Research Note

Release of Biologically Active Peptides from Grape Juice by *Oenococcus oeni* Isolated from Argentine Wine

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Abstract: The increase in biologically active peptides after proteolytic activity of *Oenococcus oeni* X_2L was studied in grape juice medium (GJM) and in GJM fermented with *Saccharomyces cerevisiae* mc2. Sequential inoculation of *O. oeni* X_2L with proteolytic activity in GJM before ("I" medium) and after ("F" medium) yeast fermentation allowed peptide release. In the "I" medium, bacterial proteolytic activity (0.083 mM) released 3.88 mg nitrogen (N) of peptides per liter and produced an increase of 203.39 µmol/L in the ferric reducing antioxidant power (FRAP) and 16.49% in the angiotensin I-converting enzyme inhibitory (ACE-I) activity after 48 hrs of incubation. In the "F" medium, a higher proteolytic activity was evidenced after 48 hrs of incubation (0.179 mM), releasing 0.87 mg N of peptides/L. The released peptides in the "F" medium produced an increase in ACE-I activity (22.15%). Calculation of the specific activity (activity expressed per mg N of peptide released) of FRAP, 1, 1-diphenyl-2-picrylhydrazyl scavenging, and ACE-I after 48 hrs of incubation revealed a higher activity in the "F" than in the "I" medium. This finding indicates a higher efficiency of the proteolytic system of *O. oeni* X_2L on grape juice proteins in the release of bioactive peptides after yeast growth.

Key words: biological activities, *Oenococcus oeni*, peptide, proteolysis, *Saccharomyces cerevisiae*, wine

During alcoholic fermentation (AF) in winemaking, *Saccharomyces cerevisiae* transforms grape juice sugars into ethanol and carbon dioxide. At the end of AF, lactic acid bacteria, mainly *Oenococcus oeni*, carry out malolactic fermentation (MLF), a process that produces the conversion of L-malic acid into L-lactic acid (Ribéreau-Gayon et al. 2000). The MLF produces stabilization, a reduction in acidity, and desirable wine aroma and flavor (Bartowsky 2014). Manca de Nadra et al. (1999, 2005), Aredes Fernández et al. (2011), and Apud et al. (2013a, 2013b) reported the effect of proteolytic activity of *O. oeni* X_2L on the macromolecular nitrogen (N) fraction of wines, which favored peptide release with biological activities. Exoprotease of *O. oeni* was partially purified and characterized by Farías and Manca de Nadra (2000). Several authors reported that food peptides exhibit particular biological activity on human health (Tripathi and Vashishtha 2006, Yalçin 2006, Hartmann and Meisel 2007, Möller et al. 2008). Aredes Fernández et al. (2011) showed that the proteolytic activity expressed by *O. oeni* during sequential inoculation of the bacterium after yeast autolysis produced a decrease in the protein concentration and an increase in the concentration of free peptides with angiotensin I-converting enzyme inhibitory (ACE-I) and antioxidant activities. This finding confirms that bioactive peptides present multifunctional activities (Di Bernardini et al. 2011, Ko and Jeon 2013).

For a better understanding of the effect of proteolytic activity by *O. oeni* on natural substrates, the current study was performed using natural grape juice medium (GJM) to determine modifications in the N profile and biological activities during sequential inoculation of *O. oeni* X_2L in GJM fermented by *S. cerevisiae* mc2.

Materials and Methods

Microbial strains and culture conditions. *S. cerevisiae* mc2 isolated from Argentine wine (GenBank Data Library, accession number FJ800031) was grown at 30°C in yeast extract-peptone-dextrose broth (YPD) medium containing: yeast extract, 10 g/L; peptone, 20 g/L; and glucose, 20 g/L. *O. oeni* X_2L isolated from Argentine wine (Strasser de Saad and Manca de Nadra 1987) was grown at 30°C in De Man, Rogosa, and Sharpe (MRS) broth with 15% (v/v) tomato juice (pH 4.8).

Sequential cultures in liquid medium. *S. cerevisiae* mc2 cells were grown in YPD medium until the exponential growth phase, then washed with sterile saline solution and resuspended in GJM containing 57 mL grape juice/L, 10 g yeast extract/L, and 1 mL polysorbate 80/L, pH 5.5. Yeast cells were inoculated at 10^8 CFU/mL in GJM and incubated at 30°C. At 0 hr ("I" medium) and after 24 hrs ("F" medium), culture aliquots were used for viable cell counts on YPD-agar medium. Cells were removed from the "I" and the "F" media by centrifugation at 7,000*g* for 10 min, and supernatants were sterilized by filtration. Aliquots of these supernatants were stored at -20°C for analytical determinations.

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An exponential culture of *O. oeni* X_2L grown in MRS broth was washed with a sterile saline solution, and cells were inoculated in 10 mL of sterile supernatants obtained from yeast cultures ("I" and "F" media) at a concentration of 10^6 CFU/mL and incubated for 48 hrs at 30°C. At different times (0, 24, and 48 hrs), viable cell counts were performed on MRS-agar plates, and aliquots of the bacterial cultures were stored at -20°C for analytical determinations.

Proteolytic activity. *O. oeni* X_2L supernatants obtained at 0, 24, and 48 hrs were used to determine proteolytic activity using autoclaved grape juice as a substrate. After 1 hr of incubation at 30°C, the reaction was stopped with 600 µL of 24% (w/v) trichloroacetic acid (TCA). Controls were obtained by precipitation with TCA before incubation. Free amino acids and peptides were quantified in 0.2 mL TCA supernatant aliquots by adding 0.4 mL of Sn-ninhydrin reagent according to Method 1 by Doi et al. (1981).

Proteins. The determination of protein concentration was carried out by the Bradford method using a calibration curve prepared with known concentrations of bovine serum albumin. The results are expressed as milligrams of N per liter (mg N/L).

Peptides and amino acids. Total free amino acid concentration was quantified in supernatants according to Method 5 by Doi et al. (1981) using Cd-ninhydrin as the reagent. Determinations of free amino acids and peptides were carried out using Sn-ninhydrin as the reagent (Doi et al. 1981, Method 1). Peptides were estimated from the difference between the results obtained with Methods 1 and 5. Results are expressed in mg N/L. L-leucine was used as the standard (14 g N/131.17 g of leucine).

ACE-I activity. ACE-I activity was determined according to the method described by Cushman and Cheung (1971), and modified by Hernández-Ledesma et al. (2003). This technique is based on the quantification of hippuric acid formed by the reaction of hippuryl-histidyl-leucine with angiotensin I-converting enzyme (ACE) in the presence and/or absence of an inhibitor. Absorbance was measured at 228 nm, and activity is expressed as the percentage of ACE inhibition.

DPPH radical scavenging activity. The DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging capacity of the samples was determined according to Von Gadow et al. (1997), using ascorbic acid as positive control. The absorbance was measured at 517 nm, and the results are expressed as the percentage of radical scavenging in samples.

Ferric reducing power. Total antioxidant capacity was determined by ferric reducing antioxidant power (FRAP) according to Benzie and Strain (1996). Absorbance was measured at 593 nm. A standard curve was constructed using $FeSO_4$ solution (100 to 1,000 µmol/L). FRAP values are expressed as $FeSO_4$ equivalents (µmol $FeSO_4$/L).

Statistical analyses. The means and reproducibility of data were calculated based on two independent experiments performed in triplicate. The experimental data were analyzed by one-way analysis of variance test. Variable means showing statistical significance were compared using Tukey's test (Minitab student R12). All statements of significance are based on the 0.05 level of probability.

Results and Discussion

In GJM, *S. cerevisiae* mc2 reached a viable cell count of 8.27 Log CFU/mL after 24 hrs of incubation (control) (Figure 1). In this medium, protein consumption and amino acid concentration increased by 7.04 mg N/L and 2.06 mg N/L after 24 hrs, respectively, whereas peptide concentration in the medium was not modified (Table 1). After this time, the microorganism reached the stationary growth phase, and no significant changes were detected in the concentration of N compounds. We concluded that lysis of the eukaryotic microorganism did not occur until 72 hrs of incubation. Figure 2 shows that the viability of *O. oeni* X_2L in the "F" medium

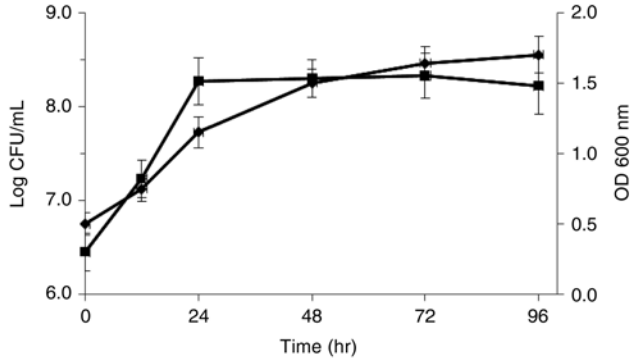

Figure 1 Growth of *Saccharomyces cerevisiae* mc2 in grape juice medium, expressed as Log CFU/mL (■) and optical density (♦).

Table 1 Change in nitrogen (N) compounds during growth of *Saccharomyces cerevisiae* mc2 in grape juice medium.

Time (hr)	Peptides (mg N/L)	Amino acids (mg N/L)	Proteins (mg N/L)
0	0.93 a[a]	1.30 a	26.19 a
24	1.00 a	3.36 b	19.15 b
48	0.98 a	3.32 b	17.32 c
72	0.97 a	3.25 b	16.57 c

[a]Values with the same letter in the same column are not significantly different ($p < 0.05$, n = 6).

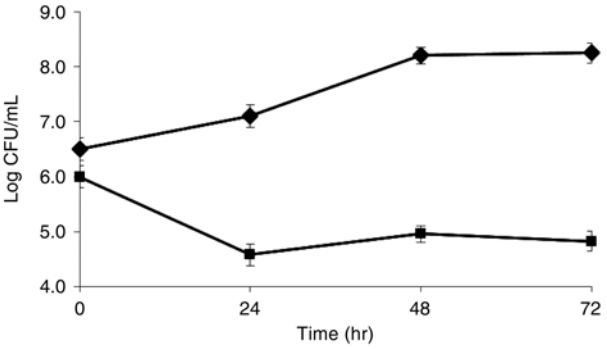

Figure 2 Viability of *Oenococcus oeni* X_2L in grape juice medium fermented with *Saccharomyces cerevisiae* mc2 (■) and nonfermented (♦), expressed as Log CFU/mL.

Table 2 Change in nitrogen (N) compounds concentration, proteolytic activity, and specific activities (ferric reducing antioxidant power [FRAP]; 1, 1-diphenyl-2-picrylhydrazyl [DPPH]; and angiotensin I-converting enzyme inhibitory [ACE-I] activity) of *Oenococcus oeni* X_2L in grape juice medium unfermented ("I") and fermented ("F") with *Saccharomyces cerevisiae* mc2.

	Nitrogenous compounds[a]				Specific activities[b]		
Medium/ time (hr)	Peptides (mg N/L)	Amino acids (mg N/L)	Proteins (mg N/L)	Proteolytic activity (mM)	FRAP (μM FeSO4/mg N)	DPPH (%/mg N)	ACE-I (%/mg N)
Unfermented ("I")							
0	0.86 a	1.97 a	27.15 a	0.025 a	–	–	–
24	3.38 b	1.19 b	21.68 b	0.074 b	285.27 ± 27.05	3.57 ± 0.32	15.97 ± 1.35
48	3.88 c	0.97 b	20.62 c	0.083 c	207.05 ± 20.20	2.47 ± 0.21	17.63 ± 1.54
Fermented ("F")							
0	1.00 a	2.94 a	19.39 a	–	–	–	–
24	1.97 b	3.87 b	15.28 b	0.030 a	470.85 ± 45.80	14.48 ± 1.21	42.91 ± 3.98
48	1.87 b	3.92 b	16.84 b	0.179 b	472.67 ± 42.30	13.86 ± 1.15	65.01 ± 6.27

[a]Values with the same letter in the same column are not significantly different ($p < 0.05$, n = 6).
[b]Specific activities were determined as the ratio between the absolute value of the activity and the concentration of released peptides. Values are expressed as mean ± standard deviation.

decreased 1.5 log cycles after 48 hrs of incubation. In the "I" medium, the bacterium was able to grow and reached a concentration of 8.20 Log CFU/mL at the end of the exponential growth phase (48 hrs). These results are in accordance with Nehme et al. (2010), who determined that the biomass of *O. oeni* X decreased in medium fermented with *S. cerevisiae*. Farías et al. (2003) reported that *O. oeni* X_2L cells decreased 0.84 log cycles after 72 hrs in medium prefermented with *Hanseniaspora uvarum* ca12.

Table 2 shows that, in the "I" medium, proteolytic activity of *O. oeni* X_2L increased to 0.049 mM and 0.083 mM after 24 and 48 hrs of incubation, respectively, while the protein concentration decreased after 24 hrs with a peptide release of 2.52 mg N/L and amino acid consumption of 0.78 mg N/L. After 48 hrs, the peptide concentration reached its maximum (3.88 mg N/L), but the amino acid N concentration was not altered. With respect to the "F" medium, proteolytic activity increased after 24 and 48 hrs of incubation, reaching 0.030 and 0.179 mM, respectively. At 24 hrs of incubation, protein N consumption was 4.11 mg N/L, with an increase in peptide and amino acid N concentration (0.97 mg N/L and 0.93 mg N/L, respectively). Aredes Fernández et al. (2011) demonstrated that during sequential inoculation of *O. oeni* X_2L in synthetic wine medium and after autolysis of *S. cerevisiae* mc2, bacterial proteolytic activity reached values similar to those ascertained in the current study. Apud et al. (2013a) determined that *O. oeni* m1 expressed proteolytic activity that produced the release of peptides from a wine protein fraction.

Figures 3, 4, and 5 show the changes of FRAP, DPPH, and ACE-I activities, respectively. In the medium fermented by *S. cerevisiae*, peptide release was not evidenced and no significant changes were detected in either of the biological activities determined during the entire incubation time (48 hrs). However, Alcaide-Hidalgo et al. (2007) found that peptides released during accelerated autolysis of *S. cerevisiae* produced an increase in antioxidant activity. Similar results were obtained by Aredes Fernández et al. (2011), who reported that after accelerated yeast autolysis, the peptide release produced

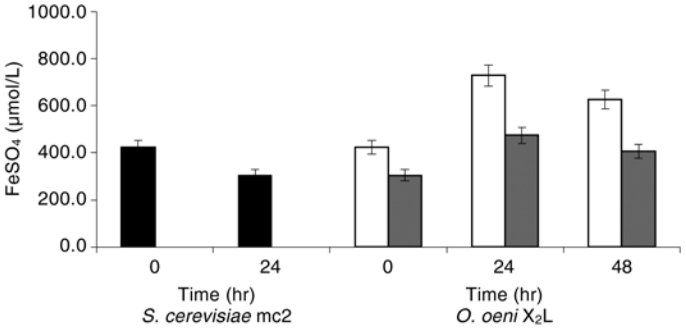

Figure 3 Change in antioxidant activity determined by reduction of the Fe^{+3}-TPTZ complex (ferric reducing antioxidant power; FRAP) in grape juice medium during growth of *Saccharomyces cerevisiae* mc2 (black bars) and *Oenococcus oeni* X_2L before (white bars) and after (gray bars) fermentation with the eukaryotic microorganism.

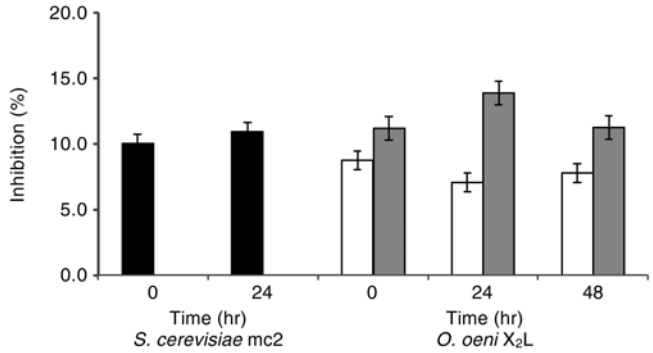

Figure 4 Change in antioxidant activity determined by radical scavenging activity (1, 1-diphenyl-2-picrylhydrazyl; DPPH) in grape juice medium during growth of *Saccharomyces cerevisiae* mc2 (black bars) and *Oenococcus oeni* X_2L before (white bars) and after (gray bars) fermentation with the eukaryotic microorganism.

an increase in FRAP activity. After inoculation of *O. oeni* in "I" medium, an increase in FRAP activity of 305.07 μmol/L was observed after 24 hrs of incubation. Nevertheless, no significant changes were detected in DPPH activity. The ACE-I activity increased 16.49% after 48 hrs incubation. In the "F"

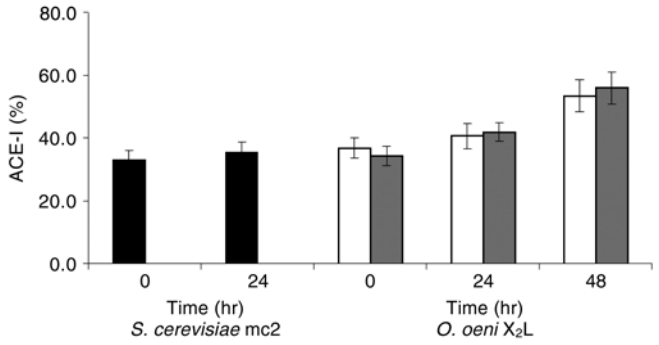

Figure 5 Change in angiotensin I-converting enzyme inhibitory (ACE-I) activity in grape juice medium during growth of *Saccharomyces cerevisiae* mc2 (black bars) and *Oenococcus oeni* X_2L before (white bars) and after (gray bars) fermentation with the eukaryotic microorganism.

medium, an increase in FRAP activity of 169.48 µmol/L was observed after 24 hrs of incubation, and an increase in ACE-I activity of 22.15% was shown after 48 hrs; however, no significant difference was detected in DPPH activity. Aredes Fernández et al. (2011) determined that peptides released by *O. oeni* X_2L after accelerated yeast autolysis showed a slightly higher antioxidant activity (FRAP and DPPH), but ACE-I activity was four-fold lower compared to the current study. This finding is probably because peptides released from the grape juice proteins present higher ACE-I activity compared to the peptides released from yeast autolysis. Adding to the known beneficial biological activities of peptides from wine or yeast autolysis, our results confirm the important role of peptides in such activities in fermented grape juice. Peptides from wine, grape juice, or yeast autolysis present beneficial biological activities (Touyz 2004, Alcaide-Hidalgo et al. 2007, Aredes Fernández et al. 2011, Jang and Lee 2011, Apud et al. 2013a, 2013b).

To establish the relationship between the concentration of peptides released and antioxidant and ACE-I activities assayed, specific antioxidant and ACE-I activities were determined, as shown in Table 2. In the "F" medium, *O. oeni* exhibited higher specific FRAP, DPPH, and ACE-I activities than in the "I" medium, despite the decrease in both bacterial viability and peptide release with respect to the "I" medium (Table 2). The higher specific activities observed in the "F" medium would be related to the greater efficiency of the proteolytic system of *O. oeni* to release bioactive peptides after yeast fermentation. These results suggest that peptides released from a natural protein source such as grape juice would have higher antihypertensive activity than those obtained from yeast autolysis. Although further studies are necessary to elucidate the mechanisms involved in the biological activities, our study confirms the efficiency of the proteolytic system of *O. oeni* X_2L to release peptides with ACE-I and antioxidant activities.

Conclusions

Growth of *O. oeni* X_2L in GJM or in GJM fermented with *S. cerevisiae* mc2 produced an increase in antioxidant (determined with FRAP and DPPH scavenging assays) and ACE-I activities. This behavior is most likely related to the presence of bacterial proteolytic activity that enabled the release of bioactive peptides. The yeast-fermented medium demonstrated higher biological activities produced through the presence of released peptides by *O. oeni* X_2L compared with the nonfermented medium. This effect is probably a result of a different exoprotease (stress exoprotease) expressed under unfavorable environmental conditions. Presence of this enzyme enables peptide release with higher and/or more specific biological activities. Our results confirm the important role of proteins derived from grape juice in the release of peptides with beneficial biological activities.

This study contributes to a current but underexplored topic by providing new knowledge on the role of a proteolytic *O. oeni* strain isolated from Argentine wines in the release of the bioactive peptides that would enable incorporation of additional value to regional wines.

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