151 GVGPVDLTQG TVSGLSTVPT FLDNLKSQGS ITSEVLGVYF KPESGSDDND
201 TNGELTLGGV DTTKYTGTLT YFPKATSGDA SYYWGISIAG FTYGSTSLAT
251 SASGIVDTGT TLIYIPTAAY NKFLTAAGGK TDSSSGLAVF TTKPTSNFGI
301 KFGSTTYTLT PAQYLVPTAQ YSEFGLSSGK YYAWINDGGA SGVNTIIGQK
351 FLEQYYSVFD TTNSRIGFAT AAHHHHHH
```

Figure 2: Amino acid sequence of BcAP8 with key features marked.

Underlined: prodomain as determined by N-terminal sequencing; Inverted colour, active site residues; shaded, predicted N-linked glycosylation motifs, the N-terminal one of which is most likely to have to contributed to the glycosylated form of *P. pastoris* expressed BcAP8 .

Table 1: Enrichment table of BcAP8 purification and activation.

BcAP8 was purified from 36 mL of *P. pastoris* expression culture supernatant. *Activity values obtained immediately following nickel purification were negatively affected by the elution buffer.

Step	Total protein (mg)	Specific activity (mM.sec-1.mg-1 protein)	Total activity (mM.sec-1)	Yield (%)	Enrichment (-fold)
Initial	17.194	1.741	29.941	100	1.00
Nickel*	12.049	1.580	19.034	64	0.91
Dialysis	9.625	2.560	24.642	82	1.47
Washing	8.603	2.813	24.202	81	1.62

BcAP8 was obtained, a purified protein yield of approximately 240 $mg.L^{-1}$ culture supernatant. This compares favourably with porcine pepsinogen expressed in *P. pastoris* which has been purified with a yield 30 mg protein per litre of supernatant (Yoshimasu et al., 2002).

Effect of pH on BcAP8 activation

Aspartic proteases are generally produced as inactive zymogens containing an N-terminal inhibitory prodomain. Under acidic conditions, these zymogens are activated through autocatalytic cleavage of the prodomain. Activation of zymogenic BcAP8 was optimised with regard to pH and time by incubating in activation buffers between pH 1 and 7. Samples were collected at various time points and activation assessed by SDS-PAGE (Figure 3). Attempts to monitor activation using activity assays were confounded by the fact that incompletely activated samples continued to activate during assays at pH 3.5.

The three forms of BcAP8 previously discussed were identified during the activation process: glycosylated and non-glycosylated zymogen, and the mature form. Activation could be observed as the transition from the zymogenic species to the 33 kDa active protein. At 12ºC, complete activation occurred immediately at pH 2, within 30 minutes at pH 3 and 3.5, and after one hour at pH 4. Further proteolysis products were generated at pH 4 and below, presumably through autolysis. Complete activation occurred within 24 hours at pH 5 with the development of relatively few proteolysis products, likely due to sub-optimal BcAP8 activity at this pH. Activation did not occur within 24 hours at pH 6 or 7. It is noteworthy that *B. cinerea* secretes oxalic acid when the pH of its environment is above 5 (Manteau et al., 2003), correlating with the pH required for activation of BcAP8 *in vitro*. This suggests that the purpose of this acidification is to activate secreted aspartic proteases.

The apparent molecular weight of a small proportion of BcAP8 secreted by *P. pastoris* into the crude supernatant coincides with that of autocatalytically activated BcAP8 at 33 kDa. N-terminal sequence analysis of these two 33 kDa proteins revealed different prodomain cleavage sites. We have found here that autocatalytic activation of BcAP8 is achieved through cleavage of the Asp43-Lys44 bond

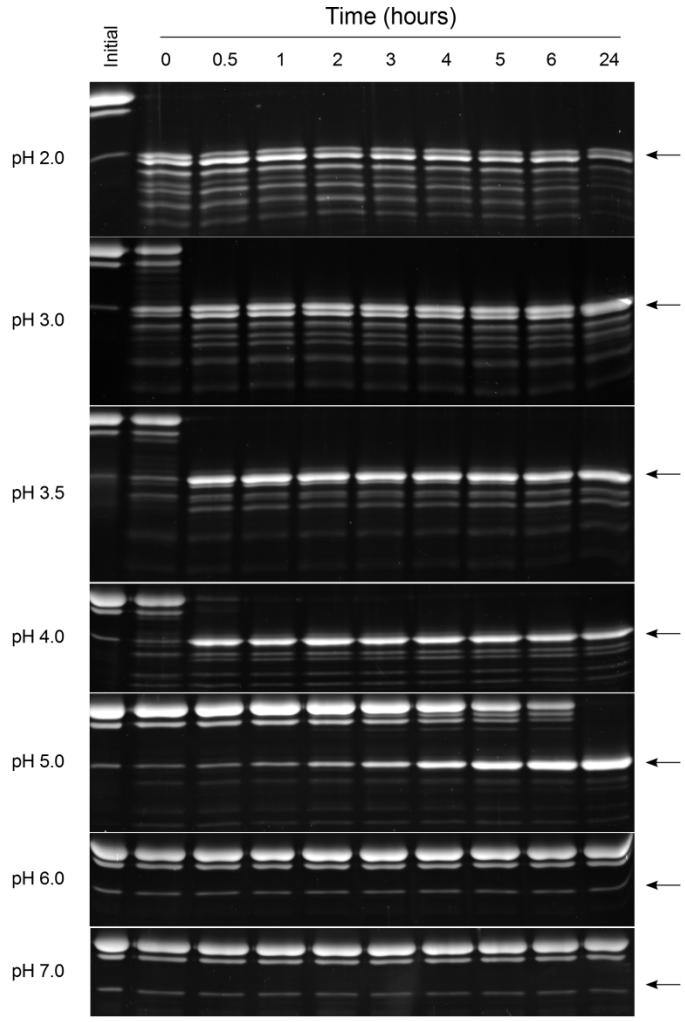

Figure 3: Effect of pH and time on activation of BcAP8.

Purified, zymogenic BcAP8 was incubated in buffers from pH 2-7 and incubated at 12ºC for 24 hours. Activation was monitored by SDS-PAGE as the conversion from the zymogenic form to the mature form, indicated by arrows.

(Figure 2), releasing a 43 residue prodomain. This differs from the 45 residue prodomain predicted by ten Have et al. (2010). The 34 kDa protein present in crude *P. pastoris* supernatant did conform to this prediction, the result of cleavage of the Arg45-Ala46 peptide bond. This difference in cleavage specificity, combined with the observation that BcAP8 is not activated at pH 6, suggests that the mature form seen initially in crude supernatant is the result of an endogenous *P. pastoris* protease, either secreted or released from dead cells. The autocatalytic activation of BcAP8 also differs from that of pepsin, which is achieved through cleavage between two hydrophobic residues (leucine and isoleucine), whereas BcAP8 cleaves between acidic and basic residues (aspartic acid and lysine). This suggests differences between the substrate specificities of these two enzymes, a fact that will be confirmed later.

At each pH, the first time point to reach complete activation as determined by SDS-PAGE was analysed for activity (data not shown). BcAP8 activated at pH 5 had the highest level of activity, with other treatments containing between 85 and 91% of the maximum. For this reason, and based on the relative lack of proteolysis products visible by SDS-PAGE, a 24 hour activation at pH 5 was adopted for all subsequent experiments.

Effect of pH on BcAP8 activity

The activity of BcAP8 was determined between pH 1 and 7 (Figure 4), demonstrating activity between pH 2 and pH 5.5, with the highest activity observed at pH 3.5. Activity reduced uniformly on either side of the pH optimum, to 88% at pH 3 and 4, and approximately 70% at pH 2.5 and 4.5. Activity at pH 6 and above was less than 5% of the maximum, in agreement with the observation that BcAP8 does not perform autocatalytic activation at pH 6.

Despite the fact that activation of BcAP8 occurs faster at pH 2 than any other pH, activity at this level was only 35% of the maximum. It is possible that BcAP8 is maximally active at this low pH, but that it self-degrades prior to activity measurement, artificially reducing activity levels. This is not supported by SDS-PAGE analysis of activation samples, in which protein at the active size is visible at pH 2 even after 24 hours. An alternative is that the activity of BcAP8 in the auto-activation reaction is increased at pH 2 relative to its activity against the synthetic

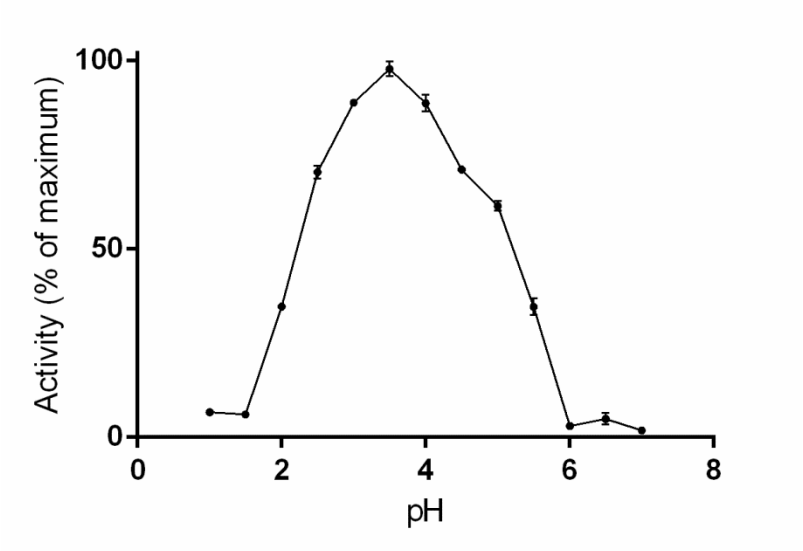

Figure 4: Effect of pH on the activity of BcAP8.

BcAP8 activity was determined at pH 1-7 using the synthetic substrate KPAEFF(NO_2)AL. Activity at each pH is expressed relative to the maximum. Error bars represent ± 1 standard deviation.

substrate, possibly indicating a preference for the Asp-Lys bond cleaved to yield the mature protein. This reaction may be favoured at lower pH due to the charge of individual amino acids under these conditions. The slow activation of BcAP8 at pH 5 is reflected in activity assays, in which this pH allowed only 60% of the maximum activity.

The BcAP8 activity profile broadly reflects that observed by Movahedi and Heale (1990b) for an aspartic protease purified from *B. cinerea*, although with the retention of more activity at pH 2 to 2.5. Although this aspartic protease was not identified, it seems likely that it was BcAP8 given that this enzyme comprises approximately 23% of protein secreted by *B. cinerea* (ten Have et al., 2010).

The maximum activity observed at pH 3.5 coincides with the pH range required for secretion of aspartic protease activity by *B. cinerea* (pH 3 to 4), and also the pH range of ripe fruit (3.32 in apples to 4.39 in tomato) (Manteau et al., 2003). This suggests that for *B. cinerea*, aspartic proteases, including BcAP8, play an important function in pathogenesis of ripe fruit.

Kinetic analysis of BcAP8

ten Have et al. (2010) produced *B. cinerea* strains lacking *BcAP1-5* or *8*, either individually, or in some cases, in pairs. None of these mutants showed altered virulence phenotype or sensitivity to grape PR proteins, suggesting that a level of redundancy exists within the *B. cinerea* aspartic protease family. We hypothesise that kinetic characterisation of each BcAP would reveal similarities and differences between these enzymes and may offer some insight into the nature of this redundancy.

Kinetic analysis of BcAP8 was performed using the synthetic substrate described (Figure 5). Rate data was analysed by non-linear regression using the Michaelis-Menten equation, yielding a K_M of 17.17 µM ±1.23, k_{cat} of 20.14 s^{-1} ±0.48 and k_{cat}/K_M of 1173 $s^{-1}.mM^{-1}$.

Porcine pepsin has been expressed in *P. pastoris* and its kinetic properties investigated with the same peptide substrate, although at 37ºC (Yoshimasu et al., 2002). At pH 3, recombinant pepsin had a K_M of 48 µM, and this increased with decreasing pH. These values are higher than the 17 µM determined for BcAP8,

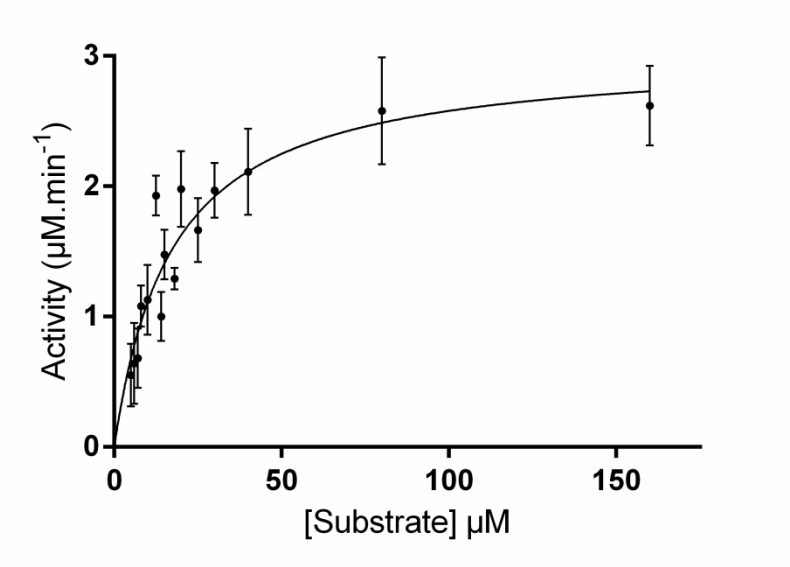

Figure 5: Michaelis-Menten curve of BcAP8 kinetics.

BcAP8 activity was determined using the synthetic substrate KPAEFF(NO_2)AL from 10-160 nM. Rate data was fit to the Michaelis-Menten equation using non-linear regression to determine K_M and k_{cat} values.

suggesting BcAP8 binds the substrate more efficiently than does pepsin. The k_{cat} of recombinant pepsin for this substrate was 183 s^{-1}, yielding k_{cat}/K_M of 3820 $s^{-1}.mM^{-1}$. These values are higher than those for BcAP8, suggesting that despite the lower K_M of BcAP8, pepsin catalyses the cleavage of this substrate with greater efficiency. The difference in temperatures used between these studies means that this comparison is only indicative of the real differences between these enzymes, and it is possible that the catalytic efficiency of BcAP8 would be altered at higher temperatures. Despite this, Dunn et al. (1986) made similar observations when comparing pepsin with fungal aspartic proteases using the same substrate. Three fungal aspartic proteases had K_M between 2 and 16 µM, compared to 35 µM for pepsin, and k_{cat} values for the fungal proteases were between 7 and 16 s^{-1}, whereas it was 97 s^{-1} for pepsin. In addition, binding and catalytic efficiency of the fungal aspartic proteases were less sensitive to substitutions in the substrate which inhibited pepsin activity, suggesting that they may be better adapted to cleavage of a broad range of substrates. It is possible that catalytic efficiency of some fungal aspartic proteases may be sacrificed in order to enable the cleavage of a broader range of peptide bonds. This is supported by the established substrate differences between BcAP8 and pepsin, demonstrated by the ability of BcAP8 to degrade grape PR proteins which pepsin lacks (Van Sluyter et al., 2013).

The kinetic parameters for recombinant pepsin determined by Yoshimasu et al. (2002) correlated well with the values for commercial porcine pepsin, demonstrating that aspartic proteases produced in *P. pastoris* are representative of their endogenously produced forms. This strongly suggests that biochemical data obtained from recombinant BcAP proteins expressed in *P. pastoris* can be used to inform about the native enzymes produced in *B. cinerea*. It was anticipated that a kinetic comparison would be conducted between BcAP8 and other *B. cinerea* aspartic proteases (1, 3, 4 and 5), however BcAP1, 3 and 4 were not successfully produced in *P. pastoris* using the same approach as BcAP8, and BcAP5 was produced in an apparent hyper-glycosylated state which was not successfully purified by nickel affinity chromatography (data not shown).

This is the first kinetic characterisation of an aspartic protease from *B. cinerea*, and represents a starting point for characterisation of the remainder of the family once suitable expression methods have been devised. Based on proteomic analysis, ten

Have et al. (2010) suggest that BcAP5, 9 and 10 may confer functional redundancy on *B. cinerea Bcap8* knockout mutants, making these interesting targets for further recombinant protein production and characterisation. Characterisation of cytosolic *B. cinerea* aspartic proteases which would likely perform different functions to the secreted proteases may offer contrast when attempting to define groups of functional similarity.

Interactions between BcAP8 and grape thaumatin-like protein

Ripe grapes contain high levels of PR proteins, including TL proteins (Tattersall et al., 1997) and chitinases (Robinson et al., 1997) which have antifungal activity (ten Have et al., 2010). As grapes ripen, they become resistant to infection by *U. necator* (Chellemi and Marois, 1992) but are still susceptible to *B. cinerea* (Derckel et al., 1998). Infection of grapes by *B. cinerea* reduces the PR protein concentration of grape juice (Girbau et al., 2004). As discussed, the pH optimum for BcAP8 activity coincides with the pH of ripe fruit, including grapes, suggesting a role for *B. cinerea* aspartic proteases in the hydrolysis of grape PR proteins in order to proceed with a successful infection of ripe fruit.

Degradation of grape PR proteins by BcAP8

BcAP8 has a demonstrated ability to degrade grape PR proteins during grape juice fermentation (Van Sluyter et al., 2013). To investigate the nature of this hydrolysis, a grape TL protein (TL-C) and chitinase (chitinase A) were purified and incubated in the presence of BcAP8 or pepsin for one or three weeks, respectively. Between 70 and 75% of these proteins were degraded by BcAP8 but were resistant to hydrolysis by pepsin (Figure 6 and Supplementary Table 1), in agreement with previous studies which have shown that pepsin is not able to hydrolyse native grape PR proteins (Van Sluyter et al., 2013).

It has previously been observed that grape PR proteins which survive winemaking are not protease inhibitors (Waters et al., 1992). This allows the investigation of these proteins as potential BcAP8 substrates. BcAP8 activity assays were conducted using the synthetic substrate and grape TL-C at equimolar concentrations. No reduction in activity towards the synthetic substrate was observed (data not shown) suggesting that TL-C does not compete with the synthetic substrate. This contrasts

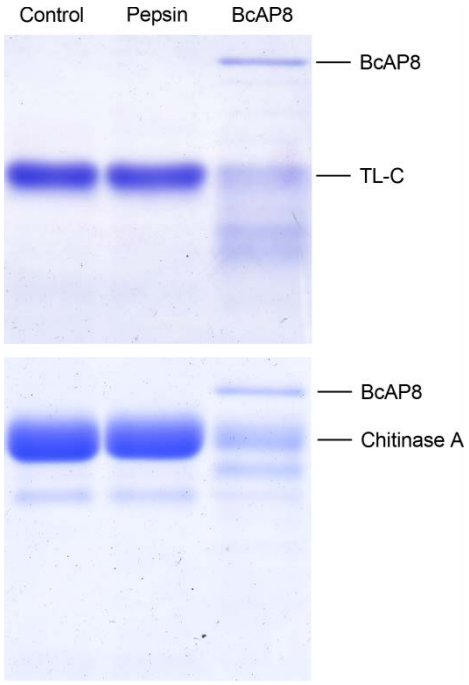

Figure 6: *In vitro* hydrolysis of grape pathogenesis-related proteins by BcAP8. Grape PR proteins were incubated at 20ºC in 100 mM sodium citrate (pH 3.5) for one week (TL-C) or three weeks (chitinase A) in the presence of pepsin or BcAP8 and separated by SDS-PAGE and stained with Commassie blue. Only BcAP8 was able to hydrolyse the grape proteins under these conditions. Pepsin was not visible due to the fact that it has a low capacity for binding Coomassie Brilliant Blue (Tal et al., 1985).

with the demonstrated ability of BcAP8 to hydrolyse TL-C. It therefore seems likely that this type of competitive assay is unsuitable for determining intact protein substrates of BcAP8, likely because a small peptide substrate optimised for aspartic protease cleavage provides a more accessible scissile bond than does a protein like TL-C in its native tertiary structure.

Substrate specificity of BcAP8 based on TL-C

Despite the characteristic resistance of PR proteins to proteolysis (van Loon, 1985), BcAP8 is capable of hydrolysing grape PR proteins whilst pepsin is not (Van Sluyter et al., 2013). This characteristic suggests differences in substrate specificity between the two enzymes crucial to performing this hydrolysis. The active site of proteolytic enzymes contains subsites which each accommodate one substrate residue. These subsites are named relative to the catalytic site, such that substrate residues P_1 and P_1' of the scissile bond reside in active site subsites S_1 and S_1' respectively (Schechter and Berger, 1967). The substrate specificity of pepsin has been thoroughly investigated, demonstrating a preference for hydrophobic residues in both the P_1 and P_1' position (Powers et al., 1977).

Some fungal aspartic proteases differ from pepsin in that they have the ability to cleave a peptide bond containing lysine in the P_1 position, an ability linked to the replacement of Thr-77 by Asp and insertion of a Ser after Gly-78 (pepsin numbering) (Shintani et al., 1997). As BcAP8 does not contain these features, it is unlikely to differ to pepsin in this regard and differences in specificity must lie elsewhere.

The substrate specificity of BcAP8 was investigated in the context of grape TL-C, a putative physiological target. Thaumatin-like protein-C was reduced and alkylated prior to BcAP8 digestion and the sequences of the resulting peptides determined by mass spectrometry. This yielded 49 peptides representing 54 unique cleavage sites. As has been shown for other aspartic proteases, BcAP8 demonstrated promiscuous specificity, cleaving bonds containing all amino acids except histidine, which is not present in TL-C. Broad characteristics of this specificity were determined by comparing the frequency with which each charge class of amino acid was present in a scissile bond to its frequency in the TL-C sequence (Supplementary Table 2).

Hydrophobic and negatively charged amino acids were favoured whilst positively charged residues were cleaved in proportion to their abundance in the sequence. Polar, uncharged residues and cysteine, glycine and proline were under-represented in cleaved bonds.

Although a data set based on one protein substrate limits the ability to compare BcAP8 with the well-studied specificity of pepsin, one notable observation may offer a point of differentiation. Lys and Arg in P_3 have a strong negative effect on the ability of pepsin to cleave any bond (Powers et al., 1977), however six bonds cleaved by BcAP8 contained this feature, representing 11% of all bond cleavages identified.

The structure of TL-C was modelled on that of grape TL protein VVTL1 (accession AAB61590) (Marangon et al., 2013). All bonds cleaved by BcAP8 were mapped on to the sequence along with secondary structural elements (Figure 7). Whilst cleavage of bonds contained within α-helices and β-sheets is possible, it has been shown that between 72 and 84% of bonds cleaved by caspase exist in regions free from secondary structure (Song et al., 2012). Thus, cleaved peptide bonds of TL-C located outside of these secondary structures were mapped on to the three dimensional model (Figure 8) and 22 were visually assessed as likely to be accessible to BcAP8 based on their location on the surface of the molecule. Hamuro et al. (2008) studied the specificity of pepsin by digesting 39 proteins, yielding 1830 cleavage points. The frequency of cleavage between each combination of amino acids in the P_1 and P_1' positions was assessed and this data was applied to the 22 bonds identified as accessible by BcAP8 (Table 2). Six of the bonds cleaved by BcAP8 are not cleaved by pepsin, and three of these are located in a single loop, positioned in the top left of the TL-C structure in Figure 8. This suggests that difficulty cleaving bonds with Gln or Pro in P_1 and Gly, Lys and Pro in P_1' may restrict the ability of pepsin to degrade TL-C. In contrast, the ability of BcAP8 to cleave these bonds is likely to destabilise the remaining tertiary structure, exposing more cleavage sites to hydrolysis and ultimately enabling complete degradation of the protein.

This study presents the first biochemical characterisation of a *B. cinerea* aspartic protease. Its activity *in vitro* appears particularly well suited to the conditions and location in which it is expressed. The pH required for activation and maximum activity aligns with that of ripe fruit, which contain high concentrations of PR

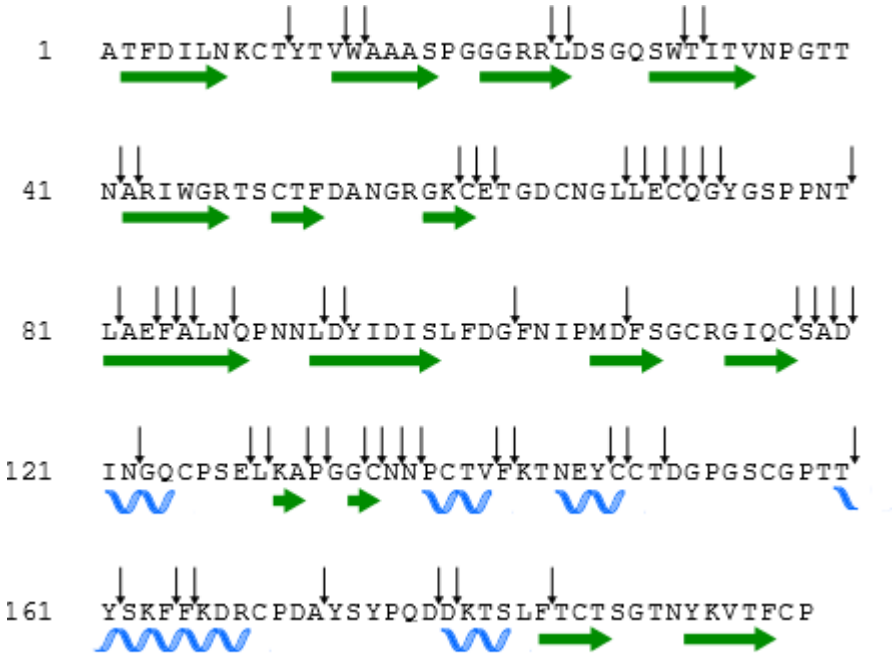

Figure 7: Digestion of grape thaumatin-like protein C by BcAP8.

Peptide sequence of grape thaumatin-like protein 'C' (accession 7406716) showing the cleavage sites and peptides derived from BcAP8 proteolysis. Peptides were determined by MS and cleavage sites mapped on to the sequence (black arrows). Regions of secondary structure based on VVTL1 are indicated by green arrows (β-sheet strands) and blue helices (alpha helices).

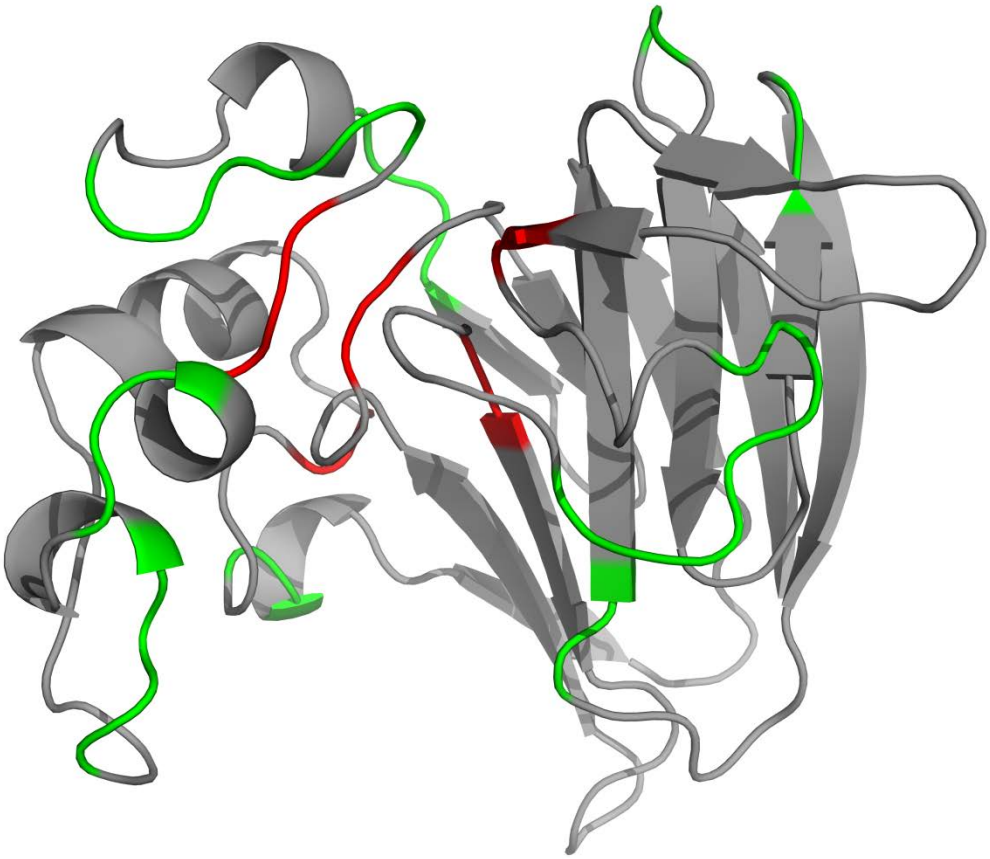

Figure 8: Model of the three dimensional structure of grape thaumatin-like protein C based on the structure of VVTL1

The structure of grape TL-C was modelled on that of VVTL1 (Marangon et al., 2013). Peptides from TL-C digestion with BcAP8 were used to determine cleavage sites, and those located outside of the secondary structure regions are indicated. Green colouring indicates regions where bonds are presumed to be accessible to BcAP8; red indicates those which are not.

Table 2: Cleavage frequency of exposed peptide bonds on grape thaumatin-like protein C by pepsin.

Analysis of TL-C peptide bonds cleaved by BcAP8 revealed 22 likely to be accessible by a proteolytic enzyme. The ability of pepsin to cleave these bonds was investigated using substrate specificity data from Hamuro et al. (2008).

P_1	P_1'	Cleavage frequency (%)
D	***I***	***0***
F	***K***	***0***
A	***P***	***0***
Q	***G***	***0***
P	***G***	***0***
L	***K***	***0***
N	A	4
T	Y	4
S	A	5
D	D	5
G	Y	10
L	D	12
T	D	12
A	D	13
C	S	20
E	C	20
L	E	21
C	Q	33
E	L	45
C	C	50
V	F	50
L	L	56

proteins with demonstrated antifungal capacity. Porcine pepsin is a model aspartic protease found in the gut which is unable to hydrolyse the plant-derived PR proteins studied. BcAP8 exhibits novel substrate specificity which enables it to hydrolyse at least two distinct classes of PR protein. *B. cinerea* secretes this enzyme during the initial stages of infection, strongly suggesting that its physiological role is to counteract one aspect of the host defence response.

Whilst the precise structural features of BcAP8 which allow this specificity are unknown, some of the main differences in specificity between BcAP8 and pepsin have been highlighted. Characterisation of the kinetic properties and substrate specificity of further *B. cinerea* aspartic proteases will allow the groups of functional similarity to be identified and this knowledge will help target future research into the physiological function of this family of proteases.

Acknowledgements

I would like to acknowledge Matteo Marangon for providing purified grape proteins used in substrate specificity studies. This work was conducted with the assistance of The Australian Wine Research Institute, a member of the Wine Innovation Cluster at the Waite Precinct in Adelaide supported by Australian grape growers and winemakers through their investment body, the Grape and Wine Research and Development Corporation, with matching funds from the Australian Government.

Supplementary data

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TL-C      ATFDILNKCTYTVWAAASPGGGRRLDSGQSWTITVNPGTTNARIWGRTSCTFDANGRGKC
VVTL1     ATFDILNKCTYTVWAAASPGGGRRLDSGQSWTITVNPGTTNARIWGRTSCTFDANGRGKC
          ************************************************************

TL-C      ETGDCNGLLECQGYGSPPNTLAEFALNQPNNLDYIDISLFDGFNIPMDFSGCRGIQCSAD
VVTL1     ETGDCNGLLECQGYGSPPNTLAEFALNQPNNLDYIDISLVDGFNIPMDFSGCRGIQCSVD
          ************************************.*****************.*

TL-C      INGQCPSELKAPGGCNNPCTVFKTNEYCCTDGPGSCGPTTYSKFFKDRCPDAYSYPQDDK
VVTL1     INGQCPSELKAPGGCNNPCTVFKTNEYCCTDGPGSCGPTTYSKFFKDRCPDAYSYPQDDK
          ************************************************************

TL-C      TSLFTCTSGTNYKVTFCP
VVTL1     TSLFTCPSGTNYKVTFCP
          ****** ***********
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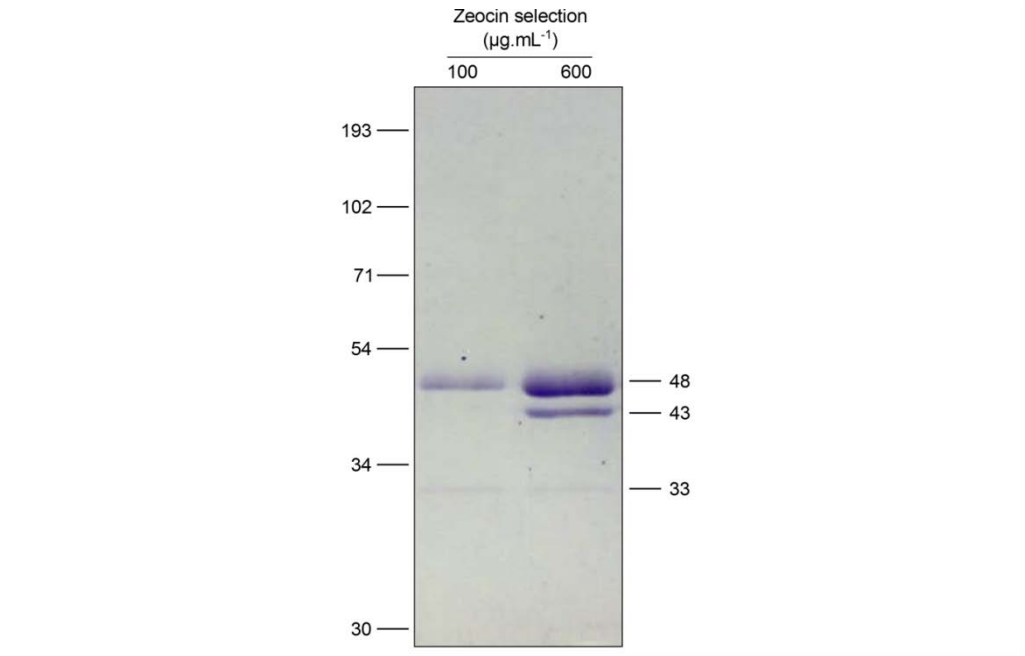
Supplementary Figure 1: Alignment of the amino acid sequences of grape thaumatin-like protein C (TL-C) and VVTL1.

The grape thaumatin-like protein C is 98% identical to VVTL1 allowing the experimentally determined structure of VVTL1 (Marangon et al., 2013) to be used in the generation of a model structure for TL-C. Alignment generated using Clustal Omega (Sievers et al., 2011).



Supplementary Figure 2: Expression of BcAP8 from *P. pastoris* transformants selected on different levels of Zeocin.

P. pastoris transformed with the BcAP8 expression vector was selected on 100 or 600 µg.mL^{-1} Zeocin. Expression culture supernatants were analysed by SDS-PAGE. Selection on higher Zeocin identified a transformant expressing 3.2-fold more BcAP8 than a transformant selected on 100 µg.mL^{-1} Zeocin.

Supplementary Table 1: Quantification of intact grape thaumatin-like proteins after digestion with BcAP8 and pepsin.

Grape thaumatin-like protein C and chitinase A were incubated for one week (TL-C) or three weeks (chitinase A) in 100 mM sodium citrate at 20ºC in the presence of pepsin or BcAP8 and separated by SDS-PAGE and stained with Commassie blue. Intact grape PR proteins were quantified by densitometry compared to a control which did not contain enzyme. Curve areas are represented by arbitrary units based on image intensity.

Treatment	TL-C		Chitinase A	
	Curve area	% of control	Curve area	% of control
Control	17489	100	23572	100
Pepsin	16968	97	23034	98
BcAP8	5374	31	5669	24

Supplementary Table 2: Substrate specificity of BcAP8.

Grape thaumatin-like protein was digested with BcAP8, yielding 54 unique cleavage sites. The amino acids at each cleavage subsite were categorised and compared to their occurrence in the TL-C amino acid sequence.

	Scissile bond %				
Amino acid class	**P_1**	**P_1'**	**Average**	**TL-C composition (%)**	**Difference**
Positive	3.70	9.26	6.48	7.58	-1.09
Negative	16.67	12.96	14.81	9.60	5.22
Polar, uncharged	22.22	16.67	19.44	28.28	-8.84
Hydrophobic	40.74	42.59	41.67	28.28	13.38
Other (C, G, P)	16.67	18.52	17.59	26.26	-8.67

Chapter 3: *Botrytis cinerea* aspartic protease hydrolysis of grape pathogenesis related proteins can improve bentonite fining efficiency

This chapter represents a manuscript intended for publication. I have included the author list and affiliations and obtained feedback and recommendations from some of these people, however I am the sole author of this manuscript.

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Abstract

Grape pathogenesis-related (PR) proteins which persist after fermentation can aggregate to form haze in white wine. These proteins are removed by bentonite fining in commercial production, a method which has several negative effects including wine volume and quality losses. Although PR proteins are inherently resistant to proteolysis, BcAP8, an aspartic protease from *Botrytis cinerea,* is able to degrade them. Addition of aspartic proteases in conjunction with pasteurisation of grape juice can precipitate and remove PR proteins. Here, BcAP8 is shown to be unstable during pasteurisation, precipitating before it can enhance the effects of the increased temperature on PR protein removal. A grape-derived aspartic protease does however appear to be active against PR proteins during this process.

When BcAP8 is added to juice prior to fermentation at a rate of 12.5 $mg.L^{-1}$ or higher, all haze proteins are removed, as determined by SDS-PAGE. The fermentation rate is not affected by the presence of this protease which remains active throughout the process. Wines were tested for their stability using the industry standard heat test at 80ºC. In contrast to a previous study testing wine protein stability at 55ºC, haze formation was increased by the addition of BcAP8. This occurs, presumably, through the formation of wine protein proteolysis products which form more haze per mass protein at 80ºC than do their intact parent proteins. Despite this, these proteolysis products appear more amenable to removal by bentonite, offering a potential improvement the in efficiency of this standard process and, potentially, the sensory quality of the finished wine.

Introduction

Proteins present in white wine can denature and aggregate, forming a visible haze in the wine (Bayly and Berg, 1967, Hsu and Heatherbell, 1987a). Although safe for consumption, haze affected wine is unacceptable for sale for aesthetic reasons. The current commercial method for haze prevention is the use of bentonite, a negatively charged clay which removes haze-forming proteins through cation exchange (Blade and Boulton, 1988). Despite its efficiency at wine protein removal, bentonite fining has several notable drawbacks which include the reduction of wine quality (Salazar and Achaerandio, 2006) and volume (Pocock et al., 2003) and the loss of aroma compounds (Lambri et al., 2010). It has been estimated that costs associated with protein removal are up to $A50 million per annum in Australia alone (Pocock et al., 2003). For these reasons it is desirable to find alternative methods to remove haze-forming proteins from wine.

Haze-forming proteins are grape pathogenesis-related (PR) proteins, and include chitinases and thaumatin-like (TL) proteins (Waters et al., 1996). These classes of protein are involved in defending plants from pathogen attack (Salzman et al., 1998) and accumulate in the grape during maturation (Robinson et al., 1997, Tattersall et al., 1997). Whilst this appears to inhibit some pathogens (Chellemi and Marois, 1992), *Botrytis cinerea* is able to successfully infect grapes at full maturity when the levels of PR proteins are at their peak. A characteristic of PR proteins, including those from grapes, is their resistance to proteolysis (Stintzi et al., 1993, Waters et al., 1995), however it has been shown that grapes and musts infected with *B. cinerea* contain lower concentrations of PR proteins (Girbau et al., 2004), suggesting that *B. cinerea* has developed means to degrade grape PR proteins. Furthermore, the protease activity of *B. cinerea* could be completely inhibited by a specific aspartic protease inhibitor and the activity of these aspartic proteases against soluble wine proteins was confirmed, although the identity of the substrate proteins was not identified (Marchal et al., 2006). It was later demonstrated that some chitinases and TL proteins present in wine are sensitive to *B. cinerea* protease activity (Cilindre et al., 2008).

As a means of haze prevention, removal of grape PR proteins by proteolysis has been explored in several studies. Waters et al. (1995) added a commercial enzyme

preparation to fractionated wine proteins, and showed that PR proteins were unaffected by this treatment, despite BSA being rapidly degraded. Pocock et al. (2003) demonstrated that the presence of aspergillopepsin or pepsin in wine during pasteurisation at 90ºC significantly reduced chitinases and TL protein content, although this effect was reduced at 45ºC. The reduction in PR protein concentration induced a corresponding reduction in the amount of bentonite required to achieve stability.

Marangon et al. (2012b) combined pasteurisation of grape juice at 75ºC with treatment with a commercial enzyme preparation containing aspergillopepsin I and II. Wines made from these juices contained significantly reduced protein levels, which translated to reduced haze and a 96% reduction in bentonite required for heat stability. In contrast, enzymatic treatment alone decreased protein levels by around 20% when added during fermentation of unpasteurised juice. In all cases, the sensory properties of treated wines did not differ from those of the bentonite-treated control wine. This data confirmed the potential of enzymatic degradation of grape PR proteins for the reduction in bentonite requirements, but the need for elevated temperatures and specialised heating equipment remained.

BcAP8, an aspartic protease from *B. cinerea* which represents 23% of total secreted protein, and 71% of the secreted aspartic protease activity (ten Have et al., 2010), has been used successfully to degrade grape PR proteins in juice at typical winemaking temperatures (Van Sluyter et al., 2013). BcAP8 and pepsin, a well-characterised gastric aspartic protease, were added to juice prior to fermentation, and whilst pepsin had no effect, BcAP8 degraded a significant amount of chitinase and TL protein. Although the resulting wines exhibited decreased haze potential, the 55ºC heat test used, whilst perhaps accurate for predicting real haze in wine, was less robust than the standard test used in the wine industry.

The current study aims to expand on the work of Van Sluyter et al. (2013) and further explore the potential of BcAP8 as an alternative means to PR protein removal. Specifically, we will investigate any effects of BcAP8 treatment on fermentation rate, haze potential and bentonite requirement of treated wines. In addition, based on previous success combining enzymatic treatment with pasteurisation, we will determine whether pasteurisation can enhance the ability of BcAP8 to remove grape PR proteins.

Materials and Methods

Materials

A single Chardonnay juice, sourced from South Australia in 2012, was used for all experiments.

BcAP8 production

Purified recombinant BcAP8 with a C-terminal 6xhistidine tag was prepared and activated as described previously (Warnock et al., 2014).

BcAP8 activity determination

BcAP8 activity was determined as previously described (Warnock et al., 2014), using reactions containing 5 nM BcAP8 in 100 mM sodium citrate (pH 3.5) with 80 µM of the synthetic substrate The substrate Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu, where Phe(NO_2) is p-nitrophenylalanine (Biomatik, Canada). Activity was monitored at 300 nm in a FLUOstar Omega spectrophotometer (BMG Labtech, Germany) at 25ºC.

Total protein estimation

Protein content of BcAP8 preparations was determined using the Biorad Protein Assay (Biorad, USA) according to the supplied protocol. Total protein content of grape juice and wine samples were quantified in quadruplicate using the EZQ Protein Quantitation Kit (Life Technologies, Australia). EZQ assays were imaged on a ChemiDoc MP Imaging System (BioRad, USA) and images were processed using the accompanying Image Lab software. For both protein assays, a standard curve was prepared from BSA.

Intact PR protein quantitation by HPLC

Intact grape PR proteins were quantified by HPLC. Samples were diluted with an equal volume of solvent A [8% (v/v) acetonitrile, 0.1% trifluoroacetic acid]. Samples were loaded at 0.75 $mL.min^{-1}$ onto a Vydac 2.1 x 50 mm C8 column (208TP5205 Grace, USA) equilibrated in 83% solvent A, 17% solvent B [80% (v/v) acetonitrile, 0.1% trifluoroacetic acid] at 35ºC. Proteins were eluted by a gradient of solvent B from 17% to 49% in the first minute, then from 49% to 85% from 1 to 5 minutes,

then 85% to 17% from 5 to 5.5 minutes. Elution was monitored at 210 and 220 nm. Protein identity was established based on retention time by comparison to purified grape proteins. Proteins were quantified by comparison to a thaumatin standard curve.

Grape juice preparation

Juice was treated with 3 $g.L^{-1}$ pectinase and settled at 4ºC for 24 hours. The supernatant was siphoned from the solids then centrifuged at 16,000 *g* for 30 minutes at 4ºC in an Avanti J-26 XPI centrifuge (Beckman Coulter, Australia). The supernatant was filtered through Whatman #2 paper and filter sterilised through a 0.2 µm Steritop filter (Millipore, USA).

Pasteurisation

Juice was injected into silicon tubing (4 mm internal diameter) pre-equilibrated to 90ºC in a water bath. Once the juice reached 70ºC, the tubing was transferred to a 75ºC water bath and incubated for 60 seconds. After pasteurisation, the juice was transferred to a 10 mL tube stored on ice. According to the specific treatment, purified active BcAP8 (15 $mg.L^{-1}$) or buffer was added, either before pasteurisation or after the juice had cooled to 2ºC. All samples were then stored at 20ºC for 16 hours. A schematic representation of treatments is presented in Figure 1a.

Grape juice fermentation

Fermentation vessels consisted of 250 mL baffled flasks sealed with silicon bungs allowing anaerobic fermentation, a sterile sampling port and a filtered air intake which was sealed outside of sampling times. Yeast inoculum was prepared according to Liccioli et al. (2011) using EC1118 yeast (Lallemand, Canada). An overnight culture was grown in 10 mL YPD media [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose] at 25ºC at 150 rpm. This culture was used to inoculate 100 mL half strength juice [50% (v/v) sterile juice, 50% (v/v) sterile water] which was cultured overnight at 20ºC at 150 rpm before use as a starter culture for 100% juice ferments. Full strength grape juice was inoculated to an OD_{600} of 0.02 and fermentations conducted at 18ºC and 150 rpm until dry.

Dose dependency of BcAP8 treatment

Fermentation of 2012 Chardonnay was carried out with the addition of active BcAP8 at final concentrations of 0.5, 2.5, 12.5 and 62.5 $mg.L^{-1}$ in a total volume of 200 mL. A control with no BcAP8 was prepared by adding buffer, and an inhibited BcAP8 control contained BcAP8 at 12.5 $mg.L^{-1}$ with pepstatin at a 5:1 molar ratio. Fermentation was conducted as described, with periodic sampling of 10 mL.

Effect of BcAP8 on bentonite requirement

Protein from 1.5 L juice was precipitated with ammonium sulphate to 80% saturation and collected by centrifugation at 16,000 *g* for 30 minutes at 4ºC. The protein pellet was dissolved in 20 mL water and dialysed against water (3 x 2 L) for a total of 24 hours at 14ºC. This protein was added back to an aliquot of the initial juice, which was termed 'protein-spiked' juice to differentiate from the initial juice ('unmodified juice'). The unmodified juice and protein-spiked juice contained 68 and 95 $mg.L^{-1}$ grape protein respectively. Both juices were fermented in the presence of 12.5 $mg.L^{-1}$ BcAP8 or buffer.

SDS-PAGE

Protein from grape juice or wine samples was precipitated by the KDS method (Vincenzi et al., 2005) using 800 µL juice or wine, 0.1% (w/v) SDS and 200 mM KCl. Protein pellets were dissolved in 100 µL SDS-PAGE sample buffer [50 mM Tris (pH 6.8), 100 mM dithiothreitol, 2% w/v SDS, 0.1% w/v bromophenol blue, 10% v/v glycerol]. SDS-PAGE was performed according to (Laemmli, 1970). Stacking gels consisted of 4% (w/v) acrylamide, resolving gels were 12%. Proteins were stained with Coomassie brilliant blue by fixing the gel in 40% (v/v) ethanol, 10% (v/v) acetic acid for 30 minutes, staining for 1 hour in staining solution [40% (v/v) ethanol, 10% (v/v) acetic acid, 0.2% (w/v) Brilliant blue R (Sigma)], then destaining in 10% (v/v) ethanol, 7% (v/v) acetic acid.

Bentonite fining trials and heat stability testing

The amount of bentonite required for protein stability was determined according to Iland *et al* (2000), using 5 mL wine samples. Heat stability testing was performed according to Pocock and Waters (2006). Samples were heated to 80ºC for 2 hours

then cooled on ice overnight. Haze was determined as absorbance at 520 nm. Samples were considered unstable if the absorbance difference between heated sample and unheated control was greater than 0.02 absorbance units.

Haze potential of BcAP8

BcAP8 was added to a commercially produced protein-stable wine at 12.5, 25, 50 and 100 $mg.L^{-1}$. Wine samples were stored overnight at 4ºC and their haze potential assessed using the heat test as described.

Statistical analysis

Protein and haze data were analysed for statistically significant differences by ANOVA and Tukey post-hoc test. Significance was set at $p<0.05$. Analyses were performed using IBM SPSS Statistics version 22.

Results and Discussion

Removal of grape PR proteins from juice by BcAP8 and pasteurisation

It has previously been noted that BcAP8 is capable of degrading grape PR proteins at winemaking temperatures, but that chitinases appear more susceptible than are TL proteins (Van Sluyter et al., 2013). As TL proteins are also found in natural haze (Esteruelas et al., 2009), it is important to target both classes of protein with any bentonite alternative. Marangon et al. (2012b) demonstrated that combining aspergillopepsin treatment of juice with pasteurisation increased the degradation of grape TL proteins compared to either treatment alone. To determine whether the ability of BcAP8 to degrade TL proteins could be similarly enhanced, Chardonnay juice was pasteurised at 75ºC in the presence or absence of BcAP8. A schematic representation of the different treatments is shown in Figure 1a. BcAP8 was added either prior to or following pasteurisation.

Pasteurisation and BcAP8 alter juice protein profile

When unpasteurised juice was treated with BcAP8 and incubated overnight at 20ºC, intact TL protein levels were reduced by approximately 20% (Figure 1c) and this reduction was inhibited by the aspartic protease inhibitor, pepstatin. Intact chitinase levels were reduced from 23.3 to 21.1 $mg.L^{-1}$, and although this reduction was not statistically significant ($p>0.05$) it was also inhibited by pepstatin. Despite this reduction in PR proteins, total protein levels were seen to increase (Figure 1b). Some of this increase can be attributed to the addition of BcAP8, but it is also likely that proteolysis products of PR proteins remained in solution and were detected by the EZQ estimation.

Intact chitinase concentration was reduced by more than 97% by all pasteurisation treatments and TL protein concentration was reduced by approximately 42% by pasteurisation alone (Figure 1c). These reductions were reflected in the analysis of total proteins. In contrast to proteolysis, where residual peptides can be detected by the EZQ kit, pasteurisation removes proteins through precipitation.

Addition of BcAP8 prior to pasteurisation did not reduce TL protein concentration more than pasteurisation alone. There was no increase in total protein corresponding to the BcAP8 addition, thus it likely precipitated during pasteurisation and was not

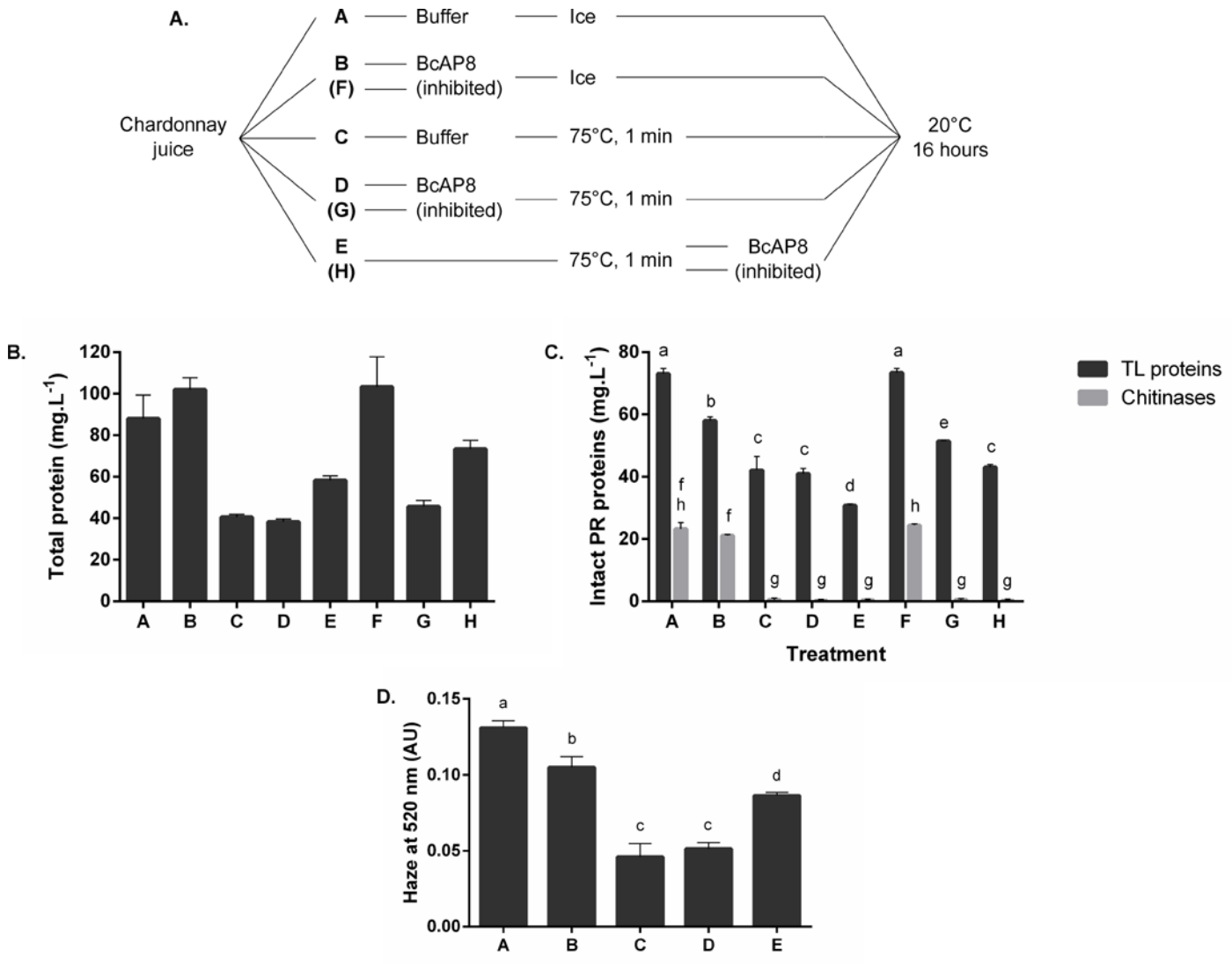

A.
Chardonnay juice
A
Buffer
Ice
B
(F)
BcAP8
(inhibited)
Ice
C
Buffer
75°C, 1 min
D
(G)
BcAP8
(inhibited)
75°C, 1 min
E
(H)
75°C, 1 min
BcAP8
(inhibited)
20°C
16 hours
B.
Total protein (mg.L-1)
C.
Intact PR proteins (mg.L-1)
TL proteins
Chitinases
Treatment
D.
Haze at 520 nm (AU)

Figure 1: Pasteurisation of grape juice in the presence or absence of BcAP8.

A) Schematic representation of treatments (A-H) used in pasteurisation experiments. BcAP8 was added to Chardonnay juice either before or after pasteurisation at 75ºC for one minute. Treatments in brackets included the aspartic protease inhibitor, pepstatin. B) Analysis of total protein in treated samples by EZQ. C) Analysis of intact PR proteins by HPLC. D) Analysis of haze at 520 nm of treated juice. In each graph, bars with different lowercase letters are significantly different, determined by ANOVA and Tukey-HSD, $p<0.05$. Error bars represent ± 1 standard deviation.

active against TL proteins before precipitating. This is supported by the fact that BcAP8 is not visible by SDS-PAGE analysis after a 55ºC heat test (Van Sluyter et al., 2013), suggesting that BcAP8 itself is not stable at elevated temperatures.

Further reduction in TL protein concentration was observed when BcAP8 was added after pasteurisation. Again, these proteolysis products and BcAP8 were detected by the EZQ assay, and proteolysis was inhibited by pepstatin.

This work demonstrates that pasteurisation at 75ºC for only one minute was sufficient to remove the majority of chitinase and a large proportion of TL protein present in this juice. A similar observation was made by Pocock et al. (2003), although combination with pepsin or aspergillopepsin treatment caused further reductions in both TL proteins and chitinases, contrary to BcAP8, whose activity was not enhanced during pasteurisation. BcAP8 is not stable at this elevated temperature, highlighting the need for a thermostable protease in order to improve the effectiveness of pasteurisation.

BcAP8 was able to degrade PR proteins in unpasteurised juice during the 20ºC incubation period. TL protein was more susceptible to this BcAP8 derived hydrolysis than was chitinase. This contrasts with previous work in which BcAP8 was more effective at degrading chitinases than TL proteins in juice at 22ºC (Van Sluyter et al., 2013). This discrepancy could arise from the presence of different PR protein isoforms in the two studies which may differ in their susceptibility to BcAP8, perhaps due to varietal differences. Another explanation is that chitinase was present at a greater concentration in the previous study, increasing the interaction frequency between BcAP8 and chitinase and allowing for greater degradation of this class of protein.

When BcAP8 and pepstatin were added prior to pasteurisation, TL-protein concentration was higher than in juice pasteurised with no addition (p<0.0005). This suggests the presence of a grape-derived aspartic protease which is active against TL proteins during pasteurisation. Tomato and tobacco have been shown to contain pepstatin-sensitive aspartic proteases capable of hydrolysing their own PR proteins, including those between 20 and 30 kDa (Rodrigo et al., 1989, Rodrigo et al., 1991). Recently, 30 *V. vinifera* aspartic proteases have been identified, 24 of which are expressed in the berry (Guo et al., 2013). The possible presence of an unidentified

grape protease could present another candidate for enzyme-mediated grape PR protein removal.

BcAP8 and pasteurisation can increase juice stability

The effect of PR protein removal by pasteurisation and BcAP8 on protein haze was investigated with a standard heat test. Juice was deemed stable if the difference in absorbance at 520 nm between heated sample and unheated control was less than 0.02 absorbance units (AU). Untreated juice was shown to be unstable, with A_{520} of 0.131 AU. Treatment of this juice with BcAP8 alone resulted in a significant reduction in haze (A_{520}=0.105 AU, p=0.002) (Figure 1d), likely through degradation of TL proteins, as chitinases were not significantly affected by this treatment.

Pasteurisation significantly reduced haze (A_{520}=0.046 AU), and addition of BcAP8 before pasteurisation had no further effect (p=0.787). This reduction in haze was achieved through the precipitation of PR proteins during pasteurisation prior to the heat test, seen as a reduction in total protein.

BcAP8 treatment following pasteurisation increased haze formation over pasteurisation alone, despite the degradation of TL proteins remaining after pasteurisation. This observation contrasts with BcAP8-treated unpasteurised juice, in which hydrolysis of TL proteins reduced haze. It is therefore likely that BcAP8 can contribute to haze. Net change in haze formation would be equal to the reduction caused by PR protein degradation plus the haze added by BcAP8. Unpasteurised juice contained more than twice as much intact PR protein than pasteurised juice, allowing greater potential for haze reduction in that treatment.

In any case, BcAP8 only improved the capacity of pasteurisation to reduce protein concentration when applied after pasteurisation, but this treatment reduced protein stability. Pasteurisation alone effectively removed chitinases and reduced TL proteins, leading to a reduction in the amount of haze formed. Marangon et al. (2012b) pasteurised Chardonnay and Sauvignon Blanc juices under similar conditions, and observed reductions in total protein of 39 and 44% respectively. Wines made from these juices produced less haze in a heat test and required less bentonite for stability.

The current study confirms that a fraction of the PR proteins precipitate during pasteurisation, and are not present to form haze during the heat test. Pasteurisation can thus be described as a controlled induction of protein haze. Whilst BcAP8 did not increase the benefits of pasteurisation, the unheated BcAP8 treatment did reduce TL protein concentration and haze formation. As BcAP8 has already been shown to degrade PR proteins during fermentation (Van Sluyter et al., 2013), it was decided to investigate the effects of this treatment in more detail. It was hypothesised that with the longer incubation times offered during fermentation, BcAP8 would degrade more of the PR proteins.

Fermentation of grape juice in the presence of BcAP8

In unpasteurised juice, degradation of TL protein by the addition of 15 $mg.L^{-1}$ BcAP8 was evident after 16 hours at 20ºC. In the previous study of Van Sluyter et al. (2013), 5 $mg.L^{-1}$ BcAP8 in an unpurified form was added to juice prior to the commencement of fermentation, a dosage which significantly reduced the PR protein content of the resulting wines. Given the reduction of TL protein concentration by BcAP8 in juice shown here, and the reduction in PR protein in the longer incubation with BcAP8 shown by Van Sluyter et al. (2013), fermentation with and without BcAP8 was investigated.

A preliminary study in which juice was fermented with different concentrations of unpurified BcAP8 suggested that PR protein degradation does not correlate in a linear fashion to BcAP8 concentration (Supplementary Figure 1). In order to determine an optimal dose for PR protein removal during fermentation, BcAP8 was added to Chardonnay juice at four different concentrations: 0.5, 2.5, 12.5 and 62.5 $mg.L^{-1}$. Two control fermentations were included: an untreated control containing buffer instead of BcAP8, and a 12.5 $mg.L^{-1}$ BcAP8 treatment in which enzyme activity was inhibited by a 5-fold excess of pepstatin. In addition, this experiment allowed investigation of the persistence of BcAP8 activity during fermentation, the effect of BcAP8 on fermentation rate, and the effect of each dosage on wine haze formation.

BcAP8 reduces intact PR protein content but not total protein content

A qualitative SDS-PAGE analysis of finished wines is shown in Figure 2. The control wines contained major proteins visible between approximately 24 and 31 kDa. The predominant protein was seen at approximately 24 kDa, followed in intensity by proteins of approximately 27 and 31 kDa. Based on previous characterisation of the migration of wine proteins by SDS-PAGE (Marangon et al., 2009), it is hypothesised that these proteins from 24 to 31 kDa are haze-forming, PR proteins. It is likely that the 24 kDa protein is a TL protein and the 27 and 31 kDa proteins are chitinases, as observed by Waters et al. (1996). Also present are proteins of 54 kDa or greater, and one of approximately 15 kDa.

Analysis of the inhibited BcAP8 control by SDS-PAGE presented a very similar profile to the untreated control, but with the inclusion of BcAP8 at approximately 32 kDa. All BcAP8 treatments substantially altered the wine protein profile. At 0.5 and 2.5 $mg.L^{-1}$ BcAP8, the 24, 27 and 31 kDa proteins were all dramatically reduced. A polypeptide of approximately 21 kDa was formed, likely a BcAP8-derived proteolysis product of one of more of these proteins. Higher and lower molecular weight proteins were largely unaffected in these treatments or in the higher BcAP8 treatments. At 12.5 $mg.L^{-1}$ BcAP8, the proteolysis product at 21 kDa decreased in intensity, suggesting further degradation of this polypeptide. In addition, the three major PR proteins were removed.

New polypeptides appeared in the higher BcAP8 treatments which likely represent proteolysis products of BcAP8. This theory is supported by the fact that the BcAP8 band in the pepstatin inhibited control is more intense than in the uninhibited treatment. Hydrolysis of BcAP8 is more obvious in the 62.5 $mg.L^{-1}$ treatment, in which all proteins between 20 and 31 kDa have been removed and replaced by BcAP8 proteolysis products. The fact that these products are the same sizes as those in the 12.5 $mg.L^{-1}$ treatment but at greater intensity supports the interpretation that these proteins are BcAP8 proteolysis products.

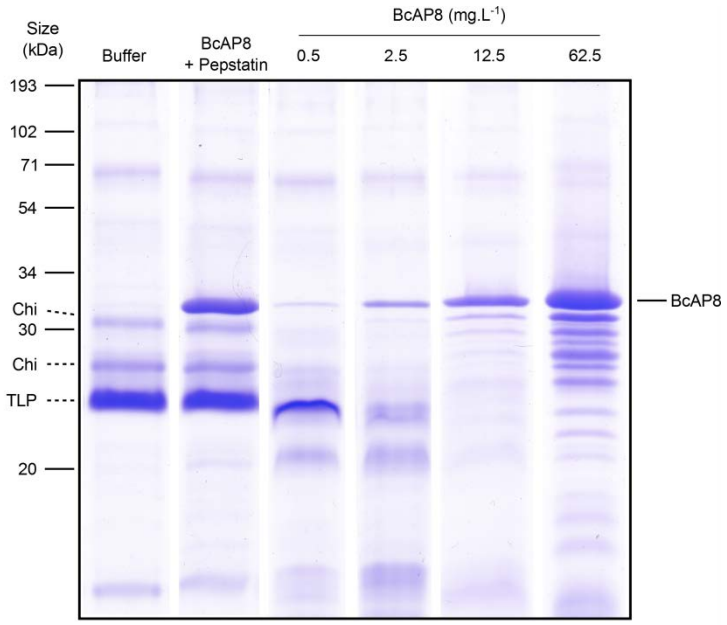


Figure 2: SDS-PAGE analysis of wine proteins.

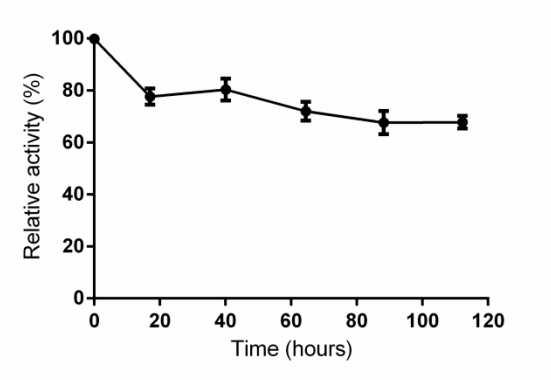
Grape juice was fermented in the presence of BcAP8 at 0.5, 2.5, 12.5 or 62.5 $mg.L^{-1}$. Total wine protein was precipitated, analysed by SDS-PAGE. Putative chitinases (Chi) and thaumatin-like proteins (TLP) are indicated on the left; BcAP8 is indicated on the right.

The effects of BcAP8 treatment seen by SDS-PAGE analysis are mirrored by HPLC quantification of intact PR proteins (Figure 3b). Chitinase levels were reduced by between 10 and 96% whilst TL proteins were reduced by 32 to 68%. These reductions are greater than those seen in the previous 15 $mg.L^{-1}$ BcAP8 treatment of juice, thus the increased incubation time compared to that used in the pasteurisation work proved beneficial. During fermentation, BcAP8 treatment at 12.5 $mg.L^{-1}$ was the minimum required to remove PR proteins visible by SDS-PAGE, and resulted in dramatic decreases in intact PR proteins determined by HPLC. These analytical methods, whilst broadly presenting the same conclusion, do not suggest the same level of intact PR protein removal. At BcAP8 concentrations of 12.5 and 62.5 $mg.L^{-1}$, SDS-PAGE demonstrates the complete removal of these proteins, whilst HPLC suggests a level of PR proteins remain. These differences are likely derived from the sample processing required for each analysis. SDS-PAGE analysis requires precipitation of the proteins, including a heating step. It is possible that further proteolysis occurs at this elevated temperature due either to BcAP8, or, more likely, a grape- or yeast-derived protease. In addition, it is possible that a proportion of total protein was not recovered from precipitation and was thus absent from SDS-PAGE analysis. Despite this inconsistency, it is clear from both methods that BcAP8 treatments induce significant reductions in the concentration of intact PR proteins.

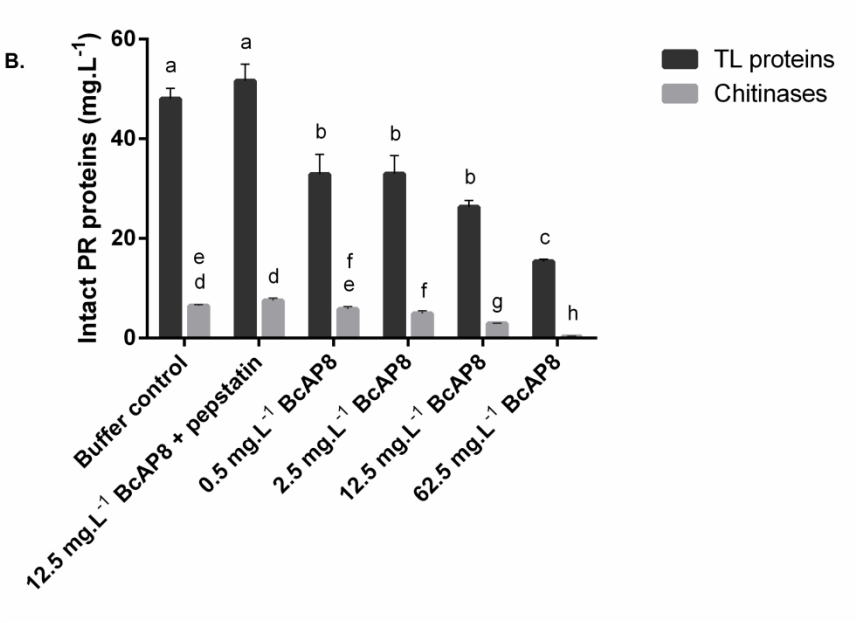
BcAP8 increases wine haze potential

The effect of each treatment during fermentation on haze formation was assessed, and the results shown in Figure 3c. Untreated wine was stable with regards to heat-induced protein haze, an unfortunate characteristic for this work, with an absorbance at 520 nm of approximately 0.011 AU. Each increasing level of BcAP8 resulted in greater haze formation, although this only became significantly different to the buffer control above 12.5 $mg.L^{-1}$ BcAP8 ($p<0.05$). This response reached a plateau in the 62.5 $mg.L^{-1}$ sample with A_{520} of approximately 0.06 AU, and the difference between the highest two BcAP8 dosages was not significant. This increasing trend in haze appears to be related to the proteolysis of PR proteins rather than BcAP8 participating directly in haze formation, as the inhibited BcAP8 treatment did not show a significantly higher level of haze than the untreated control. In addition, haze reached a plateau in the highest BcAP8 treatments, despite a 5-fold difference in

A.


Relative activity (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (hours)

B.


Intact PR proteins (mg.L-1)
60
40
20
0
TL proteins
Chitinases
a
e
a
d
b
f
b
e
b
f
b
g
c
h
d
Buffer control
12.5 mg.L-1 BcAP8 + pepstatin
0.5 mg.L-1 BcAP8
2.5 mg.L-1 BcAP8
12.5 mg.L-1 BcAP8
62.5 mg.L-1 BcAP8

C.

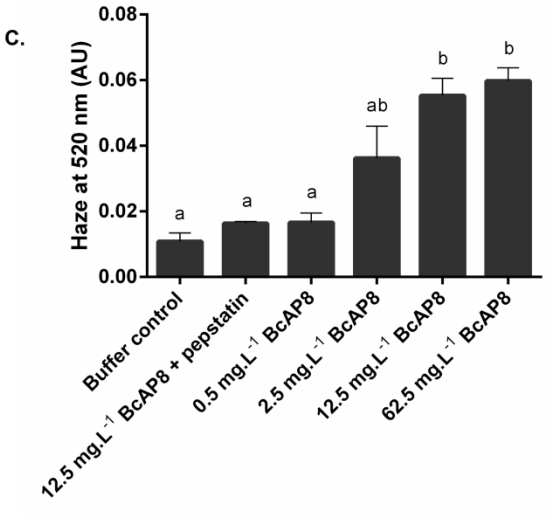

Haze at 520 nm (AU)
0.08
0.06
0.04
0.02
0.00
a
a
a
ab
b
b
Buffer control
12.5 mg.L-1 BcAP8 + pepstatin
0.5 mg.L-1 BcAP8
2.5 mg.L-1 BcAP8
12.5 mg.L-1 BcAP8
62.5 mg.L-1 BcAP8

Figure 3: Analysis of wines fermented with BcAP8.

A) Activity of BcAP8 in the 12.5 $mg.L^{-1}$ BcAP8 treatment, expressed as percentage of the initial activity, B) intact PR protein concentration following incubation with BcAP8 from 0.5 to 62.5 $mg.L^{-1}$ determined by HPLC, C) haze formation at 520 nm following heat test. In each graph, bars with different lowercase letters are significantly different, determined by ANOVA and Tukey-HSD, $p<0.05$. Error bars show ± 1 standard deviation.

BcAP8 concentration. This implies that haze is limited by the initial concentration of wine proteins, rather than the amount of BcAP8 present. Therefore, in BcAP8 treated wines, haze is a function of the concentration of wine proteins and the activity of BcAP8, with wine protein proteolysis products forming more haze than intact proteins.

The increase in haze by proteolysis of grape proteins seen here contrasts with observations in the previous study (Van Sluyter et al., 2013), in which wines fermented with BcAP8 produced less haze than controls. These differing results could be an effect of the heat tests utilised to induce haze: the previous study used a 55ºC, 18 hour test, whilst this study used an 80ºC, 2 hour test. The 80ºC test was chosen as it accurately predicts wine stability in the short to medium term (Pocock and Waters, 2006), whilst the 55ºC test was chosen as it better represents conditions to which wines are exposed during transportation (Butzke et al., 2012, Van Sluyter et al., 2013). Wine haze formation at different temperatures was investigated by Dufrechou et al. (2010), demonstrating that the mechanism of haze formation differs at 50ºC and 70ºC, with the lower temperature inducing less measurable haze with larger aggregates. In addition, different proteins had varied responses to the different temperatures, as well as to pH and ionic strength variations (Dufrechou et al., 2012). All of these factors provide points of difference between the two studies. Whilst each study compared treated wines to controls, it appears likely that the proteolysis products of wine proteins reacted differently at each temperature, thus it is possible that peptide aggregation causes less haze at 55ºC than do intact proteins, and the opposite may be true for heat tests conducted at 80ºC.

Whilst the 80ºC test is a standard test used in the wine industry to determine the effectiveness of bentonite treatments, haze formed in this test is not predictive of the amount of bentonite required for wine stability, as seen in a study by Marangon et al. (2012b). It is possible that under long term storage and transportation conditions, the haze formed during the 80ºC heat test would not form, as this heat test has been developed for analysis of wine protein haze potential only. Despite this, in the absence of long term studies of haze formation in treated wines, any bentonite replacement would likely be required to pass the industry standard test of stability.

A study of proteolytic activity secreted by different yeasts during fermentation observed that non-*Saccharomyces* yeasts could produce extracellular proteases which can degrade wine proteins (Dizy and Bisson, 2000). Whilst this resulted in wine containing less protein than *S. cerevisiae* wines, haze formation was increased. The bentonite requirement of these wines was not determined. It was suggested that these yeasts may release molecules which cause greater denaturation of the proteins present in the wine, or that the proteases secreted may themselves form haze. In this study, BcAP8 treatment does increase the level of haze, and we suggest that this occurs through the generation of unstable wine protein proteolysis products rather than direct aggregation of BcAP8.

Yeast growth is relatively unaffected by BcAP8 activity

A pepsin-like protease from *Trichoderma harzianum*, ThCAI91181 (EMBL CAI91181), has been identified as a BcAP8 homolog (ten Have et al., 2010). Expression of this protein can be induced by the presence of fungal cell wall preparations, a characteristic which suggests ThCAI91181 may play a role in mycoparasitism (Suárez et al., 2007). To investigate any negative effect BcAP8 may have on *S. cerevisiae* during fermentation, the OD_{600} of ferments was measured as a proxy for yeast biomass based on the linear correlation which has been observed between OD_{600} and *S. cerevisiae* dry cell weight (Liccioli et al., 2011). Biomass was generally lower in each treatment than the buffer control, although the difference was less than 10% at each time point (except one time point of the 62.5 $mg.L^{-1}$ treatment which was 12% lower). Biomass changes over time are shown in Supplementary Figure 2. No trend linking increasing BcAP8 concentration with lower biomass was evident, despite the 125-fold range of BcAP8 concentrations used. Biomass in the pepstatin inhibited control was also slightly lower than the buffer control, suggesting the any decrease in yeast biomass is unlikely to be caused by the activity of BcAP8. It is likely that BcAP8 treatment would not significantly affect juice fermentation rates.

BcAP8 remains active during fermentation

The duration of protease activity during fermentation was investigated by determining activity in the 12.5 $mg.L^{-1}$ BcAP8 treatment at several time points.

Activity at each point was expressed as a percentage of the initial activity (Figure 3a). Samples were diluted to achieve a BcAP8 concentration of 5 nM in the assays (approximately 70-fold dilution). Activity was also determined in the untreated and inhibited BcAP8 samples following the same dilution. BcAP8 activity was reduced to 77% of its initial activity after 17 hours in the fermentation. By the end of fermentation (approximately 88 hours), activity was reduced to 67% of the initial activity. These results demonstrate that BcAP8 remains active during fermentation. The rate at which activity was lost decreased after 17 hours, suggesting that activity may be retained well after fermentation was complete. Van Sluyter et al. (2013) observed that BcAP8 treated wines showed further reductions in PR protein concentration after storage for one year at 4ºC, although protease activity was not determined during this time. The extended activity of BcAP8 may allow lower additions of the enzyme to fermentations whilst continuing to degrade the same amount of PR proteins, albeit over a longer time period.

The decrease in activity over time is likely caused predominantly by BcAP8 autolysis, as BcAP8 degradation products are evident in SDS-PAGE analyses. Proteolysis of grape proteins would disrupt their tertiary stability, exposing more cleavage sites to hydrolysis. This creation of alternate substrates would lead to a decrease in BcAP8 autolysis, slowing the rate of BcAP8 activity loss.

No proteolytic activity was detected in either control treatment, despite the fact that aspartic protease activity has been detected in grape juice fermentations (Dizy and Bisson, 2000). It is probable that the large dilution required to attain an appropriate level of BcAP8 activity prevented detection of endogenous activity.

BcAP8 can form haze in the absence of wine proteins

Despite evidence provided here suggesting that BcAP8 added during fermentation does not participate directly in haze formation in finished wine, it was thought prudent to test this hypothesis in a model system. A commercial, protein-free Chardonnay wine was purchased and spiked with BcAP8 at increasing concentrations before being subjected to a heat test.

BcAP8 formed haze in a dose-dependent manner in the absence of wine proteins, conforming to an exponential equation (Figure 4). This response contrasts with that seen in wines fermented with BcAP8, in which haze was limited by the PR protein

concentration of the juice rather than the BcAP8 concentration (Figure 3c). The addition of purified BcAP8 to protein-free wine represents a model system for BcAP8 self-aggregation in the absence of grape protein, however there is no practical situation where BcAP8 would be added to a juice or wine which was free of other protein. Protein aggregation in a model system does not necessarily reflect that in a real wine. Whilst TL-proteins have been found in naturally occurring wine hazes (Esteruelas et al., 2009), purified TL-protein unfolded in a reversible manner in a model system, and aggregation was not observed (Falconer et al., 2010). This indicates that proteins in wine may interact differently according to the protein composition, and suggested that the less stable chitinases may facilitate the aggregation of TL-proteins in real wine. Whilst self-aggregation of BcAP8 molecules may have contributed to haze seen in BcAP8-treated ferments, this appears to be a minor effect compared to that of PR proteins and their proteolysis products.

Interaction between BcAP8 and PR protein concentration

In order to determine the effect of BcAP8 treatment on bentonite requirements, a second fermentation containing 12.5 $mg.L^{-1}$ BcAP8 was performed. Two forms of a single Chardonnay juice were fermented: an unmodified batch, and a batch which was spiked with additional grape protein. The unmodified juice had an initial total protein concentration of approximately 68 $mg.L^{-1}$, whilst the spiked juice had a concentration of 94 $mg.L^{-1}$. Initial juice protein concentration, bentonite-fined wine protein concentration, haze formation and bentonite requirement are summarised in Table 1.

PR protein proteolysis products form more haze than intact proteins

Heat tests showed that the control wine prepared from unmodified juice was stable whilst the corresponding BcAP8 treated wine was unstable, with an A_{520} of 0.0575. The control wine prepared from spiked juice was unstable, with A_{520} of 0.262, significantly higher than the unmodified control wine ($p<0.05$). The BcAP8 treated wine prepared from spiked juice formed more haze than both its control and the BcAP8 wine prepared from unmodified juice (A_{520} of 0.0794). As both BcAP8 treated wines contained the same concentration of BcAP8, the hypothesis that wine haze potential is limited by the initial grape protein concentration is supported. In

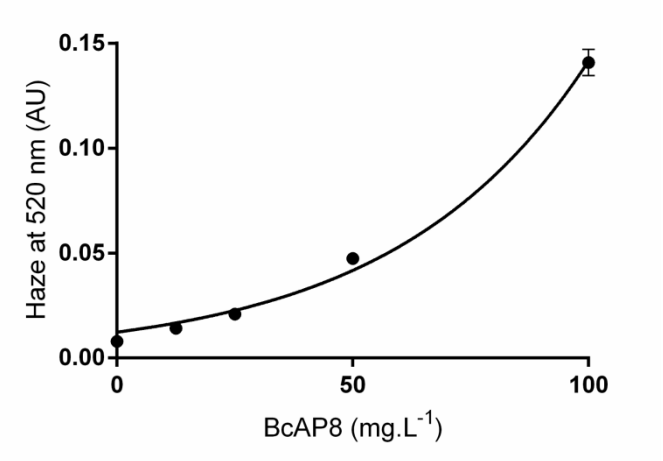


Figure 4: Heat induced haze produced by BcAP8.

BcAP8 was added to a commercial heat-stable wine to final concentrations of 12.5, 25, 50 and 100 mg.L^{-1}. Samples were subjected to a heat test and haze determined as absorbance at 520 nm. Error bars represent ± 1 standard deviation.

addition, this provides confirmation that grape protein proteolysis products are less thermostable than their intact counterparts.

BcAP8 increases bentonite efficiency

Bentonite fining trials were conducted to determine the amount of bentonite needed to render the wines protein stable. As stated, the control wine made from unmodified juice was stable, so did not require any bentonite. All other wines required 0.1 $g.L^{-1}$ to induce stability.

The total protein concentration of fined wines was quantified using the EZQ kit (Table 1). The control wine from unmodified juice was also fined with 0.1 $g.L^{-1}$ bentonite for this analysis in order to serve as a control for the BcAP8 treated wine made from this juice. After fining, the unmodified control contained 18 $mg.L^{-1}$ total protein and the spiked control contained 33 $mg.L^{-1}$, whilst neither of the BcAP8 treated wines contained any quantifiable protein. Analysis of fined wine samples by SDS-PAGE supported the total protein analysis. No protein was detectable in the 'unmodified' BcAP8 wine, and only very faintly stained protein bands were visible in the spiked BcAP8 wine (Figure 5). In contrast, proteins are visible in both controls, including between 24 and 31 kDa representing putative PR proteins.

These results are consistent with Hsu and Heatherbell (1987b), who also observed residual proteins of these sizes in bentonite-fined, stable wine. These protein analyses suggest that although BcAP8 treatment increases haze formation, it can increase the efficiency of bentonite fining, given that all wines were fined at the same level. Although the wines used in this analysis were inherently quite stable, this improved efficiency could make a significant difference to the bentonite requirement of less stable wines. This conclusion is supported by the findings of Marangon et al. (2012b), who fermented grape juice in the presence of aspergillopepsin and observed varied effects on protein content and haze. One wine contained 17% less total protein than untreated wine, including reductions in TL proteins and chitinases. Despite this, haze was not reduced, although bentonite requirement was reduced 33%. Another wine experienced similar protein reductions, and a 29% reduction in haze, but bentonite requirement was only reduced 8%. This demonstrates that haze intensity is not correlated with bentonite requirement. In fact, the increased haze produced in BcAP8 treated wines may serve as an indicator of the generation of PR protein

Table 1: Summary of protein content and bentonite requirement of normal juice and protein-spiked juice fermented in the presence or absence of BcAP8.

After fining to stability with bentonite, BcAP8 treated wines contained no detectable protein. *The control wine made from juice containing 68.26 $g.L^{-1}$ protein was stable without bentonite fining, but was fined to serve as a control.

Treatment	Initial grape protein ($mg.L^{-1}$)	BcAP8 ($mg.L^{-1}$)	Haze at 520 nm (AU)	Bentonite required ($g.L^{-1}$)	Protein after bentonite ($mg.L^{-1}$)
Control	68.26	0	0.0188	0.1*	18.38 ± 0.7
BcAP8		12.5	0.0575	0.1	0
Control	94.74	0	0.0262	0.1	33.05 ± 5.59
BcAP8		12.5	0.0794	0.1	0

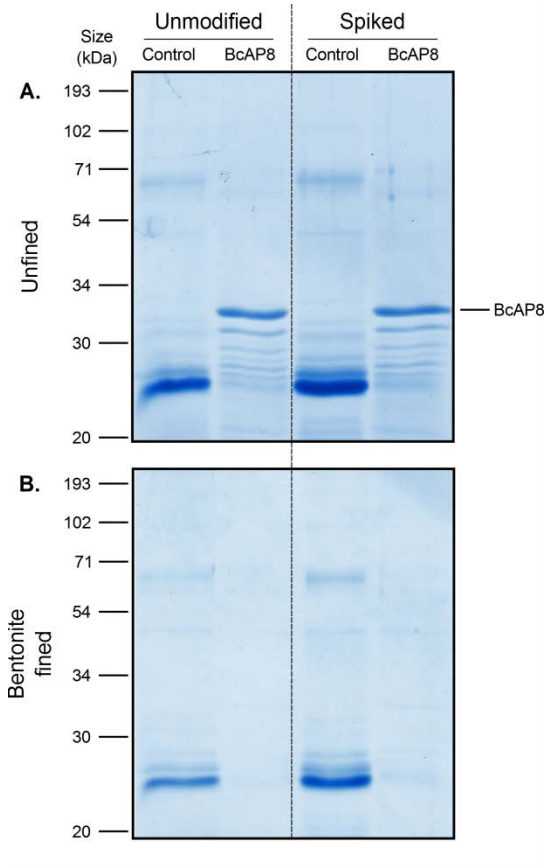


Figure 5: Effect of BcAP8 during fermentation on wine protein composition. Unmodified juice (left) and juice spiked with grape protein (right) were fermented with or without BcAP8 at 12.5 $mg.L^{-1}$. Total wine proteins were precipitated and analysed by SDS-PAGE. A) Wine protein composition at the end of fermentation, B) Wine protein composition after bentonite fining to protein stability (0.1 $g.L^{-1}$ in each case).

proteolysis products which are more amenable to removal by bentonite than are intact proteins.

This report demonstrates a potential improvement to the efficiency of bentonite fining that could translate into significant economic benefits. Whilst pasteurisation has the potential to reduce haze formation and bentonite requirements (Marangon et al., 2012b), we report here the ability of BcAP8 to improve bentonite efficiency without the need for additional expensive infrastructure or processes. This work also suggests the presence of a thermostable grape aspartic protease capable of degrading grape PR proteins at winemaking temperatures. This provides an alternative candidate for enzymatic degradation of these proteins, either alone or in combination with BcAP8.

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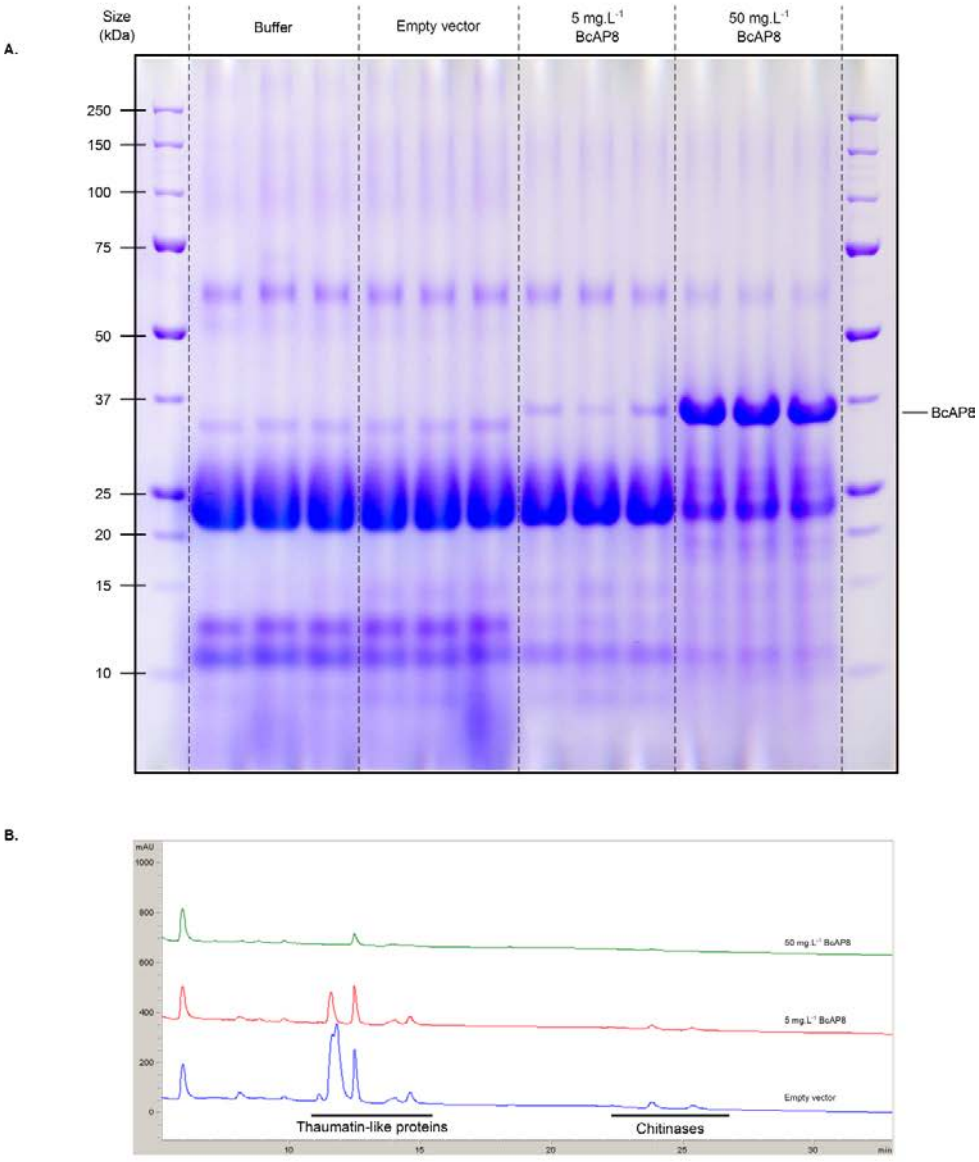
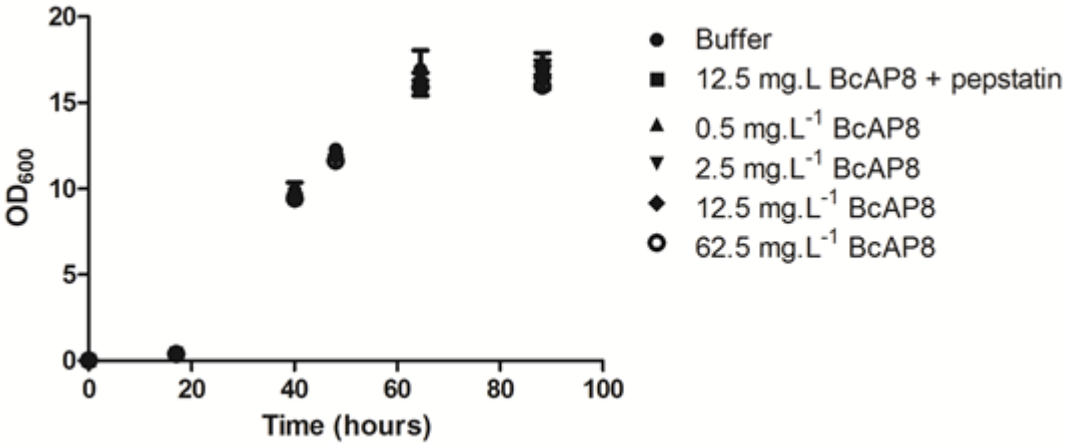
Supplementary figures



Supplementary Figure 1: Analysis of wines made with a crude BcAP8 preparation added during fermentation.

BcAP8 was added to juice at 5 or 50 mg.L^{-1}. Controls contained equivalent buffer or empty vector additions. A) Proteins were precipitated by ethanol and analysed by SDS-PAGE, B) Chromatogram of wine samples analysed by HPLC to quantify intact pathogenesis-related proteins according to the method of Van Sluyter et al. (2009), C) Intact PR protein concentration in all wines. Fermentations were conducted in triplicate. Error bars represent ± 1 standard deviation.



Supplementary Figure 2: Investigation of the effect of BcAP8 on yeast biomass during fermentation.

BcAP8 was added to juice at 0.5, 2.5, 12.5 or 62.5 $mg.L^{-1}$. Control treatments contained added buffer instead of BcAP8 or BcAP8 at 12.5 $mg.L^{-1}$ inhibited with pepstatin. OD_{600} was measured as a proxy for yeast biomass.

Final Discussion

Aspartic protease activity has been implicated as a *B. cinerea* virulence factor based on the fact that it is secreted during pathogenesis, and that its putative targets have antifungal activity. Mutant *B. cinerea* strains in which some of the fourteen aspartic protease genes had been knocked out failed to demonstrate altered virulence phenotypes, including the *Bcap8* knockout which lost the majority of its secreted aspartic protease activity (ten Have et al., 2010). Despite this, *B. cinerea* expends a great deal of resources in producing this enzyme during infection, which accounts for approximately one quarter of the secreted protein, suggesting it is likely to have an important role early in pathogenesis. BcAP8 is able to hydrolyse grape chitinase and TL protein in juice, PR proteins which otherwise remain in wine after surviving the fermentation process (Van Sluyter et al., 2013). Whether these proteins are the intended physiological substrates for BcAP8, or whether this ability is conferred by a broad substrate specificity often associated with aspartic proteases, is unknown.

In an effort to answer this and other questions, BcAP8 was produced in a heterologous system and purified to homogeneity. The N-terminal prodomain of zymogenic BcAP8 was glycosylated, however the mature and active form of the enzyme was not. General biochemical analyses demonstrated that the conditions required for activation and maximum activity of this enzyme *in vitro* coincide with conditions within fruit which hosts *B. cinerea*. This fungus also possesses the ability to alter the pH of its environment towards these conditions (Manteau et al., 2003). BcAP8 was found to activate through an autocatalytic process, cleaving its N-terminal prodomain at a different location than had been predicted by ten Have et al. (2010). This suggests a substrate specificity different to pepsin, which does not appear to cleave Asp-Lys bonds (Hamuro et al., 2008).

Further differences between BcAP8 and pepsin were identified by kinetic analysis. BcAP8 has a lower K_M for the substrate studied than does pepsin (Yoshimasu et al., 2002) suggesting that BcAP8 binds the substrate more efficiently. The k_{cat} of BcAP8 for this substrate is also lower than that of pepsin, indicating that BcAP8 is less efficient at cleaving the Phe-Phe bond in this case. Several other fungal aspartic proteases share similar kinetic parameter values with BcAP8, which all appear to be more tolerant of amino acid substitutions within the substrate than is pepsin (Dunn et

al., 1986). This suggests that fungal aspartic proteases may compromise on catalytic efficiency in favour of developing broader substrate specificity than pepsin, a trait which BcAP8 may share.

To further characterise the kinetic properties of *B. cinerea* aspartic proteases and help to define groups of functional redundancy within this family of proteases, expression of BcAP1, 3, 4 and 5 in addition to BcAP8 was attempted. Only BcAP8 was suitable for further analysis. Regardless, it is possible that direct comparison of the interactions between these aspartic proteases and a single substrate would have provided limited information regarding functional redundancy. Comparison of k_{cat}/K_M between two enzymes is only valid when the ratio between each Michaelis-Menten curve is constant at each substrate concentration, rarely the case even when using two enzymes of the same k_{cat}/K_M (Eisenthal et al., 2007).

To avoid relying too heavily on this kinetic data, a more informative approach was pursued, confirming that BcAP8 is able to hydrolyse grape PR proteins *in vitro*, and using one of these to investigate substrate specificity. BcAP8 was able to hydrolyse a grape chitinase and TL protein, while pepsin was not. Thaumatin-like protein C from grape was digested with BcAP8 and the peptides sequenced, identifying almost 50 sites of cleavage in the peptide chain. Whilst this does not represent a statistically large number of bonds in terms of a full characterisation of BcAP8 substrate specificity, investigation of a likely physiological substrate protein elucidated specific features of this enzyme which give it this unique ability. Comparison of this data with information regarding substrate specificity of the gastric aspartic protease, pepsin, revealed that the inability of pepsin to cleave bonds with Gln or Pro in P_1 and Gly, Lys and Pro in P_2 (Hamuro et al., 2008) may prevent it hydrolysing PR proteins.

This inability is in itself intuitive: a protease from an organism which is not targeted by antifungal plant PR proteins is not able to digest these proteins, whilst a fungus that is confronted by these proteins during infection, is. The fact that *B. cinerea* is able to infect grapes at full maturity when PR proteins are at their most abundant, whilst other generally successful pathogens such as *U. necator* cannot, further correlates these novel aspartic proteases like BcAP8 to the enhanced pathogenesis of *B. cinerea*.

Despite this, the role of this enzyme as a virulence factor has not been incontrovertibly demonstrated, and it is unlikely such a link can be made solely using *in vitro* analyses described here. One of the aims of this work was to begin characterising groups of redundancy within *B. cinerea* aspartic proteases to help guide *in vivo* experimentation, however this was prevented by the inability to produce other members of this family in the same manner as BcAP8.

In order to unambiguously define the roles of these aspartic proteases, other members must first be purified, either directly from *B. cinerea* or from heterologous systems. Given the large number of *B. cinerea* aspartic proteases, their relatively low expression *in vivo* compared to BcAP8 and their secretion as active proteases from the native fungus, recombinant methods are likely to enable more reproducible purification strategies that will purification from *B. cinerea* culture. *P. pastoris* has in this case produced a functional *B. cinerea* aspartic protease, confirming the suitability of this host. Expression and purification of other BcAP proteins may succeed from intracellular expression in *P. pastoris*, particularly those which are not predicted to be secreted by *B. cinerea*. BcAP5 has been produced at low levels in *P. pastoris* in a variable and hyper-glycosylated state (Reid, 2010). The polyhistidine tag used for immunohistochemical detection and purification may be blocked by carbohydrate, possibly explaining the inefficiency of nickel affinity chromatography of BcAP5. Expression of BcAP5 in *P. pastoris* may benefit from intracellular expression to avoid N-linked glycosylation, or expression in a different host organism such as *E. coli*.

Purification of other BcAP enzymes will allow characterisation of their biochemical properties including substrate specificity, which will likely be the most direct method of identifying subsets of functionality. Initially, such experiments could be investigated with PR protein digest assays as have been presented in this study of BcAP8. If the physiological role of some of these enzymes is to hydrolyse PR proteins, these assays may be sufficient to identify proteins with differing function. An alternative method of characterising specificity would be to use a library of synthetic peptide substrates to determine specificity at each subsite of individual enzymes, although this data may not help to elucidate function given how far this type of analysis is removed from a physiological situation.

Expression of *B. cinerea* aspartic protease genes could also be investigated through qPCR, determining the timing and magnitude of any changes. It is logical that functionally related enzymes would be expressed a similar manner. This potentially represents a simpler method to classify enzymes to inform further studies of *in vivo* function. It would also be interesting to investigate expression of these genes in *B. cinerea* isolates which differ in virulence toward a single host. Reduced levels of aspartic protease activity have been associated with reduced *B. cinerea* virulence (Zalewska-Sobczak et al., 1981) and isolates with reduced virulence enhance the accumulation of host PR proteins (Derckel et al., 1999). It is likely that accumulation of these PR proteins may be enabled by these less virulent isolates secreting less aspartic protease activity capable of degrading these PR proteins. In this case, a reverse approach to identifying the function of these proteases could utilise genetic transformation of *B. cinerea* to increase the secreted aspartic protease activity of a less virulent strain, determining any changes in virulence phenotype.

Independent of method chosen for subsequent research, the initial focus should be on the remaining BcAP proteins predicted to be secreted, namely BcAP5, 7, 9 and 10. Inclusion of *Bcap8* would also be prudent in any analysis of gene expression during pathogenesis by different isolates. It would also be beneficial to investigate the function of intracellular aspartic proteases, which are less likely to have developed activity specifically against PR proteins. This would provide a control which would allow confidence in any results suggesting that the ability of other aspartic proteases to degrade PR proteins is physiologically relevant.

Having defined functionally similar proteins, their effect on *B. cinerea* virulence, and other aspects of the fungal life cycle, can be investigated following a multiple-knockout strategy as begun by ten Have et al. (2010). That study used plate assays to investigate the effects of purified PR proteins on *B. cinerea* growth, attempting to identify differences in sensitivity between the knockout mutants. The wild-type strain used was already sensitive to these PR proteins, and this was not increased in any of the knockouts. The fact that *B. cinerea* is able to mount successful infection of grape containing a concentration of PR proteins which is capable of inhibiting the fungus *in vitro* (Derckel et al., 1998) suggests that this assay may not be representative of *in vivo* interactions between the fungus and plant defences. Whilst this method is easy to perform and control, it may be more informative to investigate the effect of *Bcap*

knockouts by quantifying PR proteins *in vivo* in infected plants. This will also ensure the correct environmental conditions for secretion of aspartic proteases (Manteau et al., 2003) so that their true effect on virulence can be observed.

Identification of a role for specific *B. cinerea* aspartic proteases in pathogenesis could provide targets for inhibitors to be used to control this pathogen in an agricultural setting

The other major aim of this work was to investigate the potential for *B. cinerea* aspartic proteases to be used to degrade grape PR proteins which form haze in white wine. It has previously been observed that *B. cinerea* aspartic proteases can degrade grape PR proteins *in vivo* (Girbau et al., 2004) and that BcAP8 is responsible for the vast majority of the proteolytic activity secreted by *B. cinerea* (ten Have et al., 2010), suggesting that BcAP8 may possess the majority of this unique ability. BcAP8 expressed during this study was used to investigate the hydrolysis of wine proteins both in grape juice and during small scale fermentation (Van Sluyter et al., 2013). BcAP8 was active against TL proteins and chitinases in both situations and decreased haze formed in wine. This study confirmed the ability of BcAP8 to hydrolyse these proteolysis-resistant PR proteins, however the application to winemaking required further investigation, hence the work presented here.

Pasteurisation of grape juice has been investigated for its ability to stabilise wine without the use of bentonite (Pocock et al., 2003). Proteins are removed through precipitation by pasteurisation at 90ºC, especially chitinases which do not have the ability to return to their native state after thermal denaturation (Falconer et al., 2010). This protein removal translated into a reduction in the amount of bentonite required to stabilise wine (Pocock et al., 2003). Attempts have been made to enhance the effects of pasteurisation through addition of proteolytic enzymes, specifically pepsin and aspergillopepsin (Pocock et al., 2003, Marangon et al., 2012b). Aspergillopepsin has proven particularly useful in allowing the removal of greater amounts of protein during pasteurisation and further reducing the amount of bentonite required, although its effects without pasteurisation are more modest.

As BcAP8 has demonstrated activity against grape PR proteins in their native conformation (Van Sluyter et al., 2013), it was hypothesised that its presence during pasteurisation would improve the efficiency of PR protein removal. Testing of this

hypothesis demonstrated that whilst BcAP8 could reduce intact PR protein concentration without pasteurisation, it is not sufficiently thermostable to survive pasteurisation, precipitating during the process without offering any improvement. During the course of this investigation, inhibition of BcAP8 with pepstatin also appeared to inhibit endogenous grape aspartic protease activity and its ability to degrade TL proteins. *Vitis vinifera* expresses 30 aspartic proteases, up to 24 of which can be found in the berry (Guo et al., 2013). It is possible that one or several of these proteases are active against TL proteins, thus the grape itself could represent a future source of novel aspartic proteases for use in wine protein stabilisation. The production of aspartic proteases capable of degrading endogenous PR proteins is not novel. Tomato (Rodrigo et al., 1989) and tobacco (Rodrigo et al., 1991) both produce such enzymes, and the proteins degraded by the latter are TL proteins (Vigers et al., 1992). It has been hypothesised these proteases play a role in PR protein turnover in plants (Rodrigo et al., 1991).

Preliminary studies of BcAP8 activity against PR proteins during grape juice fermentation suggested a non-linear correlation between BcAP8 dosage and PR protein degradation. Based on this finding, Chardonnay juice was fermented in the presence of BcAP8 at five different concentrations in order to identify the optimal dosage for this juice. After one week, addition of 12.5 $mg.L^{-1}$ BcAP8 was sufficient to completely digest PR proteins visible by SDS-PAGE. Lower addition rates also digested a significant amount of intact PR protein, and a strong dose-dependency was observed in which PR degradation efficiency decreased as BcAP8 concentration approached PR protein concentration.

The best measure of an optimal BcAP8 addition rate is the stability of the final wine. In heat tests, BcAP8 treatment increased haze through the generation of proteolytic peptides with increased haze potential. This is in contrast to the reduction in haze achieved in the previous study (Van Sluyter et al., 2013). This varying response is due to use of different heat tests; the study presented here used an 80ºC, 2 hour test compared to the 55ºC, 18 hour test used by (Van Sluyter et al., 2013). It has been demonstrated that the 80ºC test accurately predicts haze formation in wine over short to medium term storage (Pocock and Waters, 2006), however there is no published data supporting the accuracy of a 55ºC heat test. It seems logical however that haze formed in a heat test in which PR proteins reach their melting temperature is likely to

form by a different mechanism to natural haze, and that heat tests below these temperatures may prove more accurate.

The application of new methods to haze prevention necessitates a more thorough investigation of alternate methods of haze prediction. The benefits of this will be two-fold: not only will the efficacy of new haze prevention methods be accurately determined, but more accurate heat tests can be applied to determine bentonite fining rates in commercial settings, in order to minimise excessive fining as a result of the over-zealous standard heat test. New predictive methods should be correlated to wines stored under ideal conditions and those which simulate real world heat exposure experienced by wines during transportation and storage. It will be beneficial to include wines produced with the aid of BcAP8 in this analysis to determine whether it does in fact prevent or reduce the formation of natural haze.

Although BcAP8 increases the haze formed in heat tests, it appears to increase the efficiency of haze protein removal by bentonite. Investigation of this increased efficiency was restricted to wine which was inherently quite stable, but could represent a significant improvement over unassisted bentonite fining. This represents the most significant application presented from this study and is worthy of further investigation. A reduction in the amount of bentonite used would likely improve most aspects of the treatment, including: (i) reduction in non-specific removal of aroma compounds and associated quality downgrades, (ii) reduction in wine volume unrecoverable from bentonite lees and (iii) a reduction in bentonite waste. All of these improvements would bring large economic benefits to the stabilisation process. To determine whether increased protein removal efficiency does translate into decreased bentonite requirements, and the scale of this reduction, wines made with and without BcAP8 addition need to be analysed for their bentonite requirement. To gain a comprehensive picture of the effect of BcAP8 in wine stability, these wines could be the same as those used for long term storage trials to determine natural haze formation with and without addition of BcAP8.

The data presented here demonstrates that BcAP8 possesses novel substrate specificity which supports the ability *B. cinerea* to infect fruit containing high concentrations of antifungal PR proteins. This same characteristic can be harnessed in wine production as BcAP8 has the potential to reduce the amount of bentonite required for protein stabilisation and thus mitigate the negative effects of this

standard expensive and wasteful treatment. I believe that the work presented here represents a viable and effective addition to methods of protein haze removal from wine and is worthy of further investigation in a commercial setting.

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