Effect of Wine Inhibitors on the Proteolytic Activity of Papain from *Carica papaya* L. Latex

Ilaria Benucci, Marco Esti, and Katia Liburdi
Dept. for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, S. Camillo de Lellis, 01100 Viterbo, Italy

DOI 10.1002/btpr.2015
Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com)

The influence of potential inhibitors naturally present in wine on the proteolytic activity of papain from Carica papaya latex was investigated to evaluate its applicability in white wine protein haze stabilization. Enzymatic activity was tested against a synthetic tripeptide chromogenic substrate in wine-like acidic medium that consisted of tartaric buffer (pH 3.2) supplemented with ethanol, free sulfur dioxide (SO_2), grape skin and seed tannins within the average ranges of concentrations that are typical in wine. The diagnosis of inhibition type, performed with the graphical method, demonstrated that all of tested wine constituents were reversible inhibitors of papain. The strongest inhibition was exerted by free SO_2, which acted as a mixed-type inhibitor, similar to grape skin and seed tannins. Finally, when tested in table white wines, the catalytic activity of papain, even when if it was ascribable to the hyperbolic behavior of Michaelis-Menten equation, was determined to be strongly affected by free SO_2 and total phenol level. © 2014 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2014
Keywords: latex papain, wine inhibitors, tartaric buffer, synthetic peptide substrate, inhibition study

Introduction

Native plant cysteine proteases, particularly those of the tropical plants *Carica papaya* (papain, chymopapain, caricain and glycil endopeptidase), *Ananas comosus* (fruit bromelain, stem bromelain, ananain, and comosain), and *Ficus glabrata* (ficin), are of considerable commercial importance due to their strong proteolytic activities against a broad range of protein substrates and their activity over broad ranges of pH and temperature.[1] Plant proteases have been used in medicine, detergent manufacturing and food science for many years.[1] These enzymes are applied in baking industry to facilitate the preparation of the baking mass and gluten hydrolyzates are currently under investigation to generate new products with higher-added value.[2–4] Moreover, the use of exogenous proteases to improve meat tenderness has attracted much interest recently,[5] as well as for the production of fish protein hydrolyzate.[6]

The application of proteolytic enzymes to chillproof beer was patented by Leo Wallerstein in 1911.[7] Currently, papain (from *Carica papaya*), as well as bromelain[8] and ficin,[9] is used in the brewing industry to prevent chill-haze formation and to obtain beer with good colloidal properties at low temperatures.[10,11]

Moreover, in recent years, papain has been immobilized onto various supports and through different methods because of its great industrial applications.[12–14]

In winemaking, the mechanism of protein haze formation is not fully understood, despite the major worldwide investigation into this topic.[15] These proteins have been identified as pathogenesis-related (PR) proteins (i.e., chitinases and thaumatin-like proteins),[15] and their proteolytic digestion by enzymes may be suitable as a selective and "mild" treatment and an alternative to the traditional method, which is based on bentonite fining. Most notably, the cationic exchange properties of this clay lead to the adsorption of positively charged wine proteins and other soluble cationic constituents that induce unwanted amino acids, bioamines, polyphenols and the depletion of aroma compounds.[16–18] To date there is still a need to formulate specific methods for white wine protein stabilization, despite numerous procedures, including proteolytic enzymes (grape, yeast, and exogenous proteases), have been investigated as alternative to bentonite.[19–21]

Stem bromelain, papain from ripe fruit and papain from papaya latex have recently been characterized under wine-like conditions, and these proteases have been found to have potentially productive biotechnological applications in winemaking.[22,23]

The ethanol content, the free sulfur dioxide (SO_2) and the interactions between the phenolic compounds and proteins of wine are known to be responsible for the inhibition of several enzymes.[24] To the best of our knowledge, the effects of these compounds, which are usually present in wine, on papain activity are still unknown. Thus, the goal of the present paper was to assess the inhibition of papain (from *Carica papaya* L. latex) activity by expected inhibitors in wine (ethanol, free SO_2, grape skin and seed tannins) over the average ranges of their respective concentrations in wine. The results are compared with those of a previous inhibition study that was performed by our group on bromelain from pineapple stem.[25] Finally, papain activity was also tested in real commercial white wine.

Correspondence concerning this article should be addressed to I. Benucci at ilaria.be@tiscali.it.

Materials and Methods

Enzyme, chemicals, and wine Samples

Papain by papaya latex (EC 3.4.22.2) was supplied from Sigma-Aldrich (Milan, Italy). Synthetic tripeptide chromogenic substrate Bz-Phe-Val-Arg-p-nitroaniline (pNA) was purchased from Bachem, (Bubendorf, Switzerlan). Grape skin and seed tannins (SKT and SET, respectively) were kindly supplied by EVERINTEC (Venice, Italy) as preparations intended for enological use. All other reagents (tartaric acid, NaOH, EtOH, and sodium bisulfite) were obtained from Sigma-Aldrich (Milan, Italy). Two different commercial white table wines produced from grapes of the 2012 vintage in the Lazio region of Italy were used as samples, and their physicochemical parameters were determined according to official Office International de la Vigne et du Vin (OIV) methods.[26]

Enzymatic activity assay

Papain activity was assayed according to the methods previously described for the assay of stem bromelain[25] to facilitate comparison of the results obtained from these two proteases. Specifically, the activity was tested using Bz-Phe-Val-Arg-pNA as substrate (0–510 μM) in tartaric buffer (TB: tartaric acid/Na tartrate at 0.03 M, pH 3.2), in tartaric buffer ethanol added (TB-EtOH: TB with 12% v/v ethanol) and in white wines that had previously been filtered through a PES membrane filter (0.45 μm pore size, Sartorius Stedim Biotech Gmbh, Antella (FI), Italy), in the presence of papain (0.02 mg mL^{-1}).

Substrate cleavage results in the release of free pNA, which was detected colorimetrically at 410 nm (UV-visible spectrophotometer, Shimadzu UV 2450, Milan, Italy). Specific activities were estimated based on the linear portions of the curves (which are indicative of the steady states) and were calculated in International Units of produced pNA ($\varepsilon_{410nm} = 8.48$ mM^{-1} cm^{-1}) and are expressed as I.U. mg^{-1} of protein.[27] A blank correction was made using a sample that did not contain enzyme. All measurements were made in triplicate, and the standard deviations are reported.

Determination of the kinetic parameters

The kinetic parameters (K_M, k_{cat}, and K_a) were determined according to the Michaelis-Menten equation by fitting the experimental data with a global nonlinear regression procedure and the best-fit value, for each data set, was attested by the squares of the correlation coefficients (r^2) (GraphPad Prism 5.0, GraphPad Software).

Inhibition study

The papain inhibition study was performed according to the procedures that were previously used for stem bromelain.[25] Preliminary tests, carried out at free SO_2 levels higher than 5 mg/L, proved that at these concentrations papain activity was not detectable. For this reason, the inhibition type as well as the degree of inhibition was evaluated at low free SO_2 levels.

Therefore, papain inhibition study was performed in TB containing different concentration and mixtures of the following potential inhibitors: ethanol (EtOH, 0, 6, 9, 12, 15% v/v), free SO_2 (SO_2, 0, 2, 3, 4, 5 mg L^{-1}), a skin tannin preparation (SKT, 0, 0.15, 0.30, 0.50, 0.99 g $L^{-1}_{gallic\ acid\ eq}$) and a seed tannin preparation (SET, 0, 0.15, 0.30, 0.50, 0.99, 1.20 g $L^{-1}_{gallic\ acid\ eq}$).

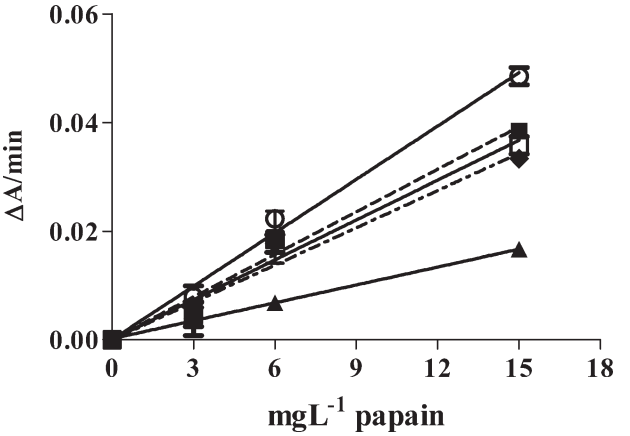

Figure 1. **ΔA/min versus papain concentration (mg L^{-1}) toward Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2) in the absence (control, O) and in the presence of different inhibitors: ethanol (12% v/v, □), free SO_2 (2 mg L^{-1}, ▲), skin (0.50 g $L^{-1}_{gallic\ acid\ eq}$, ◆) and seed (0.15 g $L^{-1}_{gallic\ acid\ eq}$,■) tannins.**

Results and Discussion

Wine compounds can affect enzymatic activity via different inhibitory mechanisms. An "inhibitor" can be defined as any substance that reduces the velocity of an enzyme-catalyzed reaction. The interactions between a reversible inhibitor (I) and a free enzyme (E), or an enzyme-substrate complex (ES) can be described by different inhibition models (competitive, uncompetitive and mixed-type inhibition) under the assumption that only a single substrate is involved in the reaction and that only one type of inhibitor is present at any time. The inhibition constant (K_i) reflects the concentration of an inhibitor that decreases the rate of an enzyme-catalyzed reaction by 50%.[28,29]

To examine the potential future biotechnological applications of papain in winemaking, the influences of the potential inhibitors that are naturally present in wine on protease activity were investigated to identify the natures of the inhibitions.

Before studying the degrees of inhibition, the type (reversible or irreversible) was identified using the graphical method, which involves plotting ΔA/min versus $[E_t]$.[28,29] The "inhibitor added" curves, all of which passed through the origin, exhibited smaller slopes than the control slope (Figure 1), which demonstrates that all compounds tested were reversible inhibitors of papain activity as was previously ascertained for stem bromelain.[25]

Moreover, papain inhibition studies were performed in pH 3.2 TB that had previously been supplemented either EtOH or SO_2 (basic elements of wine) and another study was subsequently performed in which these elements were combined with grape tannins.

Inhibitory effect of ethanol

The sensitivity of papain to EtOH was investigated in TB across varying amounts of EtOH (0, 6, 9, 12, 15% v/v) in

Table 1. Kinetic Parameters of Papain Versus the Bz-Phe-Val-Arg-pNA Substrate in Tartaric Buffer (TB) with Different Amounts of Added Ethanol (EtOH) and Free Sulfur dioxide (free SO_2) and in Tartaric Buffer Ethanol Added (TB-EtOH) with Different Amounts of Added Free SO_2, Grape Skin (SKT) and Seed Tannins (SET)

	Inhibitor	Content	$V_{max(app)}$ (I.U. mg^{-1})	$K_{M(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a ($min^{-1}\mu M^{-1}$)	r^2*
TB	EtOH (% v/v)	0	7.53 ± 0.23	234 ± 15	23826 ± 1	101.6 ± 6.5	0.993
		6	7.15 ± 0.50	332 ± 42	22599 ± 1	68.2 ± 6.5	0.981
		9	7.25 ± 0.53	428 ± 52	22922 ± 2	53.6 ± 5.8	0.986
		12	7.47 ± 0.49	583 ± 60	23611 ± 1	40.5 ± 4.2	0.992
		15	6.84 ± 0.39	712 ± 51	21629 ± 1	30.4 ± 2.4	0.997
	Free SO_2 (mg L^{-1})	2	0.290 ± 0.005	180 ± 7	916 ± 1	5.1 ± 0.2	0.997
		3	0.215 ± 0.008	162 ± 15	680 ± 2	4.19 ± 0.37	0.983
		4	0.162 ± 0.007	151 ± 16	513 ± 1	3.40 ± 0.34	0.985
		5	0.111 ± 0.006	116 ± 18	351 ± 1	3.0 ± 0.5	0.940
TB-EtOH	Free SO_2 (mg L^{-1})	0	7.47 ± 0.49	583 ± 60	23611 ± 1	40.5 ± 4.2	0.992
		2	0.57 ± 0.05	725 ± 88	1793 ± 1	2.5 ± 0.3	0.991
		3	0.36 ± 0.06	752 ± 76	1136 ± 2	1.51 ± 0.14	0.981
		4	0.27 ± 0.06	765 ± 68	840 ± 2	1.10 ± 0.09	0.992
		5	0.21 ± 0.02	760 ± 90	676 ± 1	0.9 ± 0.1	0.995
	SKT (g $L^{-1}_{gallic\ acid\ eq}$)	0.15	4.91 ± 0.4	470 ± 40	15548 ± 2	33.1 ± 2.6	0.991
		0.30	2.53 ± 0.16	458 ± 45	7997 ± 2	17.5 ± 1.6	0.992
		0.50	2.47 ± 0.23	481 ± 75	7794 ± 1	16.2 ± 2.5	0.981
		0.99	0.70 ± 0.07	449 ± 74	2223 ± 1	4.9 ± 0.8	0.973
	SET (g $L^{-1}_{gallic\ acid\ eq}$)	0.15	2.78 ± 0.19	503 ± 55	8797 ± 1	17.5 ± 1.9	0.988
		0.30	1.62 ± 0.07	348 ± 29	5129 ± 1	14.7 ± 1.3	0.999
		0.50	1.23 ± 0.07	337 ± 37	3883 ± 2	11.5 ± 1.2	0.981
		0.99	0.60 ± 0.04	313 ± 30	1889 ± 1	6.0 ± 0.5	0.986
		1.20	0.84 ± 0.03	203 ± 17	2665 ± 1	13 ± 1.1	0.986

*Freedom degree = 25.

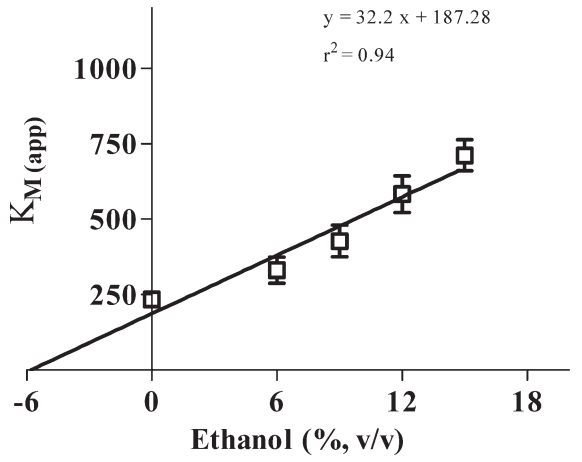

Figure 2. Competitive inhibition of the proteolytic activity of papain toward the Bz-Phe-Val-Arg-pNA substrate (0–510 μM) by ethanol (0–15% v/v) in tartaric buffer (pH 3.2). The secondary plot of $K_{M(app)}$ versus ethanol concentration was created to determine the K_i value (5.8 ± 0.3% v/v).

kinetic studies. The estimated kinetic parameters (Table 1) revealed that the $V_{max(app)}$ is similar in presence of alcohol increasing amount. In contrast, the strong increase in $K_{M(app)}$ with increased EtOH concentrations indicated that the presence of EtOH hindered the function of the ES complex and reduced the enzyme's affinity toward the substrate as indicated by the decreases in K_a. Based on these data, as with stem bromelain, EtOH should be considered to be a competitive inhibitor of the activity of papain.[25]

It has been shown that alcohol induces structural changes in proteins, including bromelain[30] and papain.[31] For both of these proteases, the conformational rearrangements that occur in the presence of ethanol have been observed to result in an increased amount of α-helical structural elements,[30,31] which explains the decrease in enzyme affinity toward the substrate that was revealed by the increase in $K_{M(app)}$.

The K_i value, as determined by a secondary plot of $K_{M(app)}$ versus the amount of EtOH (Figure 2) within the linear range of its concentration ($r^2 = 0.94$), was 5.8 (± 0.3)% v/v, which indicates that at this concentration, approximately 50% of papain remained in its free active form.

Sulfur dioxide inhibitory effect

In TB with different amounts of added free SO_2, all of the kinetic parameters decreased (Table 1), which confirms that SO_2 is a mixed-type inhibitor of papain. The K_i value, as determined by a secondary plot of $K_{M(app)}/V_{max(app)}$ versus [I], was 0.56 (± 0.05) mg L^{-1} (Figure 3A). Another secondary plot of $1/V_{max(app)}$ versus [I] (Figure 3B) was used to determine the K_i', which was 0.029 (± 0.002) mg L^{-1}. These two inhibitory constant values showed that free SO_2 strongly inhibited papain activity.

Papain inhibition studies were performed by testing compounds at different concentrations and in different mixtures in TB-EtOH that contained ethanol in the amount that is average for wine (12% v/v).

Inhibitory effect of sulfur dioxide-ethanol mixtures

As recently proven, SO_2 is a mixed-type inhibitor of stem bromelain in TB with added ethanol.[25] Based on these data, the effects of both basic wine elements on papain activity were also investigated.

The kinetic parameters obtained in TB-EtOH with two free SO_2 levels (2, 3, 4, and 5 mg L^{-1}) are summarized in Table 1. The decreases in $V_{max(app)}$ and K_a indicate free SO_2 also acts as a mixed-type inhibitor in the presence of 12% v/v alcohol.

The K_i value determined from a secondary plot of $K_{M(app)}/V_{max(app)}$ versus [I] was 0.0028 (±0.0004) mg L^{-1} (Figure 3A). Another secondary plot of $1/V_{max(app)}$ versus [I] (Figure 3B) was used to determine the K_i', which was 0.060 (±0.002) mg L^{-1}.

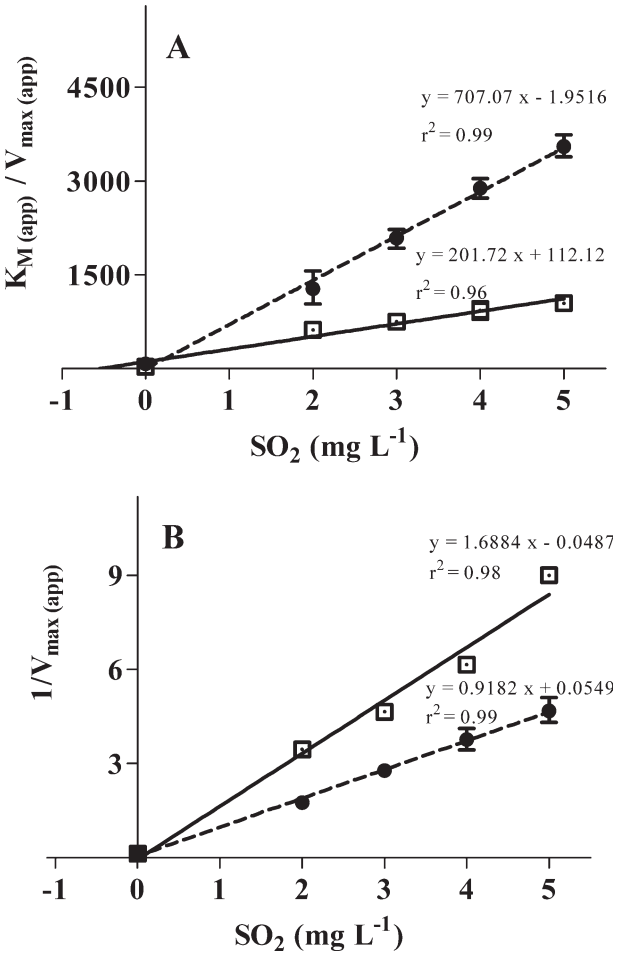

Figure 3. Mixed-type inhibition of the proteolytic activity of papain activity toward the Bz-Phe-Val-Arg-pNA substrate (0–510 μM) by free SO_2. The secondary plots of (A) $K_{M(app)}/V_{max(app)}$ and (B) $1/V_{max(app)}$ versus free SO_2 concentration were created to determine the K_i and K_i' values in tartaric buffer (□, K_i: 0.56 ± 0.05 mg L^{-1}; K_i': 0.029 ± 0.002 mg L^{-1}) and in tartaric buffer ethanol added (•, K_i: 0.0028 (±0.0004) mg L^{-1}; K_i': 0.060 (± 0.002) mg L^{-1}).

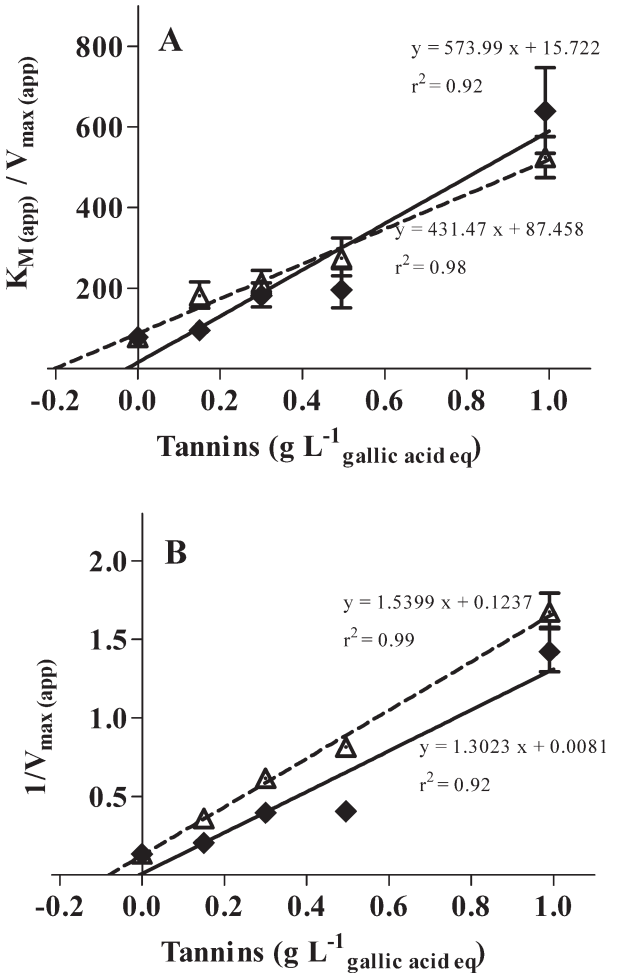

Figure 4. Mixed-type inhibitions of the proteolytic activity of papain toward the Bz-Phe-Val-Arg-pNA substrate (0–510 μM) by grape skin (SKT) and seed tannins (SET) in tartaric buffer ethanol added. The secondary plots of (A) $K_{M(app)}/V_{max(app)}$ and (B) $1/V_{max(app)}$ versus grape skin (◆) and seed (Δ) tannin concentrations were constructed to determine the K_i and K_i' values (for SKT, K_i was 0.027 ± 0.003 g $L^{-1}_{gallic\ acid\ eq}$ and K_i' was 0.006 ± 0.001 g $L^{-1}_{gallic\ acid\ eq}$; for SET, K_i was 0.20 ± 0.03 g $L^{-1}_{gallic\ acid\ eq}$ and K_i' was 0.08 ± 0.01 g $L^{-1}_{gallic\ acid\ eq}$).

These results reveal that, in the presence of alcohol amounts typical in wine, free SO_2 strongly increased the K_i' value, which indicates that SO_2 binds more easily to the free enzyme (E) than to enzyme-substrate (ES) complex.

Combined inhibitory effect of Grape tannins and ethanol

Preliminary studies proved that plant phenolic compounds react with enzymes to influence their physicochemical properties and consequently their catalytic activities. The reaction occurs at the lysine side chains of the indole rings of the tryptophan residues and at the free thiol groups of the cysteine side chains of the enzyme.[32]

Moreover, numerous studies have been performed with the aim of investigating the interaction between grape tannins and model proteins (BSA)[33,34] or enzymes, such as trypsin[35] and lysozyme;[24] these studies have identified two main types of potential interaction: hydrogen bonding and hydrophobic interactions. However, little is known about the inhibitory effects of grape skins and seed tannins on papain activity in acidic media such as TB-EtOH (pH 3.2).

Distinct kinetic curves (data not shown) were obtained in TB-EtOH supplemented with different amounts of skin tannin (0.15, 0.30, 0.50, and 0.99 g $L^{-1}_{gallic\ acid\ eq}$). In the presence of increased concentrations of inhibitor, all of the estimated kinetic parameters (Table 1) decreased, which indicates that the skin tannin acted as a mixed-type inhibitor of papain. Moreover, the grape seed tannins that were added to the TB-EtOH at different concentrations (0.15, 0.30, 0.50, 0.99, 1.20 g $L^{-1}_{gallic\ acid\ eq}$) appeared to be mixed-type inhibitors of papain based on the reduction in all of the kinetic parameters (Table 1) with increasing amounts of inhibitor, with the only exception of the highest concentration (1.20 g $L^{-1}_{gallic\ acid\ eq}$). This trend led us to hypothesize that grape tannins appear to be able to interact with both E and ES to result in EI and ESI; the EI has a lower affinity for S than E, and the ESI complex is non-productive.[28]

For SKT, a secondary plot of $K_{M(app)}/V_{max(app)}$ versus [I] within the linear range of the SKT concentrations ($r^2 = 0.92$) was used to estimate K_i (0.027 ± 0.003 g $L^{-1}_{gallic\ acid\ eq}$) as shown in Figure 4A. The other secondary plot of $1/V_{max(app)}$

Table 2. Comparison of the Inhibitory Effects of the Tested Compounds on the Activities of Papain and Bromelain[25]

		Papain	Bromelain
EtOH	Inhibition	Competitive	Competitive
	K_i	5.8 (± 0.3) % v/v	11.4 (±1.0) % v/v
Free SO_2+EtOH	Inhibition	Mixed-type	Mixed-type
	K_i	0.0028 (±0.0004) mg L^{-1}	4.55 (±1.07) mg L^{-1}
	K_i'	0.060 (±0.002) mg L^{-1}	0.4 (±0.09) mg L^{-1}
SKT+EtOH	Inhibition	Mixed-type	Uncompetitive
	K_i	0.027 ± (0.003) g $L^{-1}_{\text{gallic acid eq}}$	0.593 (±0.003) g $L^{-1}_{\text{gallic acid eq}}$
	K_i'	0.006 (± 0.001) g $L^{-1}_{\text{gallic acid eq}}$	–
SET+EtOH	Inhibition	Mixed-type	Uncompetitive
	K_i	0.20 (± 0.03) g $L^{-1}_{\text{gallic acid eq}}$	0.453 (±0.004) g $L^{-1}_{\text{gallic acid eq}}$
	K_i'	0.08 (± 0.01) g $L^{-1}_{\text{gallic acid eq}}$	–

EtOH, ethanol; SET, seed tannin preparation; SKT, skin tannin preparation; SO_2, sulfur dioxide.

Table 3. Kinetic Parameters of Papain versus the Bz-Phe-Val-Arg-pNA Substrate in Tartaric Buffer (pH 3.2) Containing the Following Three Inhibitors: A (SKT 0.99 g $L^{-1}_{\text{gallic acid eq}}$, SET 1.20 g $L^{-1}_{\text{gallic acid eq}}$, EtOH 12% v/v), B (SKT 0.99 g $L^{-1}_{\text{gallic acid eq}}$, Free SO_2 5 mg L^{-1}, EtOH 12%v/v), and C (SET 1.20 g $L^{-1}_{\text{gallic acid eq}}$, Free SO_2 5 mg L^{-1}, EtOH 12% v/v).

Combined Inhibitors		$V_{max(app)}$ (I.U. mg^{-1})	$K_{M(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a (min^{-1} μM^{-1})	r^2*
Control	EtOH	7.47 ± 0.49	583 ± 60	23611 ± 1	40.5 ± 4.2	0.992
A	SKT+SET+EtOH	6.03 ± 0.58	2291 ±258	19067 ± 1	8.3 ± 0.9	0.999
B	SKT+freeSO_2+EtOH	2.31 ± 0.23	1059 ± 141	7307 ± 1	6.9 ± 0.9	0.994
C	SET+freeSO_2+EtOH	0.18 ± 0.01	76 ± 10	575 ± 1	7.5 ± 1.1	0.976

*Freedom degree = 20.
EtOH, ethanol; SET, seed tannin preparation; SKT, skin tannin preparation; SO_2, sulfur dioxide.

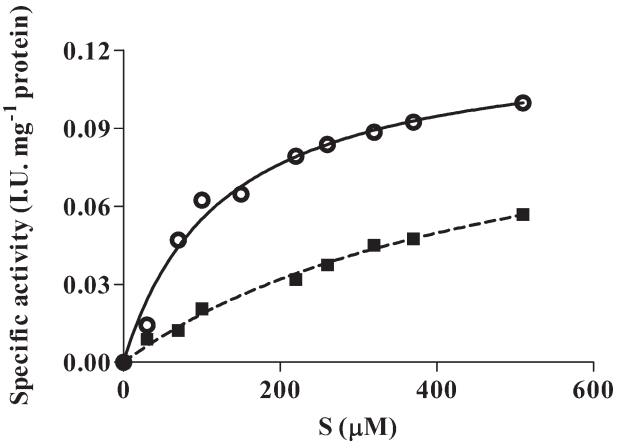

Figure 5. Specific activities (I.U. mg^{-1}protein) of papain versus the Bz-Phe-Val-Arg-pNA substrate (0–510 μM) in two different commercial table white wines: a (○) and b (■).

versus [I] (Figure 4B) within the linear range of SKT concentrations ($r^2 = 0.92$), was used to determine the K_i', which was 0.006 (± 0.001) g $L^{-1}_{\text{gallic acid eq}}$. The latter lower value suggests that the skin tannins bind more easily to ES to give rise to an ESI ternary unproductive complex.

For SET, a secondary plot of $K_{M(app)}/V_{max(app)}$ versus [I] in the linear range (0–0.990 g $L^{-1}_{\text{gallic acid eq}}$) of SET concentrations ($r^2 = 0.98$) was used to estimate K_i (0.20 ± 0.03 g $L^{-1}_{\text{gallic acid eq}}$) as shown in Figure 4A. The other secondary plot of $1/V_{max(app)}$ versus [I] (Figure 4B) within the linear range (0–0.990 g $L^{-1}_{\text{gallic acid eq}}$) of SET concentrations ($r^2 = 0.99$) was used to determine the K_i', which was 0.08 (± 0.01) g $L^{-1}_{\text{gallic acid eq}}$. These values indicate that skin tannins have a stronger inhibitory effect than do seed tannins based on the lower K_i and K_i' values.

Comparison of the papain and bromelain inhibition studies

The activity of papain was more strongly affected by all of the tested substances than was the activity of bromelain (Table 2). EtOH was found to be a competitive inhibitor of both proteases but had rather limited effects; the K_i values determined from the secondary plot were 11.4(±1.0)% v/v and 5.8(±0.3)% v/v for stem bromelain and papain, respectively. The strongest inhibitions were exerted by free SO_2 ($K_i = 4.55 \pm 1.07$ mg L^{-1}, $K_i' = 0.40 \pm 0.09$ mg L^{-1} on stem bromelain and $K_i = 0.0028 \pm 0.0004$ mg L^{-1}, $K_i' = 0.060 \pm 0.002$ mg L^{-1} on papain), which acted as a mixed-type inhibitor of both enzymes. Finally, SET and SKT were found to be uncompetitive and mixed-type inhibitors of stem bromelain and papain, respectively. The findings of this study provide useful information for future biotechnological applications of both of these proteases in the winemaking process.

Combined effect of wine inhibitors on papain activity

Finally, papain proteolytic activity was tested in a complex TB-EtOH based on TB that contained a mixture of three inhibitors: A (SKT 0.99 g $L^{-1}_{\text{gallic acid eq}}$, SET 1.20 g $L^{-1}_{\text{gallic acid eq}}$, EtOH 12% v/v), B (SKT 0.99 g $L^{-1}_{\text{gallic acid eq}}$, free SO_2 5 mg L^{-1}, EtOH 12%v/v), and C (SET 1.20 g $L^{-1}_{\text{gallic acid eq}}$, free SO_2 5 mg L^{-1}, EtOH 12% v/v). The kinetic curves followed the hyperbolic behavior of the Michaelis–Menten equation (data not shown), and the corresponding parameters (Table 3) show that all the tested inhibitor mixtures affected protease activity.

The addition of tannins (sample A) resulted in a constant $V_{max(app)}$ that was similar (6.03 I.U. mg^{-1}) to that of the control (7.47 I.U. mg^{-1}) and a significant increase in $K_{M(app)}$, (from 583 to 2291 μM), which indicates that the enzyme-substrate complex formation was sluggish. In the presence of SO_2 (sample B and C), the $V_{max(app)}$ values significantly decreased, which suggests that the product release velocity was slowed, in particular when SET were combined

Table 4. Kinetic Parameters of Papain Toward the Bz-Phe-Val-Arg-pNA Substrate in Two Different (a, b) Commercial White Wines

	$V_{max(app)}$ (I.U. mg^{-1})	$K_{M(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a (min^{-1} μM^{-1})	r^2*
Wine a	0.124 ± 0.004	126.1 ± 10.8	393.37 ± 0.01	3.12 ± 0.29	0.984
Wine b	0.113 ± 0.007	510.2 ± 53.0	357.96 ± 0.02	0.70 ± 0.08	0.992

*Freedom degree = 27.

with SO_2. Moreover, as indicated by the decrease in K_a, the enzyme's affinity toward the substrate was about 5-fold lower respect to the control in presence of all the inhibitor mixtures.

Kinetic characterization of papain in white wines supplemented with synthetic substrate

After studying the effects of wine inhibitors on protease activity in TB-EtOH, the kinetic behavior of papain was studied in two different commercial white table wines that had been supplemented with a synthetic tripeptide chromogenic substrate (Bz-Phe-Val-Arg-pNA, 0–510 μM) that was previously selected as the most suitable for the average minimum pH of wine (3.2).[23]

Papain exhibited the hyperbolic behavior of the Michaelis-Menten equation (Figure 5) in both wines despite their different physicochemical compositions. Nevertheless, comparison of the kinetic parameters (particularly k_{cat} and K_a) of the real wines (Table 4) with those of the TB-EtOH that had been supplemented with a potential inhibitor mixture (Table 3) revealed a strong reduction of proteolytic activity. Moreover, it is notable that the papain maximum velocity rate revealed in the real white wine was similar and comparable to that of the $V_{max(app)}$ that was estimated in the TB-EtOH to which the inhibitor mixture C was added (Table 3).

Furthermore, as shown in Table 4, the protease exhibited comparable V_{max} values in both wines, whereas K_a, which indicates affinity of the enzyme for the substrate, was lower in the wine with the higher levels of SO_2 and total phenols (sample "b").

Papain from papaya latex exhibited good hydrolytic activity toward a synthetic tripeptide chromogenic substrate in a TB-EtOH that had been supplemented with natural wine inhibitors (i.e., ethanol, SO_2 and grape tannins). All of the examined wine constituents were found to be reversible inhibitors. Ethanol was found to be a competitive inhibitor with a rather limited effect, whereas the strongest inhibition was exerted by sulfur dioxide, which acted as a mixed-type inhibitor.

Additionally, in both of the tested white table wines, the catalytic activity of papain was determined to be affected by SO_2 and total phenol levels.

Acknowledgment

The research was funded by the Italian Ministry of Agriculture, Food, and Forestry.

Notation

Glossary

BSA = bovine serum albumin
$[E]_{tot}$ = total enzyme molar concentration
E = free enzyme
EI = enzyme-inhibitor complex
ES = enzyme-substrate complex
ESI = enzyme-substrate-inhibitor complex
EtOH = ethanol, % v/v
I = inhibitor
I.U. = International Unit
K_a = (k_{cat}/K_M), affinity constant, min^{-1} μM^{-1}
k_{cat} = ($V_{max}/[E]_{tot}$), turnover number, min^{-1}
K_i = inhibition constant
K_M = Michaelis-Menten constant, μM
TB-EtOH = tartaric buffer added with 12% v/v ethanol
SET = seed tannin preparation, g $L^{-1}_{gallic\ acid\ eq}$
SKT = skin tannin preparation, g $L^{-1}_{gallic\ acid\ eq}$
SO_2 = sulfur dioxide, mg L^{-1}
TB = tartaric buffer (tartaric acid/Na tartrate at 0.03 M, pH 3.2)
V_{max} = maximum velocity at which enzyme catalyzes reaction, I.U. mg^{-1}

Greek symbols

ε = molar absorptivity, mM^{-1} cm^{-1}
ΔA = absorbance variation

Subscripts

(app) = referred to apparent kinetic parameters in presence of inhibitor

Literature Cited

1. Feijoo-Siota L, Villa TG. Native and biotechnologically engineered plant proteases with industrial applications. *Food Bioprocess Tech.* 2011;4:1066–1088.
2. Polaina J, MacCabe AP. *Industrial Enzymes: Structure, Function and Applications*. New York: Springer; 2007.
3. Wang YT, Yang CY, Chen YT, Lin Y, Shaw JF. Characterization of senescence-associated proteases in postharvest broccoli florets. *Plant Physiol Biochem.* 2004;42:663–670.
4. Wang JS, Zhao MM, Zhao QZ, Bao Y, Jiang YM. Characterization of hydrolysates derived from enzymatic hydrolysis of wheat gluten. *J Food Sci.* 2007;72:C103–C107.
5. Bekhit AA, Hopkins DL, Geesink G, Bekhit AA, Franks P. Exogenous proteases for meat tenderization. *Crit Rev Food Sci.* 2014;54:1012–1031.
6. Klompong V, Benjakul S, Kantachote D, Shahidi F. Use of protein hydrolysate from yellow stripe trevally (Selaroides leptolepis) as microbial media. *Food Bioprocess Tech.* 2012;5:1317–1327.
7. De Clerck J. The use of proteolytic enzymes for the stabilization of beer. *Tech Q Master Brew Assoc Am.* 1969;6:136–140.
8. Kennedy JF, Pike VW. Papain, chymotrypsin and related proteins-a comparative study of their beer chill-proofing abilities and characteristics. *Enzyme Microb Tech.* 1981;3:59–63.
9. Priest FG, Stewart GG. *Handbook of Brewing*, 2nd ed. New York: CRC; 2006.
10. Meyer AS, Koser C, Adler-Nissen J. Efficiency of enzymatic and other alternative clarification and fining treatments on turbidity and haze in cherry juice. *J Agric Food Chem.* 2001;49:3644–3650.
11. Jones BL. Endoproteases of barley and malt. *J Cereal Sci.* 2005;42:139–156.
12. Mosafa L, Moghadam M, Shahedia M. Papain enzyme supported on magnetic nanoparticles: Preparation, characterization and application in the fruit juice clarification. *Chin J Catal* 2013;34:1897–1904.
13. Sahoo B, Sahu SK, Bhattacharya D, Dhara D, Pramanik P. A novel approach for efficient immobilization and stabilization of papain on magnetic gold nanocomposites. *Colloids Surf B.* 2013;101:280–289.

14. Homaei AA, Sajedi RH, Sariri R, Seyfzadeh S, Stevanato R. Cysteine enhances activity and stability of immobilized papain. *Amino Acids*. 2010;38:937–942.
15. Waters EJ, Alexander G, Muhlack R, Pocock KF, Colby C, O'Neill BK, Høj PB, Jones P. Preventing protein haze in bottled white wine. *Aust J Grape Wine Res*. 2005;11:215–225.
16. Catarino S, Madeira M, Monteiro F, Rocha F, Curvelo-Garcia AS, Bruno de Sousa R. Effect of bentonite characteristics on the elemental composition of wine. *J Agric Food Chem*. 2008;56: 158–165.
17. Bayonove C, Cabaroglu T, Dufour C, Razungles A, Sapis JC, Baumes R, Günata Z. Influence du collage sur le potentiel aromatique variétal du vin. XXIème Congrès de la Vigne et du Vin, *Uruguay*; 1995.
18. Cabaroglu T, Canbas A, Lepoutre JP, Gunata Z. Free and bound volatile composition of red wines of *Vitis vinifera* L. cv. Okuzgozu and Bogazkere grown in Turkey. *Am J Enol Viticult*. 2002;53:63–68.
19. Dizy M, Bisson LF. White wine protein analysis by capillary zone electrophoresis. *Am J Enol Viticult*. 1999;50:120–127.
20. Modra EJ, Williams PJ, Lee TH, Wallace W. Effect of commercial peptidases on must and wine. In: Ribéreau-Gayon P, Lonvaud A, editors. *Actualités Oenologiques 89: comptes rendus du 4è symposium d'oenologie*; 15–17 juin 1989; Bordeaux. Paris: Dunod; 1990:217–221.
21. Marangon M, Van Sluyter SC, Robinson EM, Muhlack RA, Holt HE, Haynes PA, Godden PW, Smith PA, Waters EJ. Degradation of white wine haze proteins by Aspergillopepsin I and II during juice flash pasteurization. *Food Chem*. 2012;135:1157–1165.
22. Benucci I, Esti M, Liburdi K, Garzillo AMV. Pineapple stem bromelain immobilized on different supports: catalytic properties in model wine. *Biotechnol Progr*. 2012;28:1472–1477.
23. Esti M, Benucci I, Lombardelli C, Liburdi K, Garzillo AMV. Papain from papaya (*Carica papaya L.*) fruit and latex: preliminary characterization in alcoholic–acidic buffer for wine application. *Food Bioprod Process*. 2013;91:595–598.
24. Guzzo F, Cappello MS, Azzolini M, Tosi E, Zapparoli G. The inhibitory effects of wine phenolics on lysozyme activity against lactic acid bacteria. *Int J Food Microbiol*. 2011;148: 184–190.
25. Esti M, Benucci I, Liburdi K, Garzillo AMV. Effect of wine inhibitors on free pineapple stem bromelain activity in a model wine system. *J Agric Food Chem*. 2011;59:3391–3397.
26. Office International de la Vigne et du Vin. *Recueil des Méthodes Internationales d'Analyse des Vins et des Mouts*. Paris, France: OIV; 1990.
27. Hale LP, Greer PK, Trinh CT, James CL. Proteinase activity and stability of natural bromelain preparations. *Int Immunopharmacol*. 2005;5:783–793.
28. Segel IH. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. New York: Wiley-Interscience; 1975.
29. Antunes F, Marinho HS, Barreto MC, Pavão ML, Pinto RE. Diagnosis of enzyme inhibition based on the degree of inhibition. *Biochim Biophys Acta*. 2003;1624:11–20.
30. Haq SK, Rasheedi S, Sharma P, Ahmad B, Khan RH. Influence of salts and alcohols on the conformation of partially folded intermediate of stem bromelain at low pH. *Int J Biochem Cell B*. 2005;37:361–374.
31. Szabó A, Kotormán M, Laczkó I, Simon LM. Spectroscopic studies of stability of papain in aqueous organic solvents. *J Mol Catal B Enzym*. 2006;41:43–48.
32. Rohn S, Rawel HM, Kroll J. Inhibitory effects of plant phenols on the activity of selected enzymes. *J Agric Food Chem*. 2002; 50:3566–3571.
33. Schmauch G, Chamault C, Teissedre PL, Saucier C. Kinetics and stoichiometry of grape seed tannins/BSA interactions. *J Int Sci Vigne Vin*. 2010; special issue Macrowine, 67–74.
34. Carvalho E, Mateus N, de Freitas V. Flow nephelometric analysis of protein–tannin interactions. *Anal Chim Acta*. 2004;513:97–101.
35. Gonçalves R, Soares S, Mateus N, de Freitas V. Inhibition of trypsin by condensed tannins and wine. *J Agric Food Chem*. 2007;55:7596–7601.

Manuscript received Jun. 5, 2014, and revision received Oct. 4, 2014.