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Degradation of white wine haze proteins by Aspergillopepsin I and II during juice flash pasteurization

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ABSTRACT

Bentonite is commonly used to remove grape proteins responsible for haze formation in white wines. Proteases potentially represent an alternative to bentonite, but so far none has shown satisfactory activity under winemaking conditions. A promising candidate is AGP, a mixture of Aspergillopepsins I and II.; a food grade, well characterized and inexpensive protease, active at wine pH and at high temperatures (60–80 °C). AGP was added to two clarified grape juices with and without heat treatments (75 °C, 1 min) prior to fermentation. AGP showed some activity at fermentation temperatures (≈20% total protein reduction compared to control wine) and excellent activity when combined with juice heating (≈90% total protein reduction). The more heat stable grape proteins, i.e. those not contributing to wine hazing, were not affected by the treatments and therefore accounted for the remaining 10% of protein still in solution after the treatments. The main physicochemical parameters and sensorial characteristics of wines produced with AGP were not different from controls.

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1. Introduction

An essential step during white, rosé and sparkling winemaking is the removal of heat unstable grape proteins that could remain in finished wines and cause the appearance of haze during storage (Bayly & Berg, 1967; Waters et al., 2005). Grape proteins in wines, being unstable under certain conditions, can aggregate into light-dispersing particles which make wines appear turbid (Hsu, Heatherbell, Flores, & Watson, 1987; Waters, Wallace, & Williams, 1991). In particular grape pathogenesis-related (PR) proteins, namely thaumatin-like proteins (TLPs) and chitinases (Marangon et al., 2011c; Waters, Hayasaka, Tattersall, Adams, & Williams, 1998; Waters, Shirley, & Williams, 1996), are known to contribute to wine haze and need to be removed before bottling.

The most effective tool to prevent haze is treatment with bentonite, a clay cation exchanger that has been used widely in oenology as a fining agent since the 1930s (Saywell, 1934). Bentonite fining is a low cost and effective method for removing proteins from wine or grape juice. However, bentonite fining has some negative attributes including dilution of the wine by the bentonite slurry, removal of positive flavour attributes, high labour costs, handling and disposal problems associated with spent bentonite, and quality loss of wine recovered from lees (Waters et al., 2005). For these reasons, alternative methods for white wine stabilization have been extensively investigated. A variety of alternatives has been proposed, ranging from the use of other adsorbents (Cabello-Pasini, Victoria-Cota, Macias-Carranza, Hernandez-Garibay, & Muñiz-Salazar, 2005; de Bruijn et al., 2009; Salazar, Achaerandio, Labbe, Guell, & Lopez, 2006; Sarmento, Oliveira, & Boulton, 2000; Vincenzi, Polesani, & Curioni, 2005), ultrafiltration (Hsu et al., 1987), flash pasteurization (Pocock, Høj, Adams, Kwiatkowski, & Waters, 2003) and proteases (Benucci, Liburdi, Garzillo, & Esti, 2011; Waters, Wallace, & Williams, 1992), but none has proven sufficiently effective to replace bentonite.

One ideal solution to this issue would be proteolytic enzymes able to degrade the heat unstable proteins. Several authors have investigated the effects of microbial proteases such as those from *Aspergillus niger* (Bakalinsky & Boulton, 1985), *Saccharomyces cerevisiae* (Dizy & Bisson, 2000; Feuillat, Brillant, & Rochard, 1980; Lagace & Bisson, 1990; Younes et al., 2011), and *Botrytis*

Abbreviations: AGP, Aspergillopepsin I and II; PR-proteins, pathogenesis-related proteins; TLPs, thaumatin-like proteins; LTPs, lipid transfer proteins; MW, molecular weight standards; NTU, nephelometry turbidity units; Inv, vacuolar invertases; β-gluc, β-1,3-glucanase; Chit, chitinases; Barwin, Barley PR-protein.

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cinerea (Cilindre, Castro, Clément, Jeandet, & Marchal, 2007; Girbau et al., 2004; Marchal et al., 1998). However, in each study, the enzymes were not able to effectively degrade grape PR proteins because of their high proteolysis resistance and because winemaking temperature conditions are unfavourable for enzyme activity (Waters, Peng, Pocock, & Williams, 1995; Waters et al., 1992).

It has recently been discovered that the unfolding temperature of chitinases is approximately 55 °C and approximately 62 °C for TLPs (Falconer et al., 2010). Moreover, the unfolding behaviour of the two proteins is different; once heated, chitinases stay unfolded upon cooling (irreversible unfolding), while TLPs refold (reversible unfolding).

Since PR-proteins are heat unstable, several authors investigated the effects of flash pasteurization for their removal. In 1966, Ferenczy suggested that pasteurizing wines improved their protein stability but had a detrimental effect on wine quality. Later research demonstrated that heating wine for a short period at 90 °C did not have negative sensory effects (Francis, Sefton, & Williams, 1994). A study by Pocock et al. (2003) showed that combining proteases with heat treatments of wine (90 °C, 1 min) reduced bentonite requirements by 50–70% without affecting sensory profiles. Despite encouraging results, the authors concluded that more efficient proteases were needed for commercial application.

In the 1960s two extracellular acid endopeptidases (Aspergillopepsin I and II, AGP) were discovered in the culture filtrate of *A. niger* var. *macrosporus* (Koaze, Goi, Ezawa, Yamada, & Hara, 1964). Aspergillopepsin I is a typical pepsin-type aspartic proteinase, active at pH 2-4 and inhibited by pepstatin (Rao, Tanksale, Ghatge & Deshpande, 1998), while Aspergillopepsin II is a non-pepsin-type acid proteinase, resistant to the inhibitors of ordinary pepsin-type aspartic proteinases such as pepstatin (Chang, Horiuchi, Takahashi, Yamasaki, & Yamada, 1976), with optimum pH between 1.8-2.6 and optimum temperature for casein degradation at about 70 °C at pH 2.6 (Oda, 2004). Aspergillopepsin I and II are irreversibly inactivated above pH 6.0 (Fukada et al., 1995), and are usually present as the major and minor components, respectively, of the commercial crude enzyme powder named Proctase (AGP). AGP is a food grade and inexpensive enymatic preparation that is very active at wine pH and at temperatures at which grape proteins are in an unfolded state.

Since unfolded proteins are more easily cleaved by enzymes, a strategy was elaborated to exploit the ability of AGP combined with short term heat treatments to degrade haze forming proteins in grape juice. The effect of heating alone and the ability of AGP to reduce the protein content of unheated grape juice when added to a ferment before yeast inoculation were also investigated.

2. Materials and methods

2.1. Materials

A 2009 heat unstable Chardonnay juice from Langhorne Creek (South Australia) was used for preliminary experiments while 2011 Chardonnay and Sauvignon Blanc juices from the Barossa valley region (South Australia) were used for the large scale experiment. The yeast strain used was EC1118 (Lallemand, Canada). The enzyme used was Proctase (AGP, Meiji, Japan) in powder form, a preparation containing Aspergillopepsin I (EC 3.4.23.18) and Aspergillopespin II (EC 3.4.23.19). The bentonite used was a sodium–calcium bentonite (Nacalit, Erbslöh, Germany) prepared at 50 g/l in water.

2.2. Analytical methods

Alcohol, specific gravity, pH, titratable acidity, glucose/fructose and volatile acidity analysis were performed by The AWRI Commercial Services using a Foss WineScan FT 120 as described by the manufacturer (Foss, Hillerød, Denmark). Free and total SO_2 were measured by the aspiration method (Rankine & Pocock, 1970). Laccase activity was measured by Botrytest kit (Laffort Oenologie, Bordeaux Cedex, France). Brix were measured by refractometry and Baumé by densitometry.

2.3. Organic acids by HPLC

The concentrations of organic acids (citric, tartaric, malic, succinic and lactic) were determined by HPLC as described by Marangon, Lucchetta, and Waters (2011a).

2.4. Colour analyses

Wine colour was assessed by the tristimulus method (CIELAB) as described by Kwiatkowski, Skouroumounis, Lattey, and Waters (2007).

2.5. Protein content determination

Protein content was determined by EZQ® protein quantification kit (Invitrogen Australia Pty Ltd., Australia) as described by Marangon et al. (2011a).

2.6. Protein HPLC

Protein concentration and composition were determined by reverse-phase (RP) HPLC with a Vydac 2.1 × 250 mm C8 column (208TP52 Grace Davison Discovery Sciences, Australia) on an Agilent 1200 system according to the method of Marangon, Van Sluyter, Haynes, and Waters (2009) with modifications as described in Van Sluyter et al. (2009). Injection volumes were 15 µl. From the 210 nm chromatogram, protein identity was assigned by comparison to retention times of purified grape PR proteins as follows; peaks with a retention time between 12 and 18 min were assigned to the TLP class, whereas peaks eluted from 22 to 30 min were classified as chitinases. Commercial thaumatin (Sigma) was used to build a calibration curve.

2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed with NuPage 12% Bis–tris, 1.0 mm thick, 10 well gels (Invitrogen) and a XCell SureLock Mini Cell (Invitrogen) following the manufacturer's instructions. Approximately 50 mg $Na_2S_2O_5$ were added to the top reservoir prior to running to prevent cysteine oxidation. Samples were prepared by precipitating proteins with four volumes of cold ethanol from 50/200 µl of wines. The pellet was collected by centrifugation (14,000*g*, 15 min, 4 °C), and dissolved in 20 µl of loading buffer (Invitrogen NuPage recipe) with 3% 2-mercaptoethanol. Standard molecular weight was BenchMark™ Protein Ladder from Invitrogen. Proteins were stained with Pierce Imperial Protein Stain (Quantum Scientific, Sydney, NSW, Australia) according to the manufacturer's microwave instructions.

2.8. Peptide LC–MS/MS and database searching

Proteins were identified from trypsin digested gel bands according to Van Sluyter et al. (2009) with modifications. An Advance captive spray source (Michrom Bioresources, Auburn, CA) was used in place of a nano source on a Thermo LTQ XL linear ion trap mass spectrometer. The spray voltage was 1.6 kV, ion transfer tube temperature 250 °C, and the instrument was tuned using angiotensin (Sigma). Fast chromatography was performed on a 0.25 × 40 mm,

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