JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Aspartic Acid Protease from *Botrytis cinerea* Removes Haze-Forming Proteins during White Winemaking

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Supporting Information

ABSTRACT: White wines suffer from heat-induced protein hazes during transport and storage unless the proteins are removed prior to bottling. Bentonite fining is by far the most commonly used method, but it is inefficient and creates several other process challenges. An alternative to bentonite is the enzymatic removal of haze-forming grape pathogenesis-related proteins using added proteases. The major problem with this approach is that grape pathogenesis-related proteins are highly protease resistant unless they are heat denatured in combination with enzymatic treatment. This paper demonstrates that the protease BcAP8, from the grape fungal pathogen *Botrytis cinerea*, is capable of degrading chitinase, a major class of haze-forming proteins, without heat denaturation. Because BcAP8 effectively removes haze-forming proteins under normal winemaking conditions, it could potentially benefit winemakers by reducing bentonite requirements.

KEYWORDS: *wine protein haze, Botrytis cinerea, aspartic protease, pathogenesis-related protein, chitinase, Vitis vinifera, bentonite alternative*

INTRODUCTION

Vitis vinifera grape berries contain high levels of pathogenesis-related (PR) proteins that persist through winemaking and cause commercially unacceptable heat-induced hazes if not removed before bottling.[1] The majority of commercial wineries use the clay cation exchanger bentonite to remove PR proteins from wine, but the process results in wine losses, is laborious, and can negatively affect wine sensory properties.[2]

An alternative to protein removal by bentonite is the enzymatic hydrolysis of PR proteins by proteases. However, PR proteins are highly resistant to enzymatic proteolysis, and no commercially viable enzymatic methods are currently available for use at typical winemaking temperatures. A recent enzymatic alternative to bentonite uses the combination of a heat-tolerant fungal protease preparation with flash pasteurization.[3] Combined heat denaturation of PR proteins and enzymatic hydrolysis effectively reduces or prevents wine protein haze, but incurs the costs of specialized equipment and heating energy. An ideal enzymatic alternative to bentonite would not require heating. The present study demonstrates the effectiveness of a fungal protease in removing haze-forming proteins during winemaking at typical temperatures.

The secretion of proteases by *Botrytis* during infection has been extensively observed.[4−6] More recent studies have identified the secreted proteases as primarily aspartic proteases,[6−9] a serine protease, and an unusual glutamic acid protease.[10] Marchal et al.[11] proposed that *Botrytis* proteases are responsible for negatively affecting sparkling wine foam properties by removing proteins and subsequently characterized wine protein changes induced by *Botrytis* infection, suggesting a role of *Botrytis* proteases in wine protein degradation.[12−14] However, a direct causal relationship between *Botrytis* proteases and wine protein content was not demonstrated.

The aspartic acid family of *Botrytis* protease has been characterized by sequence analysis and expression studies,[6,15] but without evidence for a biological role of the enzymes. Ten Have et al.[15] characterized the *Botrytis cinerea* aspartic acid proteases by sequence analysis and knockout mutants. The mutants were grown on several types of plant materials and in the presence of grape PR proteins on agar plates, but no phenotypic differences were observed between the wild type and mutants.

One of the findings presented in ten Have et al.[15] was that a particular aspartic protease, BcAP8, is the predominant protein secreted into liquid media. Even though BcAP8 knockout mutants did not display a different phenotype, the high levels of production of the enzyme in the wild type were the motivation for the current study.

In this study, BcAP8 was added to juice prior to inoculation with yeast and successfully removed chitinases under normal winemaking temperatures and conditions. Thaumatin-like

Received: June 24, 2013
Revised: September 4, 2013
Accepted: September 6, 2013
Published: September 6, 2013

proteins (TLPs) were mostly unaffected. Even though BcAP8 only partially reduced PR protein levels in the finished wines, the impact on heat-induced haze was significant. Chitinases have recently been shown to contribute to wine haze more so than TLPs.[16−18] It is possible that by selectively removing chitinases from wines, BcAP8 could either heat stabilize wines or at least dramatically reduce the requirement for bentonite treatment.

MATERIALS AND METHODS

***Botrytis* Aspartic Protease BcAP8 Heterologous Production in *Pichia pastoris*.** BcAP8 was transformed into and produced in *P. pastoris* according to the methods of Schmidt et al.[19] The predicted BcAP8 amino acid sequence from ten Have et al.[15] was used to create the corresponding cDNA, which was codon optimized for expression in *P. pastoris* and synthesized by GeneArt (Regensburg, Germany). *Not*I and *Xho*I restriction sites were included in the synthetic cDNA and used by GeneArt for insertion in a pPICZα C vector (Invitrogen). The expression vector and BcAP8 gene sequence are described in Supporting Information Figures S1 and S2. A successful *P. pastoris* transformant was selected by zeocin resistance and grown, and BcAP8 expression was induced with methanol addition, according to the method of Schmidt et al.[19] Secreted BcAP8 in synthetic medium PNB1[20] was acidified to promote autohydrolysis of the propeptide inhibitor domain. Acidified and activated BcAP8 was 0.17 mg protein/mL and produced a single band by SDS-PAGE and was not purified further. Culture supernatant from the same *P. pastoris* strain transformed with the empty expression vector was used as a control on a volume basis.

Assessment of BcAP8 Activity in Grape Juice. BcAP8 and pepsin (Sigma) were added at 5 mg/L to Australian Semillon and Sauvignon blanc juices (200 μL per reaction + 1 mM sodium azide to prevent microbial growth). Pepsin (Sigma-Aldrich, Australia; P6887) was from a 0.1 mg/mL stock solution (A_{278}, ε = 51300 M^{-1} cm^{-1}, 34620 Da, values from Sigma product information) in 50 mM KH_2PO_4, pH 2, with H_3PO_4. Culture supernatant from empty vector transformed *P. pastoris* was used at the same rate as BcAP8 (34-fold dilution) as a negative control. Pepstatin A (2 mM in DMSO) was added to inhibitor treatments at 20 μM; 1% DMSO was added to treatments without pepstatin. Once all components were added to the juices, 100 μL of each was used for reactions at 22 °C for 21 days and the remainder incubated at 40 °C for 18 h. Following incubation, total protein was methanol/chloroform extracted according to the method of Wessel and Flügge[21] and used for SDS-PAGE with NuPage gels according to the manufacturer's instructions (Invitrogen). Gels were stained with Coomassie Brilliant Blue R-250 (Pierce Imperial Stain, Thermo Scientific) according to the manufacturer's instructions. The lower of the two major bands in the 20−25 kDa range was considered putative TLP and the higher of the two bands, putative chitinase, on the basis of previous studies in which SDS-PAGE bands were subjected to protein identification by mass spectrometry.[3,18,22]

Assessment of BcAP8 Activity in Fermentations. Yeast cultures were started with Zymaflore VL3 yeast (Laffort) added to 5 mL of yeast peptone dextrose broth, followed by incubation with mixing at 30 °C for 24 h. The VL3 culture was added to 10 mL of synthetic juice (1× Difco yeast nitrogen base (Becton Dickinson), 100 g/L glucose, 100 g/L fructose, 4 g/L tartaric acid, 4 g/L malic acid, adjusted to pH 3.5 with KOH) supplemented with 10 g/L ammonium sulfate, and that mixture was incubated at 28 °C in a 50 mL flask on an orbital shaker at 180 rpm overnight.

Two Australian *Vitis vinifera* juices, a Sauvignon blanc (21.8 °Brix, pH 3.36, titratable acidity (TA) to pH 8.2: 5.2 g/L as tartaric acid) and a Semillon (18.8 °Brix, pH 3.30, TA: 5.3 g/L), were syringe filtered (32 mm 0.8/0.2 μm Acrodisc PF filters, Pall Corp., Australia) and used for BcAP8 ferments. To account for the possibility that either phenolics or other components of grape juice might inhibit BcAP8, total juice protein ammonium sulfate precipitated (80% saturation) from a second Semillon juice was added to 300 mg/L in synthetic juice. BcAP8 was added at 5 mg/L to the three musts while they were on ice; culture filtrate from empty vector transformed *Pichia* was added in equal volume (34-fold dilution) to controls. The musts were incubated at 17 °C for 17 h and then inoculated with 10^6 cells/mL from the yeast starter cultures. Diammonium phosphate was added to the two authentic juices to increase available nitrogen by 150 mg/L. Inoculated musts were split among three formats: 8 wells per treatment of 200 μL each in clear 96-well plates for biomass assays (A_{600}); 4 wells per treatment of 1.5 mL in a 96-well 2 mL Masterblock (Greiner Bio One, Australia) for enzymatic sugar assays; 4 mL of Sauvignon blanc and Semillon, 3 mL for the synthetic juice treatments, all in triplicate, in 5 mL polypropylene screw-cap sample tubes (Sarstedt).

The 96-well plates were sealed with Breathe-Easy membranes (Sigma-Aldrich), the screw caps left loose, and the ferments incubated at 18 °C, 2.3−2.5% O_2, in a Cytomat incubator (Thermo Scientific) interfaced with a Freedom Evo liquid handling robot (Tecan Australia). The robot was configured to measure A_{600} of the 200 μL ferments every 2 h for the first 48 h. Samples were taken manually from the Masterblock ferments at 2, 3, 4, and 9 days and frozen, and glucose and fructose were assayed enzymatically with a hexokinase method enzyme kit (Roche Diagnostics, Australia).[23]

Protein Analysis by Reversed-Phase (RP) HPLC. Ferments conducted in the 5 mL sample vials were analyzed, and TLPs and chitinases quantified as cytochrome *c* equivalents,[24] by RP-HPLC 14 days postinoculation according to the method of Van Sluyter et al.,[22] but with an increased flow rate of 0.5 mL/min. Following storage at 4 °C, samples were analyzed again by RP-HPLC 360 days post-inoculation. Peaks eluting between 6 and 9 min were considered TLPs, and peaks between 11 and 15 min were considered chitinases, on the basis of chromatograms of purified proteins and well-characterized juices and wines in previous studies.[3,22,24]

Heat Stability Tests. Heat tests were performed on 200 μL samples in a 96-well plate with a silicon mat lid at 55 °C in a humid chamber for 18 h, followed by cooling on ice for 5 h. Haze was expressed in mAU as A_{540} following heating minus A_{540} measured prior to heating. Samples with and without 20 μM pepstatin were incubated to test for residual BcAP8 activity.

Statistical Analysis. Haze and protein concentration data were analyzed by one-way ANOVA followed by Tukey's test using InStat 3.1 (GraphPad Software, Inc.). *P* values <0.01 were considered significant.

RESULTS AND DISCUSSION

Recombinant BcAP8 Effects on Grape Proteins. Several studies have demonstrated that wines from *Botrytis*-infected grapes contain lower levels of PR proteins, raising the possibility of *Botrytis* proteases act on grape PR protein substrates.[11−14] Because BcAP8 is the most abundant *B. cinerea* protein in liquid culture,[15] the gene for BcAP8 was expressed in *Pichia* to investigate its activity directly against grape proteins. BcAP8 was secreted in sufficient quantities so that purification was not required. Because BcAP8 was added to juice as culture supernatant, control treatments contained equivalent volumes of culture supernatant from *P. pastoris* transformed with the empty expression vector. As a comparison to BcAP8, pig pepsin was considered to be an example of a typical aspartic protease because it is commercially available and its activity against wine haze proteins has been previously studied.[25,26] Although the pH optimum of pepsin is often considered to be pH ≤2, the actual optimum is pH 3.5,[27] the approximate pH of grape juice and wine.

Grape PR proteins have been shown to be resistant to hydrolysis by fungal proteases,[28] and pepsin has been demonstrated to be ineffective in preventing wine haze, although it does remove some proteins at 37 °C.[25] Pocock et al.[26] demonstrated that pepsin and fungal protease are effective in removing PR proteins from wine at 90 °C. As demonstrated

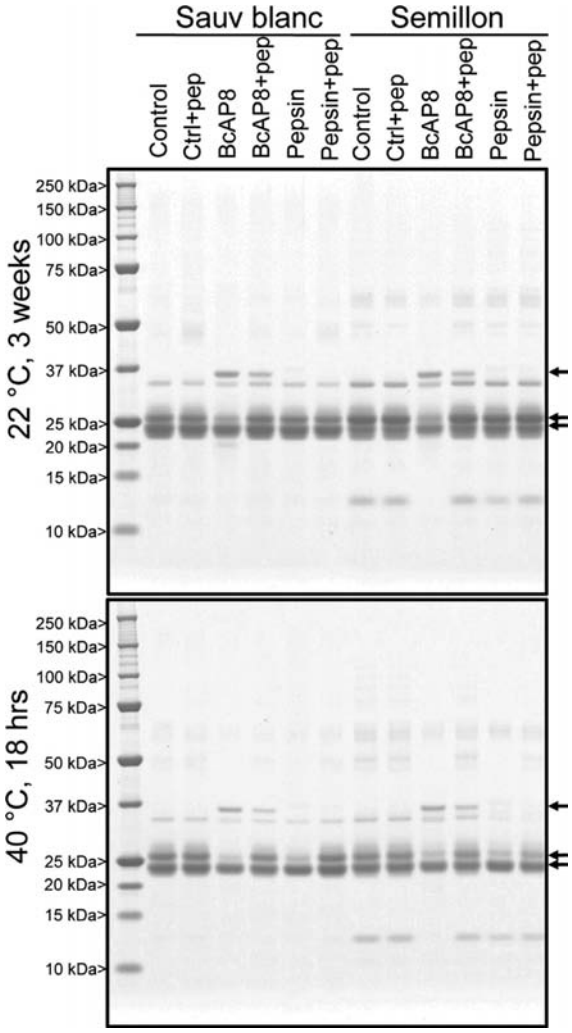

Figure 1. SDS-PAGE analysis of proteins extracted from juices treated with either BcAP8 or pepsin, with and without inhibition by pepstatin. At 40 °C the activities of the two enzymes against apparent chitinases at 26 kDa are similar. At 22 °C pepsin does not appear to destroy chitinase, but BcAP8 does. Top arrows, BcAP8; middle arrows, putative chitinase; bottom arrows, putative thaumatin-like protein.

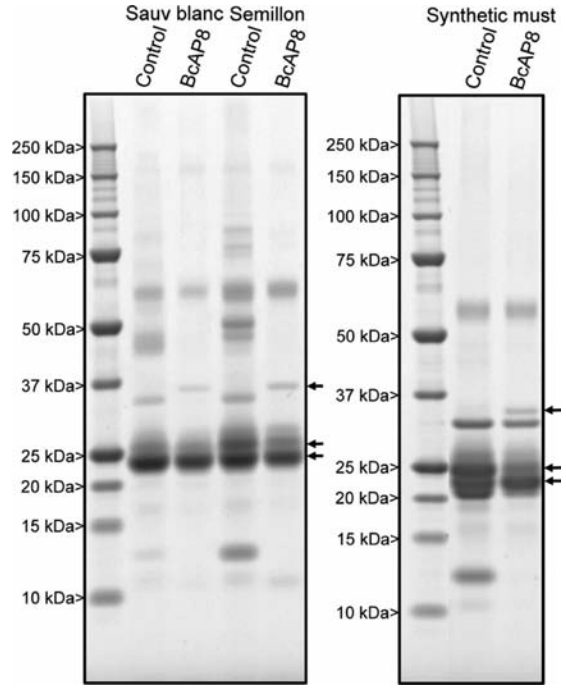

Figure 3. SDS-PAGE analysis of proteins extracted from wines made from must with BcAP8 added, the same wines as in Figure 2. Top arrows, BcAP8; middle arrows, putative chitinase; bottom arrows, putative thaumatin-like protein.

by Marangon et al.,[3] denaturing PR proteins with heat treatment at 75 °C to make them susceptible to fungal proteases is an effective way to remove them, but an ideal protease treatment should be effective at winemaking temperatures.

In the present work, pepsin had no effect against grape PR proteins at 22 °C, but did hydrolyze an apparent chitinase at 40 °C, proteolysis that was inhibited by pepstatin (Figure 1). In comparison, BcAP8 demonstrated pepstatin-sensitive proteolysis of the same apparent chitinase at both 22 and 40 °C, demonstrating that it could be an effective enzyme without heat-denaturing PR proteins, that is, under typical winemaking

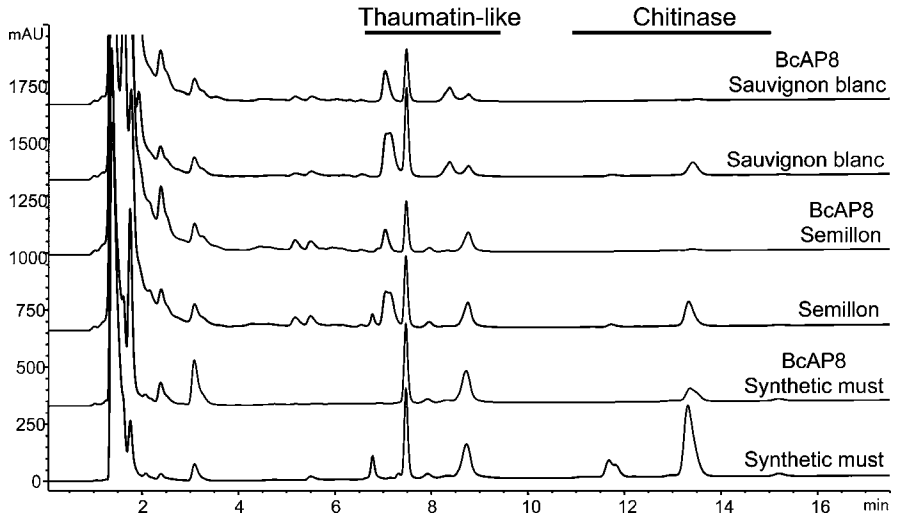

Figure 2. RP-HPLC chromatograms of three wines with BcAP8 added prior to inoculation and three control wines. All three BcAP8 wines show dramatic decreases in chitinases and some decreases in TLPs.

dx.doi.org/10.1021/jf402762k | J. Agric. Food Chem. 2013, 61, 9705−9711

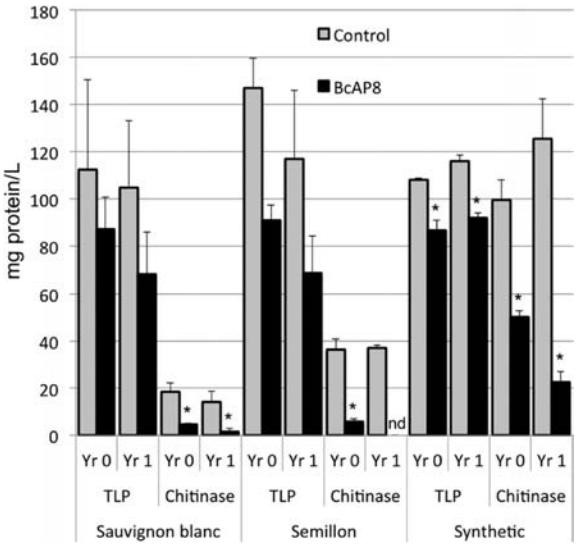

Figure 4. Thaumatin-like protein and chitinase concentrations in wines treated with 5 mg/L BcAP8 prior to inoculation. Controls were treated with *Pichia* culture filtrates from an empty vector transformant. Protein concentrations were determined by RP-HPLC ($n = 3$, ±SD) at the end of fermentation and again after 1 year at 4 °C. BcAP8 treatments that resulted in significantly less protein than the corresponding control treatments are indicated by asterisks ($P < 0.01$, Tukey's test; nd, not detected). Full statistical results are available in Supporting Information Table S1.

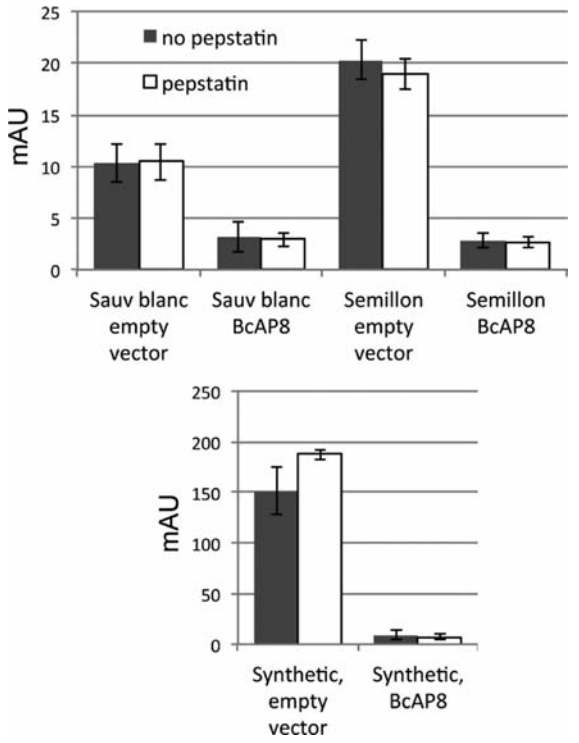

Figure 5. Heat-induced haze in wines treated with 5 mg/mL BcAP8 and empty vector controls. Haze was measured by absorbance at 540 nm following 18 h at 55 °C. Pepstatin was included as a control to inhibit possible residual BcAP8 activity during the heat tests. In all three wines BcAP8 produced a significant reduction in haze relative to the corresponding controls ($P < 0.01$). In no case did pepstatin have a significant effect ($P > 0.01$). Full statistical results are available in Supporting Information Table S2.

conditions. In both the 22 and 40 °C treatments, BcAP8 removed proteins associated with bands at 12 and 60 kDa, possibly lipid transfer protein (LTP) and invertase, respectively, although pepsin did not. Because LTP and invertase are involved in promoting positive sparkling wine foam properties,[12,14] it is possible that the proteolytic activity of BcAP8 demonstrated in Figure 1 is responsible for the reduced foam of sparkling wines from *Botrytis*-infected grapes. In the gels in Figure 1, BcAP8 appeared as a band at 36 kDa, but pepsin did not, which is typical of pepsin because it binds an unusually low amount of Coomassie stain per molecule.[29]

BcAP8 Effects on Grape Proteins under Winemaking Conditions. Although the addition of BcAP8 to sparkling wine could be detrimental to foam properties, it could be beneficial in still wines. To test the ability of BcAP8 to reduce PR proteins during winemaking, the protein was added to two grape juices and one synthetic juice including crude grape juice protein. The juices were inoculated and fermented at 17 °C. Figure 2 contains the RP-HPLC chromatograms of the resulting wines. In both of the authentic juices BcAP8 effectively eliminated chitinase and very slightly reduced the levels of TLPs. In the model juice a similar trend was evident, but some of the major chitinase remained.

SDS-PAGE results for the same wines are shown in Figure 3. BcAP8 appeared as a band slightly lower than the 37 kDa standard. Below that, at approximately 35 kDa, was a potential glucanase that was removed from Semillon and Sauvignon blanc by BcAP8, but was less affected in synthetic must. Reductions in chitinases were apparent in the authentic musts, with slight reductions in TLPs. Chitinase reduction in the synthetic must was more dramatic than in the authentic juices, and the reduction in what could be a TLP, the band slightly higher than the 20 kDa standard, was substantial.

Unlike the results in uninoculated juice, BcAP8 did not remove the band at 60 kDa. BcAP8 removed one potential LTP at approximately 13 kDa, which was particularly evident in the Semillon and synthetic must treatments. It is possible either that the higher temperatures of the juice experiments (22 and 40 °C) promoted BcAP8 activity against invertase or that BcAP8 becomes less active as stabilizing sugars are converted to denaturing alcohol. In that case, the longer exposure time of BcAP8 to substrates in uninoculated juice (3 weeks at 22 °C), versus the shorter exposure time at 17 °C before the alcohol became inhibitory, could explain the presence of potential invertase in the wines. In *Botrytis*-infected fruit, however, BcAP8 is possibly secreted into berries at high levels for substantial amounts of time before the fruit is picked, let alone inoculated with yeast, and that exposure of grape proteins to BcAP8 could potentially affect foaming properties in sparkling wines.

To test the long-term, possibly protective effect of BcAP8 on protein instability, wines were stored at 4 °C for 1 year and analyzed a second time by RP-HPLC. Results for TLPs and chitinases are shown in Figure 4. Among the controls, the changes in TLPs and chitinases were minimal. In contrast, the BcAP8 treatments, all of which have lower levels of TLPs and much lower chitinase levels shortly after fermentation, showed additional reductions in both classes of proteins and in all wines over the year of storage. The continued activity of BcAP8 after fermentation demonstrates that the enzyme is still active in the absence of sugar and in the presence of ethanol.

Effects of BcAP8 on Wine Protein Heat Stability. Because BcAP8 added before inoculation remains active in the

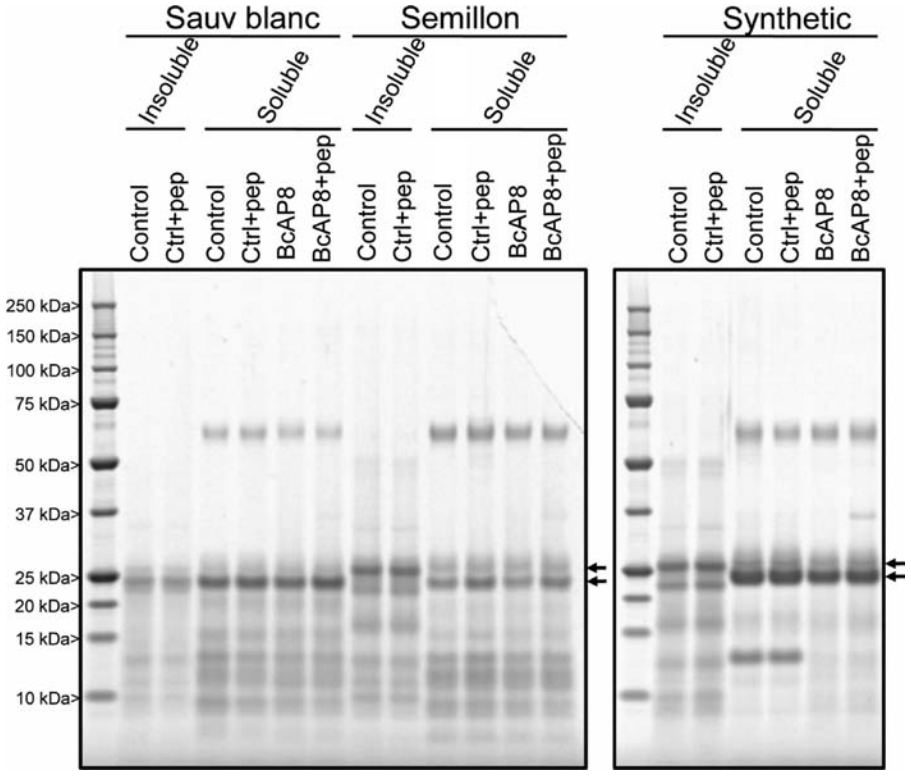

Figure 6. SDS-PAGE analysis of proteins following 55 °C, 18 h heat tests. Control wines containing no BcAP8 were centrifuged and the resulting protein pellets used for the insoluble samples; supernatants were used for soluble samples. The BcAP8 wines did not contain visible haze and were not separated into fractions prior to SDS-PAGE. Top arrows, chitinases; bottom arrows, TLPs.

resulting wine, at least for a short period of time, it is possible that it could hydrolyze proteins either as they denature naturally or in response to heat during transport or storage. To assess that possibility, small volumes of each wine, with and without the addition of pepstatin, were heated to 55 °C for 18 h in a 96-well plate and the resulting hazes measured spectrophotometrically in a plate reader (Figure 5). Pepstatin was included in the 55 °C tests to establish at what point the BcAP8 activity was destroying haze-forming proteins, before the heating or during the heating. The results were very similar between the BcAP8 treatments with and without pepstatin, suggesting that BcAP8 was not active at the end of the year of storage.

Thus, this experiment also effectively served as a heat stability test, except at conditions less stringent than normal heat stability tests,[30] but more closely resembling maximum temperatures during wine transport and storage.[31] There were dramatic differences in haze between the empty expression vector controls and BcAP8-treated wines, particularly in the synthetic must. In all of the control treatments hazes were visible; in the BcAP8 treatments, no hazes were visible even though the turbidity (A_{540}) values of the wines after heating were slightly greater than those of the wines before. The 55 °C for 18 h treatment simulated a real world scenario more closely than 80 °C for 6 h, and the significant reduction in haze under those circumstances illustrates the effectiveness of BcAP8 in reducing wine haze.

In all wines, and in the synthetic must treatment in particular, the differences in turbidity between control and BcAP8 treatments seem disproportionate compared to differences in protein amounts. All three BcAP8 wines contained >60 mg/L TLP, but produced no visible haze. On the other hand, the three BcAP8 wines had much lower levels of chitinases than the controls. It could be the case that chitinases contributed more to the hazes than TLPs in the controls, a result that would reflect the lower heat stability of chitinases.[16,17] Similarly, the potential glucanase at 35 kDa in Figure 3, removed from the authentic wines and reduced in the synthetic one, could contribute more to heat instability than TLPs.

The contribution of different types of proteins to the hazes in the control wines was assessed by collecting the hazes by centrifugation and analyzing by SDS-PAGE (Figure 6), along with the supernatants and total protein of the BcAP8 wines. (There was insufficient precipitated protein in BcAP8 wines for analyzing soluble and precipitated fractions separately.) The most apparent differences between the proteins isolated from heat tests versus those from wine are the apparent low molecular weight (<28 kDa) hydrolysis products. The hydrolysis products did not differ among the treatments, demonstrating that either BcAP8 or another pepstatin-inhibited protease was not responsible for hydrolysis during the heat test. Either a pepstatin-insensitive protease was active during the heat tests, possibly from yeast or even grape, or the hydrolysis was nonenzymatic. It is noteworthy that there appeared to be equal levels of hydrolysis products in insoluble and soluble fractions of authentic wines, demonstrating that although proteolysis took place, the products might be insoluble.

In the wine from synthetic must, although the levels of insoluble hydrolysis products were similar to those in the authentic wines, there were lower levels of hydrolysis products in the soluble fractions, suggesting that the synthetic must might lack a component of the authentic juice that enhances protein and peptide stability.

The roughly 10-fold differences in haze between the synthetic control wines and the authentic wines could be caused either by the lack of a stabilizing factor in synthetic must or by the higher chitinase levels in synthetic control wine versus authentic control wines. Falconer et al.[16] and Sauvage et al.[17] suggest that chitinases are the major heat-unstable proteins in white wines, and the present study is in agreement with them: the Semillon chitinases at 26 kDa seemed to be the major heat-unstable proteins in the Semillon and synthetic control wines (Figure 6).

The heat-precipitated protein from Sauvignon blanc wine presented a less clear scenario. It is possible that the major heat-precipitated protein was either the major TLP that also remained in the soluble fraction or a chitinase of approximately the same migration as the major TLP precipitated. By RP-HPLC (Figure 2), BcAP8 clearly removed a chitinase from the wine, but that was not immediately apparent by SDS-PAGE of the same samples because there was not a clear chitinase band above the major TLP band. However, the major band intensity decreased with BcAP8 treatment (Figure 3), and that could indicate that the removed chitinase peak by RP-HPLC represents a protein that comigrates with the major TLP. Whatever the case, BcAP8 reduces the amount of protein represented by the major band in Sauvignon blanc wine, a component of that band becomes insoluble as a result of heating, and, as a result, BcAP8 wine is more heat stable than untreated wine.

Although it does not remove all PR protein during winemaking, BcAP8 could benefit winemakers by reducing the amount of bentonite required to heat stabilize wines. The main advantage of BcAP8 over bentonite is that it does not have to be removed from wine and it could be added as a concentrated commercial enzyme preparation. The advantage of BcAP8 over other enzymes is that it is effective at normal winemaking temperatures. The success of BcAP8 raises the possibility that other proteases from *Botrytis* might be effective against grape PR proteins and perhaps complement BcAP8.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 depicts the BcAP8 expression vector design, and Figure S2 contains the BcAP8 cDNA sequence and construct detail. Tables S1 and S2 contain full ANOVA and Tukey's test results for data presented in Figures 4 and 5, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported financially by Australia's grape growers and winemakers through their investment body, the Grape and Wine Research and Development Corporation, with matching funds from the Australian government. The AWRI is a member of the Wine Innovation Cluster located in Adelaide.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

BcAP8, *Botrytis cinerea* aspartic proteinase 8; LTP, lipid transfer protein; PR, pathogenesis-related; TA, titratable acidity; TLP, thaumatin-like protein

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