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Bromelain from pineapple stem in alcoholic–acidic buffers for wine application

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ABSTRACT

Bromelain from pineapple stem has been studied in unexplored model wine buffer (pH 3.2 tartaric acid and ethanol) in order to evaluate its applicability for white wine protein stabilization.

Bromelain proteolytic activity was studied against several synthetic peptide substrates in McIlvaine buffer covering a wide pH range. Among different synthetic substrates, Bz-Phe-Val-Arg-pNA turned out to be the most suitable one to test bromelain activity at the average minimum pH value of wine (3.2). The protease activity was significantly increased by the presence of 5 mM cysteine while no influence was exerted by EDTA. The inhibiting effect of ethanol was investigated in the range 0–25% (v/v), resulting in being rather limited at the average minimum concentration found in wine (10% v/v).

The kinetic parameters were estimated in both McIlvaine (as reference) and model wine buffers. This study showed that stem bromelain worked well at the average minimum pH value of wine, and had a significantly higher activity in model wine buffer (K_a increased of 42%). These results are the basis for suggesting a future biotechnological application of this protease in winemaking.

1. Introduction

White wine protein concentration varies from about 15 to 300 mg l^{-1} (Batista, Monteiro, Loureiro, Teixeira, & Ferreira, 2009; Ferreira, Piçarra-Pereira, Monteiro, Loureiro, & Teixeira, 2002). Though present in rather small amounts, proteins are of primary importance for the colloidal stability and clarity of white wine (Batista et al., 2009; Hsu & Heatherbell, 1987; Waters, Wallace, & Williams, 1991). Haze or deposit formation in bottled wine, due to protein aggregation during storage, is a common defect of commercial wines, rendering them unacceptable to consumers.

The traditional method of stabilizing white wine against haze formation is based on bentonite fining. The negatively charged bentonite particles interact electrostatically with the wine proteins, allowing their removal (Ferreira et al., 2002). Though effective, this treatment generates different problems because of the non-specific adsorption properties of bentonite, such that it removes various molecules or aggregates involved as aroma and flavour compounds, inducing significant aroma loss and, occasionally, colour alteration (Bayonove et al., 1995; Cabaroglu, Canbas, Lepoutre, & Gunata, 2002). Bentonite fining also causes substantial volume losses (between 3% and 10%) (Hoj et al., 2001) and the disposal of spent bentonites constitute a non-negligible source of waste. Finally, bentonite handling is also of concern on account of occupational health and safety issues. For these reasons, increasing attention is given today to the development of alternative practices for protein stabilization that would maintain quality, reduce costs and be more sustainable (Waters et al., 2005).

In order to prevent the haze problem, addition of proteolytic enzymes has become the dominant method for enhancing the protein stability of beverages, especially fruit juices and beers. Meyer, Koser, and Adler-Nissen (2001) studied the efficiency of enzymatic clarification on turbidity and haze in cherry juice using different proteases, among which was bromelain from pineapple stem. This protease, active at the normal beer pH (about 4.5), is widely used for haze prevention of this beverage (Ash & Ash, 2002). In light of these considerations, pineapple stem bromelain could be used for enzymatic hydrolysis of unstable proteins present in white wine.

Bromelain is the collective name for a group of closely related proteolytic enzymes found in the tissue of the plant family of Bromeliaceae, of which pineapple, *Ananas comosus*, is the best known. Two distinct types of pineapple bromelain are recognised: stem bromelain (EC 3.4.22.32), the major proteolytic cysteinyl protease in pineapple stem, and fruit bromelain (EC 3.4.22.33), formerly called bromelin, which is the major proteolytic component in pineapple fruit (Vanhoof & Cooreman, 1997). Stem bromelain is a member of the family of cysteine proteinases, a group of enzymes that depends on the thiol group (SH) of a cysteine residue for its activity. It is a glycoprotein with one oligosaccharide moiety per molecule, which is covalently attached to the peptide chain (Mahmood & Saleemuddin, 2007; Scocca & Lee, 1969). The total molecular weight of the glycosylated form of stem bromelain, carrying

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an oligosaccharide moiety of 1000 Da, is 23.8 kDa (Ishihara, Takahashi, Oguri, & Tejima, 1979).

Bromelain is also known for its clinical and therapeutic applications, due to its relatively non-specific action on proteins and particularly for modulation of tumour growth, third degree burns, improvement of antibiotic action and as a drug for the oral systemic treatment of inflammatory, blood-coagulation-related and some malignant diseases (Braun, Schneider, & Beuth, 2005; Lotz-Winter, 1990; Maurer, 2001). It is also used in food processing and in the baking industry (Lyons, 1982), for meat tenderization and as a dietary supplement (Ravindra, Rastogi, & Raghavarao, 2008).

In the present work, the proteolytic activity of stem bromelain was characterised under wine-like conditions in order to evaluate its applicability for white wine stabilization.

2. Experimental

2.1. The enzyme and synthetic substrates

A single lot (ref No. 068K0692) of stem bromelain (EC 3.4.22.32), obtained from Sigma–Aldrich (Milan, Italy), and containing 45% of protein w/w (Bradford), was used. Synthetic peptide substrates, Ac-Arg-p-nitroaniline (pNA), Bz-Phe-Val-Arg-pNA, H-Cys(Bzl)-pNA, $(H\text{-}Cys\text{-}pNA)_2$, H-Val-Ala-pNA, Suc-Ala-Ala-Val-pNA, Suc-Ala-Pro-Leu-Phe-pNA, Suc-Phe-Leu-Phe-pNA, Z-Arg-Arg-pNA, Z-Phe-Arg-pNA and Z-Phe-pNA were purchased from Bachem, Germany. All other reagents were obtained from Sigma–Aldrich (Milan, Italy).

In all experiments the final bromelain concentration was 0.02 mg ml^{-1} (0.84 μM). All measurements were made in triplicate and the standard deviations are reported.

2.2. Buffer preparation

Bromelain proteolytic activity was studied both in Mc Ilvaine and tartaric buffer (model wine).

The McIlvaine buffer consists of 0.1 M monohydrated citric acid and 0.2 M disodium hydrogenphosphate; these two solutions are mixed in different volume ratios to obtain the final pH, from 2.6 to 12.0, as reported by Schmidt, Azambuja, and Martini (2006). This was used as reference buffer, in presence or not of ethanol (from 0 to 25%).

In order to simulate wine conditions a model wine buffer, containing ethanol 12% v/v at the average minimum pH value in wine (3.2), was used. The model wine consisted of tartaric acid/Na tartrate 0.03 M and ethanol 12% v/v with pH adjusted to 3.2, using 1 N NaOH (Nikolantonaki, Chichuc, Teissedre, & Darriet, 2010).

2.3. Proteolytic activity assessment using synthetic substrates at different pH

Bromelain proteolytic activity was assessed against all the synthetic peptide substrates (370 μM final concentration) listed in the previous paragraph. Assays were performed in McIlvaine buffer, containing 5 mM cysteine and adjusted to various pH values (2.6, 3.2, 4.0, 5.0, 7.0, 8.0, 9.0, 10.0 and 12.0) at 25 °C.

Cleavage of the substrate results in release of free pNA that was detected colorimetrically at 410 nm. Absorbance was measured for 2 min using a Perkin-Elmer Lambda 25 UV/VIS (Beaconsfield Buks, B). Proteolytic activity was determined from the change in absorbance *vs* time, using the linear portion of the curve. Bromelain activity was calculated in I.U. of pNA produced, using a molar absorptivity of 8.480 mM^{-1} cm^{-1} at 410 nm for pNA (Hale, Greer, Trinh, & James, 2005). A blank correction was used, by a sample not containing enzyme. Specific activity (A.S.) was calculated as I.U. mg^{-1} of protein.

2.4. Bromelain assay optimisation

Assay optimisation of stem bromelain was performed by using Bz-Phe-Val-Arg-pNA as substrate (370 μM). The buffer used was the McIlvaine at pH 3.2 and 8.0, in the presence or not of cysteine (5 mM) or EDTA (5 mM). Moreover, the influence of ethanol concentration (0, 5, 10, 15, 20 and 25% v/v) on protease activity was investigated in McIlvaine buffer at the optimal pH (8.0) for this substrate.

2.5. Determination of kinetic parameters

Kinetic parameters (k_{cat}, K_M, K_a) of stem bromelain were determined according to Michaelis–Menten equation (Nelson & Cox, 2008), using a non-linear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.). The substrate used in this investigation was Bz-Phe-Val-Arg-pNA over the concentration range from 0 to 510 μM.

The kinetic study of stem bromelain was initially carried out at 25 °C in McIlvaine buffer, containing ethanol at the average concentration of wine (12% v/v), at different pH values: 3.2 (average minimum value in wines), 4.0, 5.0 and 7.0 (optimum pH). In order to evaluate the buffer composition effect on protease activity, the kinetic study was also performed comparing bromelain activity in McIlvaine (pH 3.2, ethanol 12% v/v) and in a model wine buffer (tartaric acid/Na tartrate 0.03 M, pH 3.2, ethanol 12% v/v).

3. Results and discussion

3.1. Assessment of proteolytic activity using synthetic substrates at different pH

Stem bromelain has a broad substrate specificity and hydrolyses a great variety of natural and synthetic substrates. Casein and haemoglobin are the most widely used natural substrates for this protease (Vanhoof & Cooreman, 1997). Stem bromelain rapidly digests casein over the pH range 7.0–8.5 (Minami, Doi, & Hata, 1971), while the optimum for hydrolysing haemoglobin is around pH 5.0 (Murachi, 1976). Synthetic substrates are also very useful for assaying bromelain, as reported by Vanhoof and Cooreman (1997). The most commonly used are N-benzoyl-l-Arg-ethyl ester (BAEE) and N-benzoyl-l-Arg-amide (BAA), for which the pH optimum falls in the pH range 5.0–6.0 and 5.0–8.0, respectively (Yamada, Takahashi, & Murachi, 1975).

In this work, bromelain activity *vs* pH has been determined using 11 synthetic peptide substrates, in order to select the most suitable one to then test protease activity at the average minimum pH value in wine (3.2). For each substrate the optimum pH and the specific activity, both at optimal pH and at pH 3.2, were determined (Table 1).

Among the 11 substrates tested, stem bromelain cleaved five of them (Fig. 1), showing maximal activity toward Z-Arg-Arg-pNA at the optimal pH of 8.0. No activity was shown towards the following substrates: Ac-Arg-pNA, H-Cys(Bzl)-pNA, H-Val-Ala-pNA, Suc-Ala-Ala-Val-pNA, Z-Phe-Arg-pNA and Z-Phe-pNA. Nevertheless, the highest protease activity observed at the average minimum pH value of wine (3.2) was obtained with Bz-Phe-Val-Arg-pNA, and thus this substrate has been used to study bromelain activity in all other experiments.

Table 1
Optimum pH and specific activity (A.S.) of stem bromelain (at optimal pH and at pH 3.2) tested towards synthetic substrates in McIlvaine buffer (25 °C) at different pH values.

Substrate	pH optimum	A.S. (I.U. mg^{-1}) at optimal pH	A.S. (I.U. mg^{-1}) at pH 3.2
Suc-Phe-Leu-Phe-pNA	4.0	0.73 ± 0.02	0.37 ± 0.11
Suc-Ala-Pro-Leu-Phe-pNA	5.0	0.16 ± 0.04	*No activity*
$(H\text{-}Cys\text{-}pNA)_2$	4.0	0.010 ± 0.001	0.0048 ± 0.0006
Z-Arg-Arg-pNA	8.0	2.023 ± 0.008	0.011 ± 0.002
Bz-Phe-Val-Arg-pNA	8.0	1.02 ± 0.04	0.50 ± 0.04

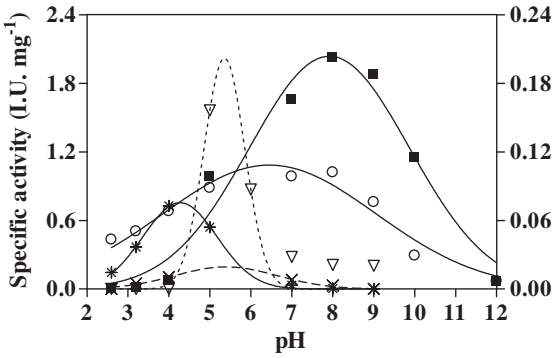

Fig. 1. Specific activity of stem bromelain in McIlvaine buffer (25 °C) *vs* pH towards five model substrates. On left *Y* axis: (○) Bz-Phe-Val-Arg-pNA, (*) Suc-Phe-Leu-Phe-pNA, (■) Z-Arg-Arg-pNA; on right *Y* axis: (×) $(H\text{-}Cys\text{-}pNA)_2$, (▽) Suc-Ala-Pro-Leu-Phe-pNA.

3.2. Optimisation of assay conditions

As previously reported, stem bromelain activity depends on the thiol group (SH) of a cysteine residue that is frequently activated by reducing agents (Minami et al., 1971). For this reason, the effect on bromelain activity of either cysteine as a reducing agent or EDTA as a metal chelator was investigated. The effect of different ethanol concentrations was also studied.

The influence of cysteine and EDTA was tested using Bz-Phe-Val-Arg-pNA as substrate in McIlvaine buffer at pH 3.2 and 8.0. The specific activity of stem bromelain increased significantly in the presence of cysteine at both pH values tested (37% and 53% at pH 3.2 and 8.0, respectively, data not shown). The enzyme was activated by cysteine, because the Cys at the active site, essential for enzyme activity, is maintained in the reduced form by the presence of free Cys in the medium (Minami et al., 1971). There was a negligible influence on protease activity by using EDTA, indicating that metals which influence bromelain activity, were not present in the buffer (data not shown).

The high natural content (up to 25% v/v) of ethanol in wines, deriving from the alcoholic fermentation of grape sugars, could also influence the enzymatic activity of steam bromelain (Haq, Rasheedi, Sharma, Ahmad, & Khan, 2005). Alcohols might denature bromelain by disrupting the side chain intramolecular hydrogen bonding. New hydrogen bonds can be formed between alcohol and protein side chains leading to stabilization of extended helical rods in which the hydrophobic side chains are exposed, whereas polar amide groups are shielded from solvents (Haq et al., 2005). For this reason the effect of different ethanol concentrations (0, 5, 10, 15, 20 and 25% v/v) on protease activity was investigated. It was found that bromelain activity was significantly reduced depending on the amount of ethanol, being more sensitive at the highest content (Table 2). Nevertheless, the ethanol effect was rather limited at a concentration closer to the average wine content (10% v/v), inducing only an 11% activity loss. A 50% activity was extrapolated for 34% v/v of ethanol (data not shown), a significantly higher content respect to the wine concentration.

Table 2
Specific activity (A.S.) of stem bromelain towards Bz-Phe-Val-Arg-pNA substrates in a McIlvaine buffer (25 °C) at pH 8.0 containing different ethanol concentrations.

Ethanol (% v/v)	A.S. (I.U. mg^{-1})	A.S. (%)
0	1.02 ± 0.04	100.0 ± 4.1
5	0.96 ± 0.05	94.3 ± 5.2
10	0.91 ± 0.04	89.1 ± 4.3
15	0.80 ± 0.02	78.5 ± 2.2
20	0.72 ± 0.13	70.4 ± 12.9
25	0.63 ± 0.02	61.7 ± 1.8

3.3. Kinetic study

A kinetic study allowed us to obtain more significant and reliable results with respect to the activity levels observed at a fixed substrate concentration. In fact, a determination of kinetic parameters, although requiring a larger number of assays, more time and higher consumption of reagents, supplies useful information relative to the whole catalytic process. 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.

For this kinetic study, the bromelain activity at different pH values (3.2, 4.0, 5.0 and 7.0) was evaluated in McIlvaine buffer in the presence of 12% v/v ethanol by using Bz-Phe-Val-Arg-pNA as substrate. Experimental data extracted using the Michaelis–Menten equation (Fig. 2A) and the curves, obtained at four different pH values, are grouped in two sets: pH 3.2–4.0 and 5.0–7.0, respectively. Kinetic parameters (Table 3), obtained by means of non-linear regression, showed similar values of k_{cat}, indicating that the product release is not greatly pH-dependent, being only slightly higher at acidic pH. However, an interesting differential behaviour was observed relative to the K_M, that reflects the enzyme-substrate complex formation; these values were about 6-fold lower at higher pH, indicating a higher affinity for the substrate at pH 5.0–7.0. Obviously, the K_a reflects the K_M differences, indicating an affinity for the substrate about 5-fold higher at pH 5.0–7.0. This may be due to the imidazole group of the His in the active site that polarises the SH-group of Cys and enables the deprotonation at neutral and weakly acidic pH (Otto & Schirmeister, 1997). Our kinetic measurements lead to the conclusion that for bromelain, as well as some other cysteine proteinases such as papain, actinidin and cathepsin, a further group with a pK_a of 4.0–5.5 influences the reactivity. This conclusion comes from the log [*S*] *vs* log K_M and log [*S*] *vs* log k_{cat} plots, that indicate a deprotonation occurring at pH 4.5 that influences both enzyme-substrate complex formation and product release (data not shown).

In terms of the possibility to utilise bromelain for removing proteins in wine applications, these results indicate that bromelain can be very effective at the average minimum pH values of wine, precisely due to its higher value of k_{cat}.

It should be noted that all these experiments were performed in McIlvaine buffer, which has a different composition relative to that of wine. In order to further evaluate the proteolytic activity of

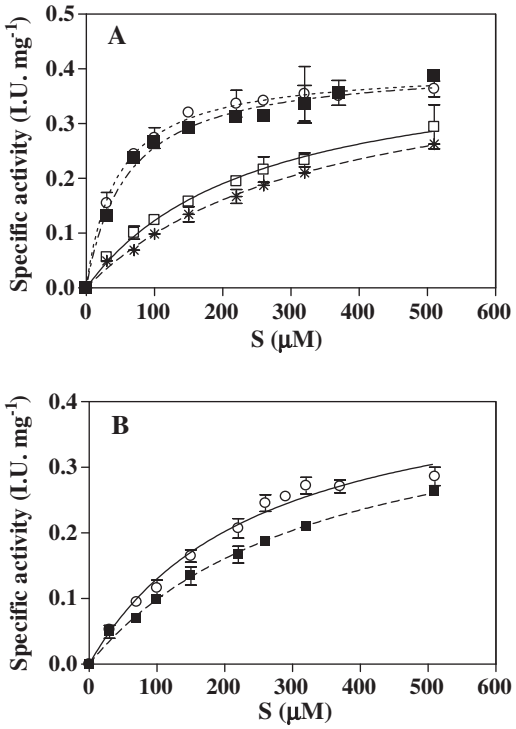

Fig. 2. Specific activity of stem bromelain towards Bz-Phe-Val-Arg-pNA substrate: (A) in McIlvaine buffer (ethanol 12% v/v), at pH 3.2 (*), 4 (□), 5 (○) and 7 (■); (B) in two different buffer solutions at pH 3.2, containing ethanol 12% v/v: McIlvaine (■) and tartaric buffer (○).

Table 3
Kinetic parameters of stem bromelain towards the Bz-Phe-Val-Arg-pNA substrate in McIlvaine buffer (ethanol 12% v/v), at different pH: 3.2, 4.0, 5.0 and 7.0.

pH	V_{max} (I.U. mg^{-1})	K_M (μM)	k_{cat} (min^{-1})	K_a (min^{-1} μM^{-1})
3.2	0.44 ± 0.02	342.6 ± 31.3	1154.03 ± 0.02	3.37 + 0.34/−0.28
4.0	0.42 ± 0.03	245.5 ± 31.0	1121.51 ± 0.03	4.57 + 0.66/−0.51
5.0	0.403 ± 0.009	45.6 ± 4.8	1065.18 ± 0.01	23.35 + 2.78/−2.24
7.0	0.41 ± 0.01	59.2 ± 6.0	1078.67 ± 0.01	18.23 + 2.07/−1.68

bromelain relative to its potential application for winemaking, a comparison between the activity in McIlvaine buffer, *vs* that in a model wine buffer was carried out.

It is known that, among the organic acids naturally present in wine, tartaric acid is the main component, contributing strongly for the overall acidity of this beverage; its concentration can vary within the average range of 1.5 –5.0 g l^{-1} (Fernandes & Reis, 2006). Thus, in order to simulate a wine medium, the tartaric acid content in the model wine buffer used in our experiments was similar to the highest average concentration found in wine. To our knowledge, no one has evaluated the activity of bromelain in a similar medium, and thus these experiments are fundamental for considering the further application of bromelain for use in wine biotechnology.

Table 4
Kinetic parameters of stem bromelain towards the Bz-Phe-Val-Arg-pNA substrate in two different buffer solutions at pH 3.2, each containing ethanol 12% v/v: McIlvaine and tartaric buffer.

Buffer, pH 3.2	V_{max} (I.U. mg^{-1})	K_M (μM)	k_{cat} (min^{-1})	K_a (min^{-1} μM^{-1})
McIlvaine	0.44 ± 0.02	342.6 ± 31.3	1154.04 ± 0.02	3.37 + 0.34/−0.28
Tartaric	0.46 ± 0.02	249.7 ± 27.1	1201.90 ± 0.02	4.81 + 0.58/−0.47

The kinetic curves obtained with two different buffers are reported in Fig. 2B. A significantly higher affinity of bromelain towards synthetic substrates was revealed in the model wine buffer, as can be seen from the K_M and K_a values reported in Table 4.

4. Conclusions

Among all synthetic substrates tested, Bz-Phe-Val-Arg-pNA turned out to be the most suitable for evaluating the pineapple stem bromelain activity at the average minimum pH value found in wine (3.2). Protease activity was significantly increased by the presence of cysteine while no influence was effected by EDTA. Moreover, the ethanol effect was rather limited at the average minimum concentration (10% v/v) of wine. The kinetic study of pineapple stem bromelain is essential for demonstrating the potential of this protease in winemaking in order to prevent unwanted protein precipitation after bottling. The enzyme demonstrated a high activity in McIlvaine buffer at the average minimum pH value of wine, but also a significantly higher activity in tartaric buffer accounted for by the interaction of enzyme affinity and turnover under varying conditions. These preliminary results show that stem bromelain could find productive biotechnological applications in winemaking. Our next goal will be to study the effect of different inhibitors of bromelain present in wines, such as sulphur dioxide and other substances such as tannins from grape skins and seeds as well as the gallic and ellagic tannins.

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