Immobilised native plant cysteine proteases: packed-bed reactor for white wine protein stabilisation

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Abstract This research presents a feasibility study of using a continuous packed-bed reactor (PBR), containing immobilised native plant cysteine proteases, as a specific and mild alternative technique relative to the usual bentonite fining for white wine protein stabilisation. The operational parameters for a PBR containing immobilised bromelain (PBR-br) or immobilised papain (PBR-pa) were optimised using model wine fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA). The effectiveness of PBR-br, both in terms of hazing potential and total protein decrease, was significantly higher than PBR-pa, in all the seven unfinned, white wines used. Among the wines tested, Sauvignon Blanc, given its total protein content as well as its very high intrinsic instability, was selected as a control wine to evaluate the effect of the treatment on wine as to its soluble protein profile, phenolic composition, mineral component, and sensory properties. The treatment in a PBR containing immobilised bromelain appeared effective in decreasing both wine hazing potential and total protein amount, while it did not significantly affect the phenol compounds, the mineral component nor the sensory quality of wine. The enzymatic treatment in PBR was shown to be a specific and mild technique for use as an alternative to bentonite fining for white wine protein stabilisation.

Keywords Native plant cysteine proteases · Chitosan beads · Covalent immobilisation · Packed-bed reactor · Wine hazing potential · Total wine proteins

Introduction

Haze or turbidity in a transparent medium is the optical phenomenon caused by the presence of small suspended particles that divert light from its regular course. During the storage of bottled white wines grape proteins, that are stable in wine at normal environmental temperatures (Falconer et al., 2010), can become insoluble thus leading to haze formation, which represents a serious quality defect for consumers (Vincenzi et al., 2011).

The mechanism of protein haze formation in white wine is not fully understood, despite the major worldwide efforts to investigate this phenomenon (Waters et al., 2005). Research has indicated that protein instability is not dependent on the total protein content of the wine (Bayly and Berg, 1967; Moretti and Berg, 1965) but rather on pathogen-related (PR) proteins, which include chitinases (CH) and thaumatin-like proteins (TLPs) (Waters et al., 2005; Le Bourse et al., 2011; Marangon et al., 2012). Recent studies proved that grape CH represents the primary cause of haze formation, confirming the key role of sulphate in the formation of visible haze aggregates (Marangon et al., 2011a, b).

The technique currently used for removal of unstable haze-forming proteins from wine, absorption onto bentonite, is non-specific and can also impair wine quality. As well as proteins, bentonite removes various molecules or aggregates involved as aroma and flavour compounds, thus causing an overall reduction of wine organoleptic properties (Ferreira et al.,

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2002; Sauvage et al., 2010). Finally, the use of different kinds of bentonite may cause significant variation in the concentration of micro-elements (Catarino et al., 2008). Furthermore the disposal of spent bentonites constitutes a non-negligible source of waste.

To date there is still a need to develop specific and effective methods for white wine protein stabilisation, despite the fact that numerous procedures, alternative to bentonite, have been investigated. These include application of other adsorbents (Gump and Huang, 1999; Cabello-Pasini et al., 2005), treatment with zirconia in continuous and batch mode (Pashova et al., 2004), ultra-filtration (Miller et al., 1985), flash pasteurization (Pocock et al., 2003), and use of proteolytic enzymes (grape, yeast and exogenous proteases) in free form (Marangon et al., 2012; Modra and Williams, 1988). Modra and Williams (1988) tested commercial proteolytic enzyme preparations as alternative to bentonite fining, including a commercial undetailed bromelain from unknown source (fruit or stem). Earlier reports on structural and kinetic analyses revealed that stem bromelain differed markedly in their enzymatic activity from fruit bromelain. From the results, it was concluded that the stem bromelain showed more enzymatic activity than fruit bromelain (Gautam et al., 2010).

This work on native plant cysteine proteases (NPCP) follows other studies in which bromelain from pineapple stem (Benucci et al., 2011; Benucci et al., 2012) and papain from *Carica papaya* latex (Benucci et al., 2013) were shown to demonstrate proteolytic activity in model wine fortified with synthetic substrate even in the presence of wine inhibitors (Esti et al., 2011). Moreover, in a preliminary screening study, stem bromelain immobilised on chitosan beads, after a daily treatment in a lab-scale stirred reactor, revealed a high capacity to reduce haze potential in wine (Benucci et al., 2014).

One ideal solution for protein stabilisation, suitable for industrial-scale use, would be the treatment of unstable white wine in a continuous flow reactor, such as packed-bed reactor (PBR), so as to assure the reuse of immobilised NPCP without a separation step. In addition a PBR is more cost effective than batch operation and the easy automation and control of the system allow a greater productivity, as well as an improved enzyme stability (Laudani et al., 2007).

This research represents a feasibility study concerning the use of a continuous PBR, containing immobilised NPCP, as a specific alternative technique to bentonite fining for white wine protein stabilisation in laboratory bench-scale apparatus. The aims of the present work are: i) to design a continuous and easily scalable PBR; ii) to optimise the reactor flow conditions; iii) to prove the efficacy of immobilised NPCP in decreasing white wine hazing potential; iv) and to compare the efficacy of PBR and bentonite treatment in enhancing the overall quality of a Sauvignon blanc.

Materials and methods

Enzyme, chemicals and wines

Two NPCP from Sigma-Aldrich (Milan, Italy), namely stem bromelain (EC 3.4.22.32) and papain (EC 3.4.22.2), containing respectively 29 % and 37 % w/w of protein (Bradford, 1976), were immobilised on commercial chitosan beads by a direct mechanism at pH 3.2 (Chitoparl BCW-3010, Wako Chemicals GmbH, Neuss, Germany) (Benucci et al., 2012). At the end of immobilisation period biocatalysts were extensively washed with 2 M NaCl to remove all non-covalently bound enzymatic proteins.

The selected synthetic tripeptide chromogenic substrate, Bz-Phe-Val-Arg-p-nitroaniline (pNA), was purchased from Bachem (Bubendorf, Switzerland). All other reagents were obtained from Sigma-Aldrich (Milan, Italy).

The wine-like acidic medium, mimicking wine conditions, was a solution of tartaric acid/sodium tartrate at 0.03 mol, pH 3.2, containing ethanol (12 % v/v). Seven different unfinned monovarietal white wines (Moscato di Terracina, Malvasia del Lazio, Malvasia di Candia, Chardonnay, Manzoni bianco, Riesling, Sauvignon blanc), produced during the 2012 vintage in Lazio (a central region of Italy), were used as samples, and their oenological parameters were determined according to European official methods of analysis (European Commission, 1990). Before testing, all wines were filtered through a PES membrane filter (0.45 µm pore size, Sartorius Stedim Biotech GmbH, Antella (FI), Italy).

Enzymatic activity assay The activity of immobilised bromelain and papain was tested, at 20 °C, in wine-like acidic medium fortified with the selected substrate (Bz-Phe-Val-Arg-pNA, 0.22 mmol). Both NPCP cleave synthetic substrate via the hydrolysis of the ester bond between amino acids in the N-terminal position and pNA, whose release was detected spectrophotometrically at 410 nm.

Immobilised NPCP activity was determined by measuring the change in absorbance vs time using an UV-visible spectrophotometer (Shimadzu UV 2450, Milan, Italy). Specific activity calculated in IU of pNA produced [$\epsilon_{\text{mmol}} = 8.480/(\text{mmol} \times \text{cm})$ for pNA.] (Hale et al., 2005) was expressed as IU/mg of immobilised protein. A blank correction was made using a sample that did not contain enzyme. All measurements were made in triplicate.

Kinetic study The kinetic study of native (free) and immobilised proteases was carried out in wine-like acidic medium fortified with Bz-Phe-Val-Arg-pNA substrate (0–0.5 mmol), using the same amount of enzymatic protein, corresponding to 53 and 20 µg BSA_{eq.} for br and pa, respectively. For immobilised proteases the kinetic study was conducted both in a well mixed batch reactor (Batch) and in packed-

bed reactor (PBR) thermostated at 20 °C. All experiments were carried out in triplicate in three different Batch and PBR in order to obtain three independent replicates. Kinetic parameters were estimated by a nonlinear regression procedure (GraphPad Prism 5.01, GraphPad Software, Inc.). The K_M value (Michaelis–Menten constant) reflects the formation of the enzyme substrate complex, whereas k_{cat} (turnover number) measures the number of substrate molecules turned over per enzyme per minute. Moreover, k_{cat} is indicative of the product release velocity, representing the maximum number of moles of substrate converted to the product per number of moles of catalyst per unit time. This parameter can be obtained from the equation $k_{cat} = V_{max}/[E]_{tot}$, where $[E]_{tot}$ is the enzyme molar concentration. In addition, K_a (affinity constant), being the ratio k_{cat}/K_M , indicates the affinity of enzyme towards the substrate. It is indicative of both reaction steps and expresses the overall catalytic efficiency.

Optimisation of packed-bed reactor parameters A PBR containing immobilised bromelain (PBR-br) or immobilised papain (PBR-pa) was used to achieve the protein stabilisation of seven different unfinned monovarietal white wines. Firstly, it was necessary to optimise the operational parameters such as flow rate (Q_v), space velocity (S_v) and residence time (τ) using model wine fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mmol).

The PBR consisted of an unbreakable glass tube with internal volume of 7.29 cm³ (11.46 cm in length with a 0.9 cm inner diameter). The column was packed with 5.3 g of biocatalyst and the lower and upper ends of the tube were layered with glass beads (2 cm in thickness). The PBR was connected to the rest of the apparatus and a peristaltic pump (Minipuls 3, Gilson, Milan, Italy) was used for feeding in the model wine, fortified with the synthetic substrate, which moved upwards in the column at varying flow rates (from 0.30 to 15.00 mL/min). Then, samples of the effluent stream were collected and analysed spectrophotometrically at 410 nm in order to evaluate the concentration of the reaction product (C_p), represented by free pNA, as well as the product-formation rate (r_p).

Wine treatment in packed-bed reactor Of each of the above-mentioned white wines, 50 mL were treated in a bench-scale PBR-br or in a PBR-pa, introducing the wine at the optimised flow rate (6.43 and 6.00 mL/min, respectively). Treatment was carried out in triplicate in three different PBR in order to obtain three independent replicates for each wine sample. Then, treated wines were analysed in triplicate order to determine both the hazing potential and the total protein content.

Stability test After the reaction ends in PBR-br or in PBR-pa the stability of immobilised proteases was evaluated by enzymatic activity assay, feeding the model wine fortified with the

synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mmol) at the optimised flow rate (6.43 and 6.00 mL/min, respectively). In order to verify that no enzyme activity was present in wine after reaction, 0.1 mL of treated wine was used for the enzymatic activity assay toward the synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mmol).

Heat test Heat test was carried out on untreated wines and on wine samples treated in PBR (20 °C), containing immobilised proteases. The hazing potential of white wines was determined by a heat test: wine samples were incubated at 80 °C for 6 h and then kept at 4 °C for 16 h (Vincenzi et al., 2011; Pocock and Rankine, 1973). After equilibration at room temperature (approximately 25 °C), haze formation was measured using an HD 25.2 turbidimeter (Delta Hom), calibrated with Formazin turbidity standard, ranging from 0 to 800 NTU (Delta Hom). Turbidity was expressed in nephelometric turbidity units (NTU). Wine stability was calculated as the difference in the wine NTU after and before the heat test. The difference in turbidity between the wine after the heat test and that of the initial wine (ΔNTU) was proportional to hazing potential; moreover, wine is considered stable if this difference does not exceed 2 NTU (Moine-Ledoux and Dubourdieu, 1999). The turbidity removal yield [TRY(%)] by immobilised NPCP was calculated as the percentage of turbidity removed from the treated wine.

Protein content determination Total protein content of the above-mentioned white wines, before and after enzymatic treatment in PBR, was determined using the potassium dodecyl sulphate method (Vincenzi et al. 2011). Proteins were precipitated from 1 mL of wine by adding 10 μ L of 10 % (w/v) sodium dodecyl-sulphate and 250 μ L of 1 mol KCl. The pellet obtained after centrifugation was then dissolved in 1 mL of distilled water by ultrasonic bath and protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, Saint Louis, USA) according to the manufacturer's instructions; bovine serum albumin was used as standard for the calibration curve.

Electrophoretic separation (SDS-PAGE) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed (Laemmli, 1970). on precast commercial gels of 4–15 % (Bio-Rad, Richmond, California, USA) using a vertical electrophoresis apparatus (Mini-Protean Tetra cell, Bio-Rad, Richmond, California, USA) and standard molecular weight (Precision Plus Protein Standards, Kaleidoscope, Bio-Rad, Richmond, California, USA) were from 10 to 200 kDa.

Samples were prepared by precipitating proteins with four volumes of cold ethanol (−18 °C) from 50 μ L of wine; the

pellet obtained after centrifugation was dissolved in 20 μL of loading buffer (4 $\times$ Laemmli Sample Buffer) containing 3 % (v/v) of β -mercaptoethanol. All samples were boiled for 5 min and 20 μL was loaded in each well. The run was done until the dye front reached the reference line (about 1 h at 200 V). The gel was fixed and stained with colloidal Coomassie Brilliant Blue G-250. The destaining was done in deionised water.

Effect on phenolic composition of white wine

UV-Visible spectra of Sauvignon blanc wine samples (unprocessed, enzymatically treated in PBR-br and bentonite-fined (300 g/hl) were recorded with a Shimadzu UV 2450 spectrophotometer (Milan, Italy), using 2-mm- path-length UVettes (Eppendorf). Samples were scanned from 230 to 460 nm at ~ 1 nm intervals and water was used for the reference scan.

Direct analysis of minerals in white wine The determination of minerals in the unprocessed, the enzymatically treated (PBR-br) and the bentonite-fined Sauvignon Blanc wine samples was carried out by use of Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES), using a SPECTRO CIROS VISION instrument (SPECTRO Analytical Instruments GmbH, Kleve, Germany). Before the ICP-OES analysis, wine samples were prepared according to the O.I.V. analytical methods (O.I.V., 2011).

Wine sensory analysis A sensory panel was selected and trained in order to determine any perceptible difference in the aroma and flavour properties of the Sauvignon blanc wine samples (unprocessed, enzymatically treated in packed bed reactor containing immobilised bromelain, and bentonite-fined ones).

A Duo-Trio test, performed according to the normative UNI ISO 10399:2004(E) (2010), was carried out by the panel composed of 10 trained individuals (6 women and 4 men, between 25 and 55 years of age) belonging to the laboratory staff.

Two samples and a reference wine were presented to panellists as 30 mL aliquots in a 3-digit-coded, covered ISO standard wine glass at 22–24 $^{\circ}\text{C}$ (4 testing section, $n = 40$ responses). The presentation order was randomized within tests and between tests, and across judges. They were asked to match the test sample with the reference.

Statistical analysis

Data were analysed by one-way completely randomised Analysis of Variance (ANOVA) with an EXCEL[®] Add-in macro DSASTAT (Onofri, 2006). This followed by Tukey Honestly Significant Difference (Tukey HSD) post-hoc test ($\alpha = 0.05$) for multiple comparisons of samples.

Results and discussion

Packed-bed reactor optimisation

Bromelain and papain were linked on commercial chitosan beads by a direct mechanism with an immobilisation yield of 30 % (± 1) and 29 % (± 1), respectively. Enzymatic activity assay and protein content determination carried out on washing solution (2 M NaCl) proved that no enzyme leakage occurred during the following treatment with immobilised proteases.

Two similar PBRs, packed with immobilised bromelain or immobilised papain were constructed as previously described (Fig. 1a). Each column was fed with model wine fortified with synthetic substrate at varying flow rates, with the aim of optimising the process conditions. These experiments, carried out for both the PBR-br and the PBR-pa (Table 1), allowed the determination of the relationship between r_p and S_v , which depends on Q_v and bed volume (7.29 mL) (Watanabe et al., 2001; Xia et al., 2004).

Data in Fig. 1b show that the relationship between r_p and S_v is represented by a bell shape curve with a maximum of free pNA formation at a S_v of 0.88 (for PBR-br) and 0.82 min^{-1} (for PBR-pa), with a τ of 1.1 and 1.2 min, respectively. The

Fig. 1 a Sketch of the packed-bed reactor apparatus: 1-model wine fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA) or unprocessed white wine; 2-peristaltic pump; 3- packed-bed reactor containing immobilised bromelain or immobilised papain; 4. butterfly valve; 5. enzymatically processed model wine or white wine; b relationship between space velocity (S_v) and product-formation rate (r_p) in a packed-bed reactor, containing immobilised bromelain (○) or immobilised papain (■), and fed at varying flow rates with model wine fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mM)

Table 1 Process parameters of the packed-bed reactor, containing immobilised bromelain (PBR-br) or immobilised papain (PBR-pa), fed at varying flow rates with model wine fortified with synthetic substrate (Bz-Phe-Val-Arg-pNA, 0.22 mmol)

	Q_V (mL/min)	S_V (1/min)	τ (min)	C_p (mmol)	r_p (mmol/min)
PBR-br	0.30	0.04	25.0	0.170 ± 0.01	0.052
	0.60	0.08	12.5	0.090 ± 0.01	0.054
	1.18	0.16	6.30	0.089 ± 0.005	0.105
	1.84	0.25	4.00	0.069 ± 0.002	0.127
	2.98	0.41	2.40	0.059 ± 0.01	0.176
	6.43	0.88	1.10	0.035 ± 0.001	0.222
	9.00	1.23	0.81	0.021 ± 0.001	0.186
	10.00	1.37	0.73	0.015 ± 0.001	0.150
	12.00	1.65	0.60	0.016 ± 0.001	0.193
	12.86	1.76	0.57	0.014 ± 0.001	0.176
	13.85	1.90	0.53	0.009 ± 0.001	0.130
	15.00	2.06	0.49	0.01 ± 0.01	0.19
	15.00	2.06	0.49	0.01 ± 0.01	0.19
PBR-pa	0.64	0.09	11.1	0.13 ± 0.01	0.09
	1.25	0.17	5.88	0.08 ± 0.01	0.10
	2.97	0.41	2.44	0.05 ± 0.01	0.16
	4.50	0.62	1.61	0.08 ± 0.01	0.40
	6.00	0.82	1.22	0.10 ± 0.02	0.62
	9.00	1.23	0.81	0.06 ± 0.01	0.52
	11.25	1.54	0.65	0.03 ± 0.01	0.39
	13.85	1.90	0.53	0.02 ± 0.01	0.22
	15.00	2.06	0.49	0.01 ± 0.01	0.19
	15.00	2.06	0.49	0.01 ± 0.01	0.19
	15.00	2.06	0.49	0.01 ± 0.01	0.19
	15.00	2.06	0.49	0.01 ± 0.01	0.19
	15.00	2.06	0.49	0.01 ± 0.01	0.19

Legend: Q_V : flow rate; S_V : space velocity; τ : residence time; C_p : concentration of the reaction product; r_p : product-formation rate

free pNA yield, obtained with higher S_V , decreased significantly for both proteases, indicating that the increase in Q_V reduced the contact time between the substrate and biocatalyst surface during passage through the column, as reported by other authors (Laudani et al., 2007). Moreover, under these conditions of medium, PBR-pa hydrolyzes the synthetic substrate more efficiently than PBR-br (C_p 0.10 and 0.035 mM, respectively); both PBR stored at 4 °C in model wine, remained stable over 4 days with a proteolytic activity retention of about 70 % for PBR-pa and 60 % for PBR-br after 7 days. Furthermore, no proteolytic activity was detected in wine after PBR process, proving that no enzyme leakage occurred during treatment.

The kinetic study of native and immobilised proteases was carried out both in batch mode (Batch) and in packed-bed

reactor (PBR) at the optimised flow rate (6.43 and 6.00 mL/min for PBR-br and PBR-pa, respectively). Kinetic parameters collected in Table 2 prove that papain was more active toward synthetic substrate respect to bromelain, both as native enzyme and in immobilised form. Moreover, the immobilisation affected kinetic properties leading to lower V_{max} and K_a values with respect to free papain, independently by the reactor configuration. Both NPCP showed similar catalytic properties in Batch and in PBR, proving that the reactor configuration did not significant affect. As it can be seen (Table 2), the V_{max} values obtained for immobilised bromelain (PBR-br and Batch-br) were comparable to those obtained for the free form (Free br) and the affinity constant (K_a) were 2.4–2.8 fold higher respect to the native enzyme.

Table 2 Kinetic study of native and immobilised proteases [bromelain (br) and papain (pa)] in batch mode (Batch) and in packed-bed reactor (PBR), toward Bz-Phe-Val-Arg-pNA substrate (0–0.5 mmol), in model wine (20 °C)

Sample	V_{max} (IU/mg _{BSEaq})	K_M (mM)	k_{cat} (min ⁻¹)	K_a (min ⁻¹ mM ⁻¹)	R^2
Free br	1.71 ± 0.34	0.38 ± 0.14	771 ± 1	2043 ± 208	0.98
PBR-br	1.77 ± 0.21	0.14 ± 0.04	797 ± 2	5685 ± 1023	0.97
Batch-br	1.53 ± 0.17	0.14 ± 0.04	689 ± 1	4962 ± 1635	0.98
Free papain	56.74 ± 4.86	0.71 ± 0.09	$65,392 \pm 5$	$92,076 \pm 12,243$	1.00
PBR-pa	9.30 ± 1.08	0.29 ± 0.06	$10,720 \pm 1$	$37,537 \pm 9513$	0.99
Batch-pa	7.56 ± 0.91	0.25 ± 0.06	8708 ± 4	$34,750 \pm 9676$	0.99

Table 3 Oenological parameters of seven unfinned monovarietal white wines, with variable native protein haze and total protein content

	Wine sample						
	Moscato di Terracina	Malvasia del Lazio	Malvasia di Candia	Chardonnay	Manzoni bianco	Riesling	Sauvignon b.
Δ NTU Index ₀	3.8	8.8	12.5	26.0	35.0	55.0	70.0
Total protein (mg/L)	61	77	90	135	165	231	281
pH	2.90	3.22	3.01	3.31	3.24	2.98	3.30
Alcohol level (% v/v)	11.93	11.21	10.95	11.67	13.23	10.92	12.90
Total acidity (g/L tartaric acid)	6.48	5.83	5.54	4.81	4.35	5.77	5.90
L(–)-Malic acid (g/L)	2.28	3.22	2.16	2.45	1.80	2.11	0.90
Total SO ₂ (mg/L)	49	73	50	68	91	73	83
Free SO ₂ (mg/L)	11	16	11	12	20	16	13
Total phenols (mg/L catechin)	1288	1328	1029	1129	1032	1154	957

Δ NTU Index₀, difference between the turbidity of the initial wine and that of the wine after the heat test

Fig. 2 **a** Hazing potential (Δ NTU Index) of wines before and after enzymatic treatment in packed-bed reactor containing immobilised bromelain (PBR-br) or immobilised papain (PBR-pa), with the corresponding turbidity removal yield (percentage of turbidity removed from the treated wine, as arabic number); **b** total protein content (mg/L) of wines before and after enzymatic treatment in packed-bed reactor containing immobilised bromelain (PBR-br) or immobilised papain (PBR-pa), with the corresponding protein removal yield (percentage of proteins removed from the treated wine, as arabic number). Reported values are mean $\pm$ 95 % confidence interval of triplicate analysis. Values, among the three experimental conditions (Control wine, PBR-br, PBR-pa) for each wine sample, with different roman letters are significantly different (Tukey's test, $p \leq 0.05$)

White wine hazing potential and protein content reduction by immobilised NPCP in PBR

Seven white wines with different native protein stability (Δ NTU Index₀) and different total protein content (Table 3) were treated in a PBR-br or in a PBR-pa, at the optimised flow rates (6.43 and 6.00 mL/min, respectively).

Preliminary trials on Sauvignon blanc wine have been carried out in order to investigate the potential absorption effect of the support, as such or covered with the proteases firstly immobilised and then deactivated with NaOH 1 N (data not shown). Because of its total protein content as well as its very high intrinsic instability, Sauvignon blanc was selected among the seven wines tested to serve as control wine. The limited absorption effect observed when deactivated proteases covered the chitosan bead (3–7 % in terms of Δ NTU and 3–4 mg/L in terms of total protein content) was subtracted from the data thereafter presented.

Applying the optimised process conditions above mentioned, and a single passage through the PBR, both NPCPs significantly reduced the hazing potential (Fig. 2a) in all tested wines, as well as reducing the protein content (Fig. 2b), with the exception of the Moscato di Terracina sample, in which the total protein content remains practically unchanged under the three experimental conditions.

Moreover, it is worth noting that the effectiveness of the PBR-br, both in terms of decreasing the hazing potential and the total protein, was always significantly higher than that of the PBR-pa (Tukey's HSD test $p < 0.05$); this behavior was the opposite with respect to those observed toward the synthetic substrate in model wine. The higher efficacy observed for bromelain with respect to papain is probably attributable to their having different affinity and selectivity towards various protein families of wine, as well as their different sensitivities to wine inhibitors. Moreover, the turbidity removal yield [TRY(%)], calculated as the percentage of turbidity removed from the treated wine, ranged from 59 to 96 % for PBR-br and from 25 to 81 % for PBR-pa (Fig. 2a). The percentage protein reduction ranged from 14 to 68 % for PBR-br and from 4 to 57 % for PBR-pa (Fig. 2b). Despite the treatment with immobilised NPCP and in particular with PBR-br, stabilized three of the seven tested wines (Δ NTU Index < 2), both proteases showed a satisfactory activity under winemaking conditions (20 °C), as recently observed for AGP protease (Marangon et al., 2012). These data proved that treatment in PBR-br could be optimized to improve its stabilization power or it could be applied as preliminary step to reduce bentonite dosage rate needed for wine stabilization.

Because of its total protein content as well as its very high intrinsic instability, Sauvignon blanc was selected among the seven wines tested to serve as a control wine against which to evaluate the effect of PBR enzymatic treatment on the soluble protein profile, the phenolic composition, the mineral component, and the properties of wines.

Figure 3 shows results from the SDS-PAGE analysis performed to investigate which proteins from the Sauvignon blanc wine were affected by the enzymatic treatment. When comparing the intensities of bands from lanes Br (immobilised bromelain) and Pa (immobilised papain) with the control lane C it appears that there was a notable decrease in protein content, especially in the band intensities in the 15–25 kDa range, that is those corresponding to TLPs and chitinases which have been identified as the main proteins responsible for haze formation (Marangon et al. 2011a, b).

Furthermore treatment in the PBR-br appeared most effective in decreasing the two protein fractions (TLPs and chitinases) confirming the preliminary observations on the reduction of both wine hazing potential and total protein amount. In light of these overall results, it was decided to investigate the effect of enzymatic treatment by immobilised bromelain in a PBR on wine phenols, on the main mineral component and on the sensory quality of Sauvignon blanc wine and to compare these results with those for the unprocessed and bentonite-fined wine.

Effect of bromelain-PBR treatment on wine phenolic composition

The UV-Visible spectra of Sauvignon blanc, either that treated in a PBR-br or bentonite-fined, were distinctly different from those of unprocessed wine (Fig. 4). The absorbance values, at 280, 320 and 420 nm, registered for both of the treated wines, were statistically lower than those seen for unprocessed sample, indicating that removal of phenolic compounds had

Fig. 3 SDS-PAGE of Sauvignon Blanc protein fractions. Proteins from 50 μ L of wine were loaded on each lane. Relative molecular mass of protein standards are given on the left side of the gel. The other lanes are as follows: C-unprocessed control; Br-wine treated in packed-bed reactor containing immobilised bromelain (two experimental replicates: Br1 and Br2); Pa-wine treated in packed-bed reactor containing immobilised papain (two experimental replicates: Pa1 and Pa2); free enzymes (bromelain: Br and papain: Pa)

Fig. 4 UV-Visible spectra of Sauvignon blanc unprocessed wine (Control wine, ♦), enzymatically treated in packed bed reactor containing immobilised bromelain (PBR-br, △) and bentonite-fined (●). Histograms in the inset represent the absorbance of wine samples at three wavelength (280, 320 and 420 nm). Reported values are

mean $\pm$ 95 % confidence interval of triplicate analysis. Values, among the three experimental conditions (Control wine, PBR-br, bentonite-fined) for each characteristic wavelength, with different roman letters are significantly different (Tukey's test, $p \leq 0.05$)

occurred. Moreover, both at 320 and 420 nm, the absorbance values appeared significantly lower in PBR-br treated Sauvignon blanc, demonstrating that the passage through the packed-bed bioreactor exerted a marked and selective depletion effect toward cinnamic acids (320 nm) and their oxidation derivatives (420 nm), whereas, bentonite fining resulted in an indiscriminate reduction of the overall wine phenolic compounds (280 nm). It is worth noting that the absorbance at 280 nm of bentonite-fined wine was significantly lower with respect to the enzymatically treated sample showing the lesser impact of PBR-br on wine total phenols.

Effect of bromelain-PBR treatment on wine mineral component

Enzymatic treatment had a small effect on the mineral content of Sauvignon blanc wine, for which Al, Si and Mg all showed lower values with respect to the unprocessed sample (Table 4). On the other hand, the effect of bentonite addition to wine resulted in a significant decrease in the amount of K (about 15 % respect to the unprocessed wine), as well as in a significant increase in the concentration of Al, Be, Fe, Si and Mg, which are probably released from the clay during the fining. These data, in accordance with findings by other authors

(Catarino et al., 2008). showed that the enzymatic treatment is of low-impact and therefore safer with respect to the conventional bentonite fining.

Effect of bromelain-PBR treatment on the overall wine sensory quality

The Duo-trio test allowed the evaluation of whether there were detectable differences between treated and unprocessed Sauvignon blanc. The results (data not shown) proved that bentonite-fined wine was perceived to be significantly different with respect to the unprocessed wine, whereas no significant difference was observed when comparing the enzymatically treated sample with the unprocessed wine. In addition the bentonite-fined wine was perceived differently from the sample treated in PBR-br, again confirming that enzymatic treatment did not affect the sensory profile of wine.

Conclusions

A continuous and easily scalable packed-bed reactor, containing native plant cysteine proteases (bromelain from pineapple stem or papain from *Carica papaya* latex) immobilised on

Table 4 Mineral components of unprocessed Sauvignon blanc (Control wine), wine enzymatically treated in a packed bed reactor containing immobilised bromelain (PBR-br) and bentonite-fined wine

Sauvignon b. wine	Al (mg/L)	Be (mg/L)	Fe (mg/L)	Si (mg/L)	K (mg/L)	Mg (mg/L)
Control wine	0.57 ^b	0 ^b	0 ^b	11.65 ^b	216 ^a	65.0 ^b
PBR-br	0.48 ^c	0 ^b	0 ^b	10.74 ^c	218 ^a	62.9 ^c
Bentonite-fined	5.80 ^a	0.023 ^a	0.31 ^a	18.80 ^a	189 ^b	76.3 ^a

Reported values are mean $\pm$ 95 % confidence interval of triplicate analysis. For each parameter, means with different roman letters are significantly different ($p \leq 0.05$)

commercial chitosan beads, was realised in a laboratory bench-scale apparatus for which the operational parameters were optimised using model wine fortified with a synthetic substrate. The effectiveness of immobilised bromelain, in terms of decreasing both hazing potential and total protein decrease, was significantly higher than that for immobilised papain, in all the seven unfinned monovarietal white wines treated. The turbidity removal yield, calculated as the percentage of turbidity removed from the treated wine, ranged from 59 to 96 % for a packed-bed reactor containing immobilised bromelain and from 25 to 81 % for packed-bed reactor containing immobilised papain, whereas the percentage of protein reduction ranged from 14 to 68 % and from 4 to 57 %, respectively.

Despite the treatment with immobilised NPCP and in particular with PBR-br, stabilized three of the seven tested wines (Δ NTU Index <2), both proteases showed a satisfactory activity under winemaking conditions. Treatment in PBR-br could be further optimized to improve its stabilization power or it could be applied as preliminary step to reduce bentonite dosage rate needed for wine stabilization.

Moreover, the most effective protein stabilisation treatment, by immobilised bromelain, did not significantly affect the phenolic composition of wine, its mineral component, nor the sensory quality. Overall, the results proved that enzymatic treatment, precisely for its efficacy in reducing turbidity potential and protein content, while also having little effect on wine quality, could serve as a specific and mild alternative technique to bentonite fining.

Glossary

BCA, Bicinchoninic acid; *CH*, chitinases; C_p , concentration of the reaction product (mmol); *ICP-OES*, Inductively Coupled Plasma - Optical Emission Spectroscopy; *NPCP*, native plant cysteine proteases; *PBR*, packed-bed reactor; *PBR-br*, PBR containing bromelain immobilised on BCW; *PBR-pa*, PBR containing papain immobilised on BCW; *pNA*, p-nitroaniline; Q_v , flow rate (mL/min); r_p , product-formation rate, calculated as $C_p \cdot Q_v$ (mmol/min); *SDS-PAGE*, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; S_v , space velocity (1/min); *TLPs*, thaumatin-like proteins; *TRY*, turbidity removal yield (%)

Greek Symbols

E , molar absorptivity (1/(mmol·cm)); τ , residence time (min)

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References

- Bayly FC, Berg HW (1967) Grape and wine proteins of white wine varieties. *Am J Enol Vitic* 18:18–32
- Benucci I, Esti M, Liburdi K (2014) Effect of free and immobilised stem bromelain on protein haze in white wine. *Aust J Grape Wine Res* 20:347–352
- Benucci I, Esti M, Liburdi K, Garzillo AMV (2012) Pineapple stem bromelain immobilized on different supports: catalytic properties in model wine. *Biotechnol Prog* 28:1472–1477
- Benucci I, Esti M, Lombardelli C, Liburdi K, Garzillo AMV (2013) Papain from papaya (*Carica papaya* L.) fruit and latex: preliminary characterization in alcoholic–acidic buffer for wine application. *Food bioprod Process* 91:595–598
- Benucci I, Liburdi K, Garzillo AMV, Esti M (2011) Bromelain from pineapple stem in alcoholic–acidic buffers for wine application. *Food Chem* 124:1349–1353
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72:248–254
- Cabello-Pasini A, Victoria-Cota N, Marcias-Carranza V, Hernandez-Garibay E, Muniz-Salazar R (2005) Clarification of wines using polysaccharides extracted from seaweeds. *Aust J Grape Wine Res* 56:52–59
- Catarino S, Madeira M, Monteiro F, Rocha F, Curvelo-Garcia AS, Bruno de Sousa R (2008) Effect of bentonite characteristics on the elemental composition of wine. *J Agric Food Chem* 56:158–165
- Esti M, Benucci I, Liburdi K, Garzillo AMV (2011) Effect of wine inhibitors on free pineapple stem bromelain activity in a model wine system. *J Agric Food Chem* 59:3391–3397
- European Commission, 1990. Commission regulation (EEC) no 2676/90 of 17 September 1990 determining community methods for the analysis of wines. *o j l* 272, 1–192.
- Falconer RJ, Marangon M, Van Sluyter SC, Neilson KA, Chan C, Waters EJ (2010) Thermal stability of thaumatin-like protein, chitinase, and invertase isolated from sauvignon Blanc and Semillon juice and their role in haze formation in wine. *J Agric Food Chem* 58:975–980
- Ferreira RB, Picarra-Pereira MA, Monteiro S, Loureiro VB, Teixeira AR (2002) The wine proteins. *Trends Food Sci Technol* 12:230–239
- Gautam SS, Mishra SK, Dash V, Goyal AK, Rath G (2010) Comparative study of extraction, purification and estimation of bromelain from stem and fruit of pineapple plant. *Thai Journal of Pharmaceutical Sciences* 34:67–76
- Gump BM, Huang CF (1999) Removal of unstable protein in grape juice and wine by adsorbents resins. California Agricultural Technology Institute, Publications n 990402
- Hale LP, Greer PK, Trinh CT, James CL (2005) Proteinase activity and stability of natural bromelain preparations. *Int Immunopharmacol* 5:783–793
- Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Laudani CG, Habulin M, Knez Z, Della Porta G, Reverchon E (2007) Immobilized lipase-mediated long-chain fatty acid esterification in dense carbon dioxide: bench-scale packed-bed reactor study. *J Supercrit Fluids* 41:74–81
- Le Bourse D, Conreux A, Villaume S, Lameiras P, Nuzillard JM, Jeandet P (2011) Quantification of chitinase and thaumatin-like proteins in grape juices and wines. *Anal Bioanal Chem* 401:1541–1549
- Marangon M, Lucchetta M, Waters EJ (2011a) Protein stabilization of white wines using zirconium dioxide enclosed in a metallic cage. *Aust J Grape Wine Res* 17:28–35
- Marangon M, Van Sluyter SC, Neilson KA, Chan C, Haynes PA, Waters EJ, Falconer RJ (2011b) Roles of grape thaumatin-like protein and chitinase in white wine haze formation. *J Agric Food Chem* 59:733–740
- Marangon M, Van Sluyter SC, Robinson EM, Muhlack RA, Holt HE, Haynes PA, Godden PW, Smith PA, Waters EJ (2012) Degradation of white wine haze proteins by aspergillopepsin I and II during juice flash pasteurization. *Food Chem* 135:1157–1165

- Miller GC, Amon JM, Gibson RL, Simpson RF (1985) Loss of aroma attribute to protein stabilization with bentonite or ultrafiltration. *Aust N Z Grapegrow Winemak* 256:52–59
- Modra EJ, Williams PJ (1988) Are proteases active in wines e juices. *Aust N Z Grapegrow Winemak* 292:42–46
- Moine-Ledoux V, Dubourdieu D (1999) An invertase fragment responsible for improving the protein stability of dry white wines. *J Sci Food Agric* 79:537–543
- Moretti RH, Berg HW (1965) Variability among wines to protein clouding. *Am J Enol Vitic* 16:69–78
- Onofri A (2006) Enhancing excel capability to perform statistical analyses in agriculture applied research. In: *In computational statistics and data analysis - statistical software newsletters*, eds. Association for statistical Computing, International
- O.I.V. Office International de la Vigne et du Vin (2011) *Compendium of international methods of wine and must analysis*. France, Paris
- Pashova V, Guell C, López F (2004) White wine continuous protein stabilization by packed column. *J Agric Food Chem* 52:1558–1563
- Pocock KF, Rankine BC (1973) Heat test for detecting protein instability in wine. *Australian Wine, Brewing and Spirit Review* 91(5):42–43
- Pocock KF, Høj PB, Adams KS, Kwiatkowski MJ, Waters EJ (2003) Combined heat and proteolytic enzyme treatment of white wines reduce haze forming protein content without detrimental effect. *Aust J Grape Wine Res* 9:56–63
- Sauvage FX, Bach B, Moutounet M, Vernhet A (2010) Proteins in white wines: thermo-sensitivity and differential adsorption by bentonite. *Food Chem* 118:26–34
- UNI EN ISO 10399 (2010). *Sensory analysis - Methodology - Duo- trio test*.
- Vincenzi S, Marangon M, Tolin S, Curioni A (2011) Protein evolution during the early stages of white winemaking and its relations with wine stability. *Aust J Grape Wine Res* 17:20–27
- Watanabe Y, Miyawaki Y, Adachi S, Nakanishi K, Matsuno R (2001) Continuous production of acyl mannoses by immobilized lipase using a packed-bed reactor and their surfactant properties. *Biochem Eng J* 8:213–216
- Waters EJ, Alexander G, Muhlack R, Pocock KF, Colby C, O'Neill BK, Høj PB, Jones P (2005) Preventing protein haze in bottled white wine. *Aust J Grape Wine Res* 11:215–225
- Xia YM, Fang Y, Xu ZG, Hu XY, Shen YA, Brown JJ (2004) A novel process for enzymatic synthesis of N-lauroyl- β -amino propionitrile using a packed bed reactor coupled with on-line separation. *J Mol Catal B Enzym* 31:111–115