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Effect of Wine Inhibitors on Free Pineapple Stem Bromelain Activity in a Model Wine System

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ABSTRACT: The influence of potential inhibitors, naturally present in wine, on the activity of stem bromelain was investigated in order to evaluate the applicability of this enzyme for protein stabilization in white wine. Bromelain proteolytic activity was tested against a synthetic substrate (Bz-Phe-Val-Arg-pNA) in a model wine system after adding ethanol, sulfur dioxide (SO_2), skin, seed, and gallic and ellagic tannins at the average range of their concentration in wine. All the inhibitors of stem bromelain activity tested turned out to be reversible. Ethanol was a competitive inhibitor with a rather limited effect. Gallic and ellagic tannins have no inhibitory effect on stem bromelain activity, while both seed and skin tannins were uncompetitive inhibitors. The strongest inhibition effect was revealed for sulfur dioxide, which was a mixed-type inhibitor for the enzyme activity. This study provides useful information relative to a future biotechnological application of stem bromelain in winemaking.

KEYWORDS: pineapple stem bromelain, wine inhibitors, model wine system

1. INTRODUCTION

Stem bromelain is a cysteine proteinase extracted from the stem of the pineapple plant, *Ananas comosus*.[1] It has multiple uses in food processing, including meat tenderization, applications in the baking industry[2] and enhancement of protein stability in beverages, especially fruit juices and beers.[3,4] To our knowledge, no one apart from us has proposed the use of pineapple stem bromelain for biotechnological application in wine. However, in a recent study there was a preliminary characterization of this protease under wine-like conditions, showing that stem bromelain might find productive biotechnological applications in winemaking.[5]

Since wine contains various compounds (such as ethanol, polyphenols and sulfur dioxide (SO_2)) that could have inhibitory effects on the enzymatic activity of stem bromelain, further studies are necessary in order to evaluate its applicability for protein stabilization in white wine. Among these potentially inhibitory compounds, ethanol is derived from the alcoholic fermentation carried out by yeasts, which convert grape sugars to carbon dioxide and alcohol. Ethanol concentration, depending on the wine style and degree of maturity of the grapes, can range from 8% to 18% v/v in dry white and red wines.[6]

Wine is also an excellent source of various classes of polyphenols, most of which originate in the grape berry; white wines contain significantly lower amounts of total phenols compared with red wines.[7] Wine tannins are phenolic compounds classically divided into condensed and hydrolyzable forms. While the first involve flavan-3-ol units with various degrees of substitution and polymerization and constitute the largest group of proanthocyanidins, the latter are composed of gallic acid and ellagic acid esters formed with glucose or related sugars.[8]

The biological activity of phenolic compounds can be summarized as comprising three main mechanisms: metal chelation, antioxidant activity and enzyme inhibition.[9] In particular, tannins have the ability to act as protein-complexing agents, inhibiting the activity of proteolytic enzymes such as trypsin.[7] Liang et al.[10] have studied the effect of polyphenols extracted from Chinese green tea on the activity of bromelain from pineapple juice, proving that tea polyphenols act as competitive inhibitors of bromelain.

Sulfur dioxide is industrially used as an antioxidant, an inhibitor of oxidizing enzymes and an antiseptic. Sulfur dioxide can be present in two different forms in wine, namely, free (as HSO_3^- or SO_2) or bound to carbonyl or unsaturated compounds and/or phenols.[11] Only free SO_2 possesses reducing and antiseptic properties. Excessive levels of SO_2 must be avoided during the winemaking process as they result in poor wine aroma and flavor. Finally, the content of this toxic substance in the end-product must comply with existing legal limits (EC regulations 1493/1999 and EC 753/2002).

Inhibition studies can tell us something about the specificity of an enzyme, the physical and chemical architecture of the active site, and the kinetic mechanism of the reaction. An "inhibitor" can be defined as any substance that reduces the velocity of an enzyme-catalyzed reaction. In order to supply a complete characterization of stem bromelain to evaluate its suitability for protein stabilization of white wine, the influence on its protease activity of potential inhibitors naturally present in wine must be determined. Ethanol, tannins and sulfur dioxide have never been tested as inhibitors of stem bromelain activity under wine like conditions. In this work the inhibitory effects of ethanol, sulfur dioxide (SO_2), grape skin, seed, gallic and ellagic tannins were investigated over the average range of their respective concentrations of wine.

2. MATERIALS AND METHODS

2.1. The Enzyme and Chemicals. Stem bromelain (EC 3.4.22.32) was obtained from Sigma-Aldrich (Milan, Italy). The synthetic peptide substrate Bz-Phe-Val-Arg-p-nitroaniline (pNA) was

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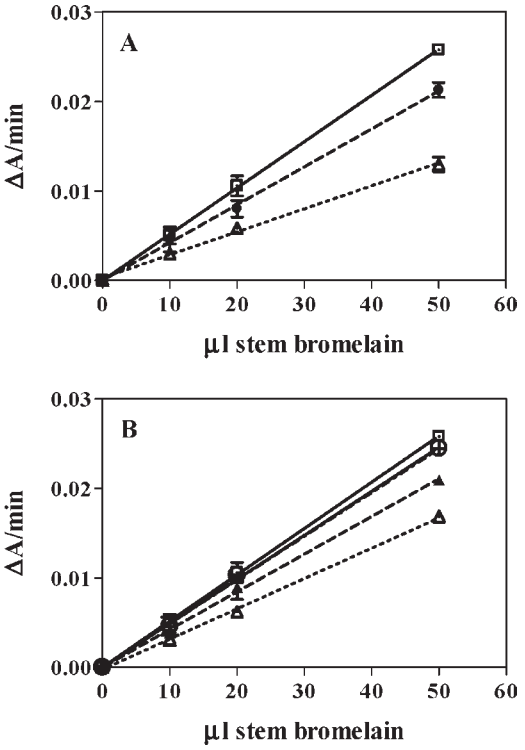

Figure 1. ΔA/min vs stem bromelain assay volume (μL) versus Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2) in the absence (control, □) and in the presence of different inhibitors: (A) ethanol (●) and SO_2 (Δ); (B) skin (▲), seed (Δ), gallic (○) and ellagic tannins (+).

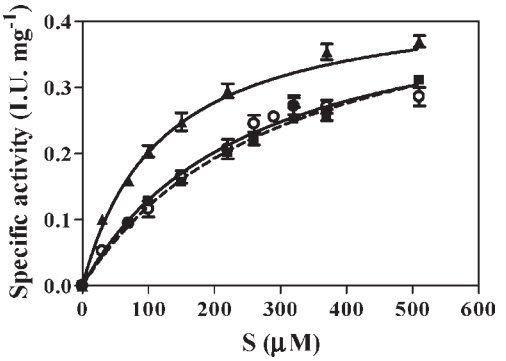

Figure 2. Specific activity (IU mg^{-1}) of stem bromelain versus Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2) in the absence (control, ▲) and in the presence of different ethanol concentrations: 12% v/v (○) and 18% v/v (■).

purchased from Bachem, Germany. Grape skin, seed, gallic and ellagic tannins, as preparations intended for enological use, were kindly supplied by EVERINTEC (Venice, Italy). All other reagents were obtained from Sigma-Aldrich (Milan, Italy).

2.2. Total Phenol Content of Tannin Preparations. The total phenol content of the above-mentioned tannin preparations was measured at 700 nm using an UV−visible spectrophotometer (Perkin-Elmer Lambda 25, Beaconsfield Buks, B) according to the Folin−Ciocalteu method.[12] Results were expressed as gallic acid equivalents (g L^{-1} gallic acid equiv). A calibration curve was constructed on the basis of solutions at known and increasing concentrations of gallic acid.

2.3. Enzymatic Activity Assay. Stem bromelain activity was assayed using Bz-Phe-Val-Arg-pNA as substrate, at 25 °C in a model wine buffer (tartaric acid/Na tartrate 0.03 M, pH 3.2, ethanol 12% v/v) containing cysteine (5 mM). Several concentrations of Bz-Phe-Val-Arg-pNA, ranging from 0 to 510 μM, were tested in the presence of 0.02 mg mL^{-1} (0.84 μM) stem bromelain.

Cleavage of the substrate results in release of free pNA that was detected colorimetrically at 410 nm. Proteolytic activity was determined from the change in absorbance vs time (3 min), using the linear portion of the curve. Bromelain activity was calculated in IU of pNA produced, using a molar absorptivity of 8.480 mM^{-1} cm^{-1} for pNA.[13] Specific activity was calculated as IU mg^{-1} of protein. A blank correction was made using a sample that did not contain enzyme.[5] All measurements were made in triplicate, and the standard deviations were reported.

2.4. Kinetic Study and Determination of Kinetic Parameters. A kinetic study was carried out by varying the substrate concentration (0−510 μM) at 25 °C in the presence of 0.02 mg mL^{-1} (0.84 μM) stem bromelain in model wine buffer, which also contained one of the following potential inhibitors at different concentrations: ethanol (0, 12, 18% v/v), free SO_2 (0, 10, 25 mg L^{-1}), skin tannin preparation (0, 0.5, 2 g L^{-1}), seed tannin preparation (0, 0.5, 2 g L^{-1}), gallic tannin preparation (0, 3, 5 g hL^{-1}) and ellagic tannin preparation (0, 5, 10 g hL^{-1}).

Kinetic curves were obtained measuring proteolytic activity at different substrate concentrations (8−10 points), making 3 turns of measurements for each concentration. The total time necessary to carry out a single kinetic curve is about 2 h (incubation time).

Kinetic parameters (k_{cat}, K_m, K_a) of stem bromelain were determined according to Michaelis−Menten equation using a nonlinear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.).

The K_m (Michaelis−Menten constant) value reflects the enzyme−substrate complex formation, 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, the K_a (affinity constant), being the ratio k_{cat}/K_m, indicates the affinity of the enzyme toward the substrate. It is indicative of both reaction steps and expresses the overall catalytic efficiency.

2.5. Inhibition Study. Irreversible and reversible inhibition may be distinguished by plotting ΔA/min vs [E_t], where [E_t] represents the amount of enzyme added to the assay. For a reversible inhibitor, the "plus inhibitor" curve has a smaller slope than the control curve and goes through the origin. If an irreversible inhibitor is present, the "plus inhibitor" curve has the same slope as the control curve, but intersects the horizontal axis at a position equivalent to the amount of enzyme that is irreversibly inactivated.[14]

The interaction between a reversible inhibitor (I) and free enzyme (E), or enzyme−substrate complex (ES) can be described by different inhibition models (competitive, uncompetitive and mixed-type inhibition), assuming that only a single substrate is involving in the reaction and that only one type of inhibitor is present at any time. K_i value (inhibition constant) reflects the concentration of an inhibitor that decreases the rate of an enzyme-catalyzed reaction by 50%. The equilibria

$$E + I \underset{K_i}{\rightleftharpoons} EI \quad (1)$$

$$ES + I \underset{K_i'}{\rightleftharpoons} ESI \quad (2)$$

are defined by the thermodynamic constants, K_i or K_i', respectively:

$$K_i = \frac{[E][I]}{[EI]} \quad (3)$$

$$K_i' = \frac{[ES][I]}{[ESI]} \quad (4)$$

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Table 1. Kinetic Parameters of Stem Bromelain versus Bz-Phe-Val-Arg-pNA Substrate in Tartaric Buffer (pH 3.2) Containing Different Concentrations of Ethanol: 0% v/v, 12% v/v and 18% v/v

ethanol (% v/v)	$V_{max(app)}$ (IU mg^{-1})	$K_{m(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a (min$^{-1}\mu$M^{-1})
0	0.44 ± 0.02	118 ± 10	1166.20 ± 0.02	9.89 +1.20/−0.97
12	0.46 ± 0.02	250 ± 27	1201.90 ± 0.02	4.81 +0.59/−0.47
18	0.48 ± 0.02	305 ± 20	1287.05 ± 0.02	4.22 +0.30/−0.26

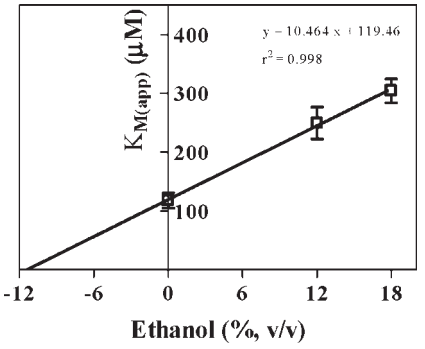


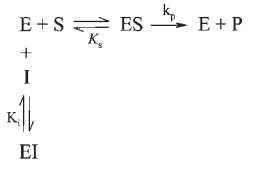
Figure 3. Secondary plot of $K_{m(app)}$ versus ethanol concentration.

Table 2. Total Phenolic Content of Enological Tannin Preparations Used, at Different Concentrations (g L^{-1}), Expressed as Grams of Gallic Acid Equivalents of Model Wine Solution (g L^{-1} gallic acid equiv)

tannins	exptl concn used (g L^{-1})	total phenolic content (g L^{-1} gallic acid equiv)
skin	0.5	0.25
	2	0.99
seed	0.5	0.30
	2	1.20
gallic	0.03	0.02
	0.05	0.03
ellagic	0.05	0.02
	0.1	0.04

These definitions and equilibria describe the different types of enzyme inhibition listed below. In each inhibition model, the kinetic equation used is a modification of Michaelis−Menten, in which the kinetic parameters K_m and V_{max} are replaced by the corresponding apparent kinetic parameters $K_{m(app)}$ and $V_{max(app)}$.[14]

2.5.1. Competitive Inhibition. A competitive inhibitor is a substance that combines with free enzyme in a manner that prevents substrate binding. That is, the inhibitor and the substrate are mutually exclusive, often because of true competition ;for the same site.



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A competitive inhibitor acts only to increase the K_m for the substrate. The V_{max} remains unchanged, but in the presence of a competitive inhibitor, a much greater substrate concentration is required to attain any given fraction of V_{max}.

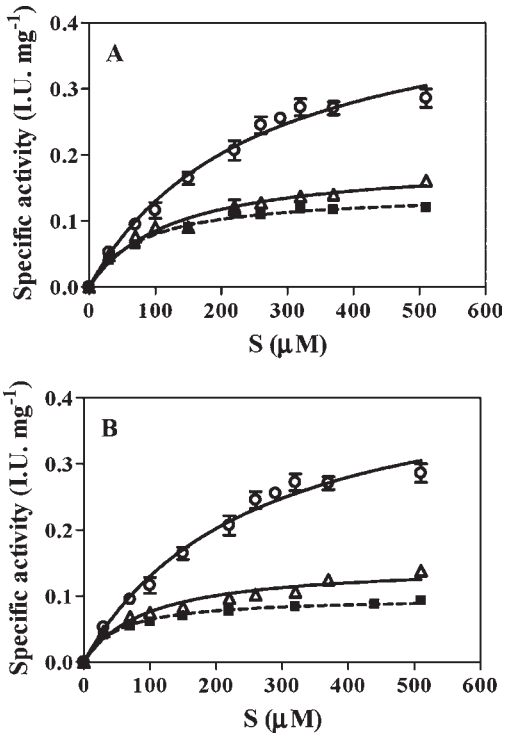
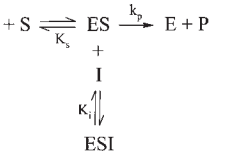


Figure 4. Specific activity (IU mg^{-1}) of stem bromelain versus Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) in the absence (control, ○) and in the presence of different concentrations of (A) skin tannins (0.25 g L^{-1} gallic acid equiv (Δ); 0.99 g L^{-1} gallic acid equiv (■)) and (B) seed tannins (0.30 g L^{-1} gallic acid equiv (Δ) and 1.20 g L^{-1} gallic acid equiv (■)).

The K_i value is determined by a secondary plot of $K_{m(app)}$ vs [I], that has intercepts of K_m (on the $K_{m(app)}$-axis) and $-K_i$ (on the [I]-axis). The lower the value of K_i, the greater is the degree of inhibition at any given [S] and [I]. The $K_{m(app)}$ is a linear function of the inhibitor concentration, as shown below:[14]

$$K_{m(app)} = \frac{K_m}{K_i}[I] + K_m \quad (6)$$

2.5.2. Uncompetitive Inhibition. An uncompetitive inhibitor binds reversibly to the enzyme−substrate complex yielding an inactive ESI complex, as described by the following equilibria:



(7)

An uncompetitive inhibitor decreases V_{max} and K_m to the same extent. A secondary plot of $1/K_{m(app)}$ vs [I], being linear with

Table 3. Kinetic parameters of stem bromelain versus Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) containing different concentration of skin and seed tannins

tannins	concn (g L^{-1} gallic acid equiv)	$V_{max(app)}$ (IU mg^{-1})	$K_{m(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a ($min^{-1}\mu M^{-1}$)
	0	0.46 ± 0.02	250 ± 27	1201.90 ± 0.02	4.81 +0.59/−0.47
skin	0.25	0.191 ± 0.008	125.2 ± 15.8	506.147 ± 0.008	4.04 +0.58/−0.45
	0.99	0.143 ± 0.003	79.14 ± 6.52	378.42 ± 0.003	4.78 +0.43/−0.36
seed	0.30	0.149 ± 0.006	95.28 ± 13.02	393.229 ± 0.006	4.13 +0.60/−0.49
	1.20	0.098 ± 0.002	52.64 ± 3.92	259.235 ± 0.002	4.92 +0.40/−0.34

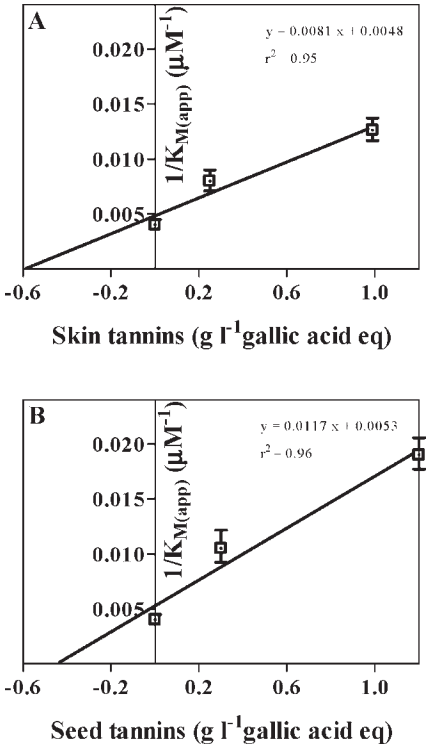


Figure 5. Secondary plot of $1/K_{m(app)}$ versus (A) skin tannin concentration and (B) seed tannin concentration.

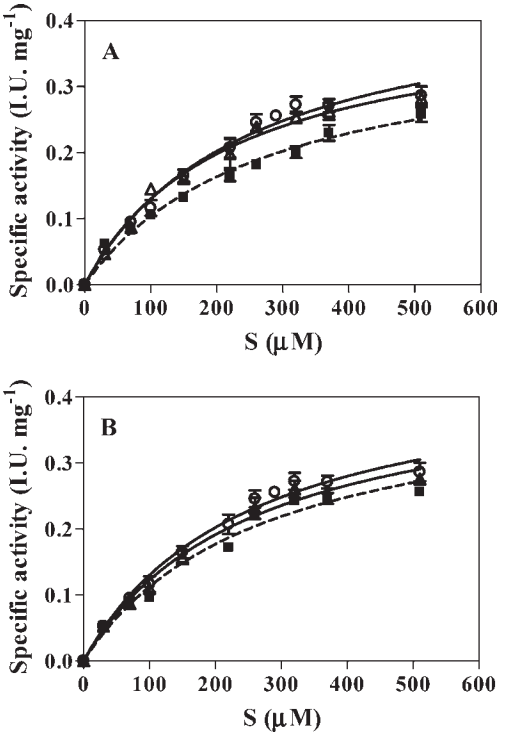
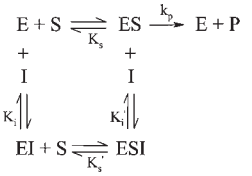


Figure 6. Specific activity (IU mg^{-1}) of stem bromelain versus the Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) in the absence (control, ○) and in the presence of different concentrations of (A) gallic tannins (0.02 g L^{-1} gallic acid equiv (Δ); 0.03 g L^{-1} gallic acid equiv (■) and (B) ellagic tannins (0.02 g L^{-1} gallic acid equiv (Δ); 0.04 g L^{-1} gallic acid equiv (■)).

intercepts of $1/K_m$ (on the $1/K_{m(app)}$-axis) and $-K_i$ (on the [I]-axis), is used for the determination of the K_i value:[14]

$$K_{m(app)} = \frac{1}{K_i K_m}[I] + \frac{1}{K_m} \quad (8)$$

2.5.3. Mixed-Type Inhibition. A mixed-type inhibitor binds reversibly both to free enzyme and to enzyme−substrate complex. EI has a lower affinity than E for S, and the ESI complex is nonproductive. The system can be considered a mixture of partial competitive inhibition and pure noncompetitive inhibition; the equilibria describing this system are shown as follows:



(9)

$$\alpha = 1 + \frac{[I]}{K_i} \quad (10)$$

$$\alpha' = 1 + \frac{[I]}{K_i'} \quad (11)$$

$$K_{m(app)} = K_m \frac{\alpha}{\alpha'} \quad (12)$$

$$V_{max(app)} = \frac{V_{max}}{\alpha'} \quad (13)$$

A secondary plot of $1/V_{max(app)}$ vs [I], being linear with intercepts of $1/V_{max}$ (on the $1/V_{max(app)}$-axis) and $-K_i'$ (on the [I]-axis), is used to determine the K_i' value. Another secondary plot of $K_{m(app)}/V_{max(app)}$ vs [I], being linear with intercepts of K_m/V_{max} (on the $K_{m(app)}/V_{max(app)}$-axis) and $-K_i$ (on the [I]-axis), is used to determine the K_i value.[14]

3. RESULTS AND DISCUSSION

Wine contains various compounds that could have an inhibitory effect on enzymatic activity. In light of these considerations and in order to propose a future biotechnological application of

Table 4. Kinetic Parameters of Stem Bromelain versus Bz-Phe-Val-Arg-pNA Substrate in Tartaric Buffer (pH 3.2, Ethanol 12% v/v) Containing Different Concentrations of Gallic and Ellagic Tannins

tannins	concn (g L^{-1}gallic acid equiv)	$V_{max(app)}$ (IU mg^{-1})	$K_{m(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a ($min^{-1}\mu M^{-1}$)
	0	0.46 ± 0.02	250 ± 27	1201.90 ± 0.02	4.81 +0.59/−0.47
gallic	0.02	0.42 ± 0.02	220.7 ± 22.5	1101.15 ± 0.02	4.99 +0.56/−0.46
	0.03	0.38 ± 0.02	270.5 ± 29.45	1016.52 ± 0.02	3.76 +0.46/−0.37
ellagic	0.02	0.44 ± 0.02	255.8 ± 29.6	1153.51 ± 0.02	4.51 +0.59/−0.47
	0.04	0.42 ± 0.03	270.2 ± 36.5	1100.09 ± 0.03	4.07 +0.63/−0.48

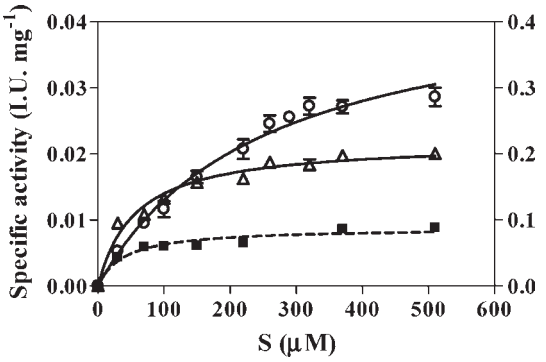


Figure 7. Specific activity (IU mg^{-1}) of stem bromelain versus Bz-Phe-Val-Arg-pNA substrate in tartaric buffer (pH 3.2, ethanol 12% v/v) in the absence (control, on the right *Y* axis, ○) and in the presence of different concentrations of free SO_2, 10 mg L^{-1} (Δ) and 25 mg L^{-1} (■), on the left *Y* axis.

stem bromelain in winemaking, the influence on protease activity of the potential inhibitors naturally present in wine was investigated.

Being a member of the protease family, stem bromelain may present self-cleaving activity. It is known[15] that the autolysis is strongly dependent on pH, and it is unimportant at acidic pH (0.8−3) in reaction time up to 4 h. Stem bromelain used was a raw preparation, containing 45% (w/w) of total proteins, which includes enzymatic and nonenzymatic ones. The presence of nonenzymatic proteins in the raw preparation may result in a protective effect on protease autolysis, since these proteins could be an alternative substrate for bromelain. Our results showed that bromelain solubilized at pH 3.2 retains 96% of its initial activity after 2 h kept in ice (data not shown), indicating that protease autolysis is undetectable in such an experimental condition.

For each compound, the first step was to identify the inhibition type: reversible or irreversible (Figures 1A and 1B) by analysis of the kinetic data as described in Materials and Methods. The "inhibitor added" curves have a smaller slope than the control curve, and they pass through the origin. The ethanol inhibitory effect was rather limited, while sulfur dioxide strongly affected protease activity. Gallic and ellagic tannins showed no significant inhibitory effect, while skin and seed tannins proved to be reversible inhibitors. In summary all inhibitors tested turned out to be reversible inhibitors of stem bromelain activity.

3.1. Inhibitory Effect of Ethanol on Stem Bromelain Activity. Ethanol in wine is derived from the alcoholic fermentation of grape sugars, and its concentration can range from 8% to 18% v/v in dry white and red wines.[6]

The kinetic curves obtained in the presence of different ethanol concentrations (0, 12, 18% v/v) are reported in Figure 2. Kinetic parameters (Table 1) show that V_{max} does not change in the presence of ethanol, while $K_{m(app)}$ increases significantly and K_a decreases. In light of these data, ethanol is shown to be a competitive inhibitor of stem bromelain activity. The K_i value was determined by a secondary plot of $K_{m(app)}$ vs ethanol concentration (%, v/v), as previously described (section 2.5.1). As shown in Figure 3, the K_i value was 11.4 (±1.0) % v/v, indicating that the ethanol inhibition effect was rather limited. In fact, at a concentration closer to the average ethanol content in wine, the ratio K_i/[I], corresponding to the ratio [E]/[EI] (eq 3), indicates that about 50% of stem bromelain remains in free active form [E]. In light of these considerations, the protease activity remained sufficient to support its use in winemaking.

3.2. Inhibitory Effect of Tannins on Stem Bromelain Activity. In order to study inhibitory effect of tannins on stem bromelain activity, different enological preparations, at various concentrations (g L^{-1}), were used. Even though dependent on the content of proanthocyanidic tannins in the grapes, the phenolic composition of a wine is strongly affected by winemaking techniques and enological practices.[16]

3.2.1. Total Phenolic Content of Tannin Preparations. Total phenolic content of the different tannin preparations, expressed as grams of gallic acid equivalents per liter of model wine solution is shown in Table 2. Among the enological preparations tested, gallic and seed tannins presented the highest total phenolic content (67% and 60%, respectively), while skin and ellagic tannins showed the lowest values (50% and 40%, respectively).

3.2.2. Skin and Seed Grape Tannins. Most of grape seed and skin proanthocyanidic tannins are oligomeric and polymeric forms of (+)-catechin and (−)-epicatechin. Moreover, some epigallocatechin is found in skin, whereas epicatechin gallate is a small but significant proportion of seed tannins.[17]

As shown in Figures 4A and 4B, distinct kinetic hyperbolic curves were obtained in the presence of different amounts of skin and seed tannins in the model wine buffer. The estimated kinetic parameters $V_{max(app)}$ and $K_{m(app)}$ decrease to the same extent; however K_a does not change, indicating that both proanthocyanidic tannins are uncompetitive inhibitors (Table 3). K_i value, determined by a secondary plot of $1/K_{m(app)}$ vs [I] was 0.593 (±0.003) g L^{-1} gallic acid equiv and 0.453 (±0.004) g L^{-1} gallic acid equiv for skin and seed tannins, respectively (Figure 5 A,B). These results indicate that the inhibitor effect of seed tannins was higher than that for those from skin. In any case the inhibitory effect of both tannins is not limiting for bromelain application in winemaking, since at the highest tannin concentration tested (1.200 g L^{-1}gallic acid equiv) about 50% of stem bromelain remains in the free active form [E] (eq 3).

3.2.3. Gallic and Ellagic Tannins. Gallotannins and ellagitannins are, respectively, gallic and ellagic acid esters with glucose or other sugars.[17] The kinetic curves (Figure 6 A,B) and the unchanged kinetic parameters V_{max}, K_m and K_a obtained in presence of different gallic and ellagic tannin concentrations

Table 5. Kinetic Parameters of Stem Bromelain versus Bz-Phe-Val-Arg-pNA Substrate in Tartaric Buffer (pH 3.2, Ethanol 12% v/v) Containing Different Concentrations of Free SO_2: 0 mg L^{-1}, 10 mg L^{-1} and 25 mg L^{-1}

free SO_2 (mg L^{-1})	$V_{max(app)}$ (IU mg^{-1})	$K_{m(app)}$ (μM)	$k_{cat(app)}$ (min^{-1})	K_a ($min^{-1}\mu M^{-1}$)
0	0.46 ± 0.02	250 ± 27	1201.90 ± 0.02	4.81 +0.59/−0.47
10	0.0218 ± 0.0006	56.3 ± 6.3	57.7282 ± 0.0006	1.03 +0.13/−0.10
25	0.0088 ± 0.0003	40.0 ± 6.5	23.2420 ± 0.0003	0.58 +0.11/−0.08

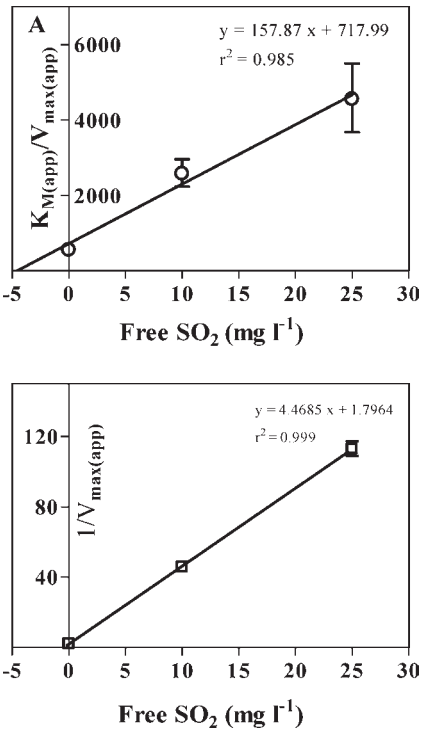


Figure 8. Secondary plots of $K_{m(app)}/V_{max(app)}$ versus SO_2 concentration (A) and of $1/V_{max(app)}$ versus SO_2 concentration (B).

(Table 4) indicate that these phenolic compounds have no inhibitory effect on stem bromelain activity. This conclusion is reached in accordance with the discussion of results represented in Figure 1, given that both the "plus gallic" and the "plus ellagic" tannin curves were about superimposable with the control one, indicating that these tannins did not have any significant inhibitory effect at the levels tested.

3.3. Inhibitory Effect of SO_2 on Stem Bromelain Activity. Sulfur dioxide is added to wine, especially white wine, during the winemaking process to prevent undesirable microbial growth and oxidation processes.[18,19] To the best of our knowledge, sulfur dioxide inhibition of bromelain in wine-like conditions is still unknown, although its inhibitory effect on the activity of various enzymes, such as trypsin and plant metabolic enzymes, has been studied.[20,21] It was demonstrated that sulfur dioxide is able to inactivate many enzymes by splitting their disulfide linkages; in particular, both sulfur dioxide and H_2SO_3 are able to convert disulfide bonds of enzymes or proteins to thiosulfonates and thiols.[21]

Hyperbolic kinetic curves for bromelain were obtained in the presence of different free sulfur dioxide concentrations in model wine buffer (Figure 7). The estimated kinetic parameters ($V_{max(app)}$, $K_{m(app)}$ and K_a) decrease, indicating that sulfur dioxide is a mixed-type inhibitor (Table 5). The K_i value, determined by a secondary plot of $K_{m(app)}/V_{max(app)}$ vs [I], was 4.55 (±1.07) mg L^{-1} (Figure 8A). Another secondary plot of $1/V_{max(app)}$ vs [I] was used to determine the K_i' value, which was 0.40 (±0.09) mg L^{-1} (Figure 8B). These K_i and K_i' values show that free sulfur dioxide strongly inhibits stem bromelain activity.

In the biological phases of winemaking (alcoholic and malolactic fermentation), before the aging and fining phases, the free SO_2 level is kept less than 25 mg L^{-1} in order to allow yeast and lactic acid bacteria metabolism. Our data indicate that stem bromelain can be suitably applied in all the early winemaking phases. In fact, the K_i value (4.55 (±1.07) mg L^{-1}, corresponding to 68 μM), indicates that just the 30% of enzyme is in the free form (eq 3); a stronger inhibitory effect was indicated by the K_i' value (0.4 (±0.09) mg L^{-1} corresponding to 0.4 μM) that implies that the ratio E/EI is strongly forced toward the EI complex (eq 4).

This study showed the influence of inhibitors naturally present in wine toward bromelain activity, giving important information about the enzyme performance for a further biotechnological application in winemaking. Our next goal will be to study the inhibition effect of these inhibitors toward immobilized bromelain. In fact, in many cases, immobilization by covalent linkage can modify the enzyme structure, and thus it might overcome the inhibitory effects. Moreover, enzyme immobilization results as very interesting in order to ensure a repeated/continuous use as well as an easy separation from the reaction mixture so as to prevent protein contamination of treated wine.

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