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Short communication

Increase in antioxidant and antihypertensive peptides from Argentinean wines by *Oenococcus oeni*

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ABSTRACT

Cells from an exponential *Oenococcus oeni* m1 culture in a grape juice medium were inoculated into a synthetic wine medium (SW) supplemented with a protein and polypeptide fraction (PPF) of high molecular weight (higher than 12,400 Da) obtained from four varietals of Cafayate Argentinean wines. *O. oeni* maintains viability after 48 h incubation time and enables the increase in extracellular proteolytic activity and the release of low molecular weight peptides by 1.067, 0.397, 0.915 and 0.705 mg N/L in the respective SW supplemented with PPF from Cabernet Sauvignon, Malbec, Tannat and Torrontés wine varietals. After 48 h incubation time, concomitantly with peptide release, an increase in antioxidant and antihypertensive activities was detected in all studied media. The highest increase was detected in the presence of PPF from Cabernet and Tannat wine varietals. Maximum increase in antioxidant activity (366.1 μmol $FeSO_4$/L in the case of ferric reducing antioxidant power and 8.9% in 2,2-diphenyl-1-picrylhydrazyl radical scavenging) was produced by the peptides released from PPF of Cabernet Sauvignon wine. The peptides released from PPF Tannat wine varietal caused the highest increase in antihypertensive activity (56.2% in angiotensin I-converting enzyme inhibitory activity). *Oenococcus oeni* m1 would provide additional benefits to wine such as an increase in bioactive peptides with multifunctional beneficial activities.

1. Introduction

The study of food-derived peptides with antihypertensive, antioxidant and other biological activities beneficial to human health is a challenge that has been taken up during the past few years (Möller et al., 2008; Sarmadi and Amin, 2010). Angiotensin I-converting enzyme (ACE) is a vital component in blood pressure regulation. In the kinin–kallikrein system, ACE inactivates the vasodilator bradykinin, while in the renin–angiotensin system, ACE acts by hydrolyzing the decapeptide Angiotensin I and releasing the octapeptide Angiotensin II, which exercises a powerful vasoconstrictive action and stimulates the secretion of aldosterone, favoring the retention of sodium and water and the consequent increase in arterial blood pressure. Therefore, ACE activity causes a rise in blood pressure by increasing vascular resistance and fluid volume (Zhao and Li, 2009) while ACE-inhibitor compounds exert an antihypertensive action. Free radicals, which are physiologically produced, can exert various functions such as signaling and providing a defense against infections. Nevertheless, an excessive amount of reactive radicals can result in cellular damage. Antioxidant peptides can prevent free radical formation and scavenge radicals or hydrogen peroxide and other peroxides (Samaranayaka and Li-Chan, 2011). Up to now, only a few papers have dealt with this subject.

With respect to wine, Alcaide-Hidalgo et al. (2007) found that peptides released during accelerated autolysis of *Saccharomyces cerevisiae* in a wine model showed inhibitory activity toward the angiotensin I-converting enzyme (ACEI activity) as well as oxygen radical scavenging capacity. Aredes Fernández et al. (2011) reported that the sequential inoculation of the proteolytic X_2L strain of *Oenococcus oeni* in a synthetic wine medium after accelerated yeast autolysis increased peptide nitrogen concentration and improved antihypertensive and antioxidant activities. Several authors have found that antihypertensive peptides with ACEI activity in fermented foods also present radical scavenging activity, suggesting the existence of multifunctional activity in these compounds (Hernández-Ledesma et al., 2005; Aredes Fernández et al., 2011).

O. oeni, the major species found in wine during malolactic fermentation, is able to hydrolyze wine proteins via extracellular enzyme activity (Manca de Nadra et al., 1999; Manca de Nadra, 2007; Folio et al., 2008). *O. oeni* m1 was previously reported as a proteolytic strain with technological properties relevant to the vinification process (Strasser de Saad and Manca de Nadra, 1987; Arena et al., 1999). The role of

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lactic acid bacteria from wine such as *O. oeni* in the production of bioactive peptides using the protein fraction of wines as a substrate has not been explored yet. This fraction contains proteins and polypeptides derived mainly from grape pulp and to a lower extent from autolyzed yeast (Ferreira et al., 2002). The current study examines the modification of the antihypertensive and antioxidant activities of peptides produced by the proteolytic activity of *O. oeni* m1 in a synthetic wine medium supplemented with the high molecular weight protein fraction of different Argentinean wine varietals.

2. Materials and methods

2.1. Microorganism

Oenococcus oeni m1 isolated from Argentine wine (Strasser de Saad and Manca de Nadra, 1987).

2.2. Wines

Commercial wine samples from Cafayate (Argentina) were obtained directly from wine stores. The red wine varietals used were Cabernet Sauvignon, Malbec and Tannat. The Torrontés varietal of white wine was also studied.

2.3. Isolation of the wine protein–polypeptide fraction

To obtain the protein–polypeptide fraction (PPF), the wines were dialyzed against distilled water for 48 h, using a dialysis cellulose membrane with a molecular weight cut-off (MWCO) of 12,400 (Sigma–Aldrich, St. Louis, MO, USA). The retentate was vacuum concentrated and stored at −20 °C (Manca de Nadra et al., 1999).

2.4. Culture medium and cultivation conditions

Oenococcus oeni m1, isolated from Argentine wine (Strasser de Saad and Manca de Nadra, 1987), was grown at 30 °C in grape juice broth described by Aredes Fernández et al. (2010). Cells were grown to the exponential growth phase ($OD_{560}=0.6$), harvested by centrifugation at 5000 ×*g* for 15 min at 4 °C, washed three times with sterile (0.85% w/v) saline solution and then suspended in synthetic wine medium (SW) at 10^8 cells/mL. SW contained: 5% (v/v) ethanol, 1.0 g glucose/L, 1.0 g fructose/L, 4 g tartaric acid/L, 3 g L-malic acid/L, 0.1 g acetic acid/L, 0.1 g K_2SO_4/L and 0.025 g MgSO4.7H_2O/L, pH 4.8. Identical inocula were performed in different SW supplemented with the PPF of the different wine varietals, as follows: Cabernet Sauvignon (SWca), Malbec (SWma), Tannat (SWtn) and Torrontés (SWto). Protein concentration in the resulting media was adjusted to the same concentration as the one determined in the commercial wines samples.

Viable cell count was determined on MRS agar (pH 4.8) at 30 °C under microaerophilic conditions. Optical density was also determined in a CECIL 2020 model spectrophotometer at 560 nm. The means and reproducibility of viability data were calculated based on three independent determinations performed in duplicate.

2.5. Proteolytic activity

At different times, proteolytic activity was determined in 0.2 mL of culture supernatant using 0.2 mL of autoclaved grape juice as substrate in the presence of 0.6 mL of citrate buffer 0.05 M, pH 5.0. After 1 h of incubation at 30 °C, the reaction was stopped by the addition of 0.7 mL 24% (w/v) trichloroacetic acid (TCA). In all cases controls were obtained by precipitation with TCA immediately before incubation. Low molecular weight nitrogen compounds (free amino acids and peptides) were quantified on an aliquot of 0.2 mL of TCA supernatants by adding 0.4 mL of Sn–ninhydrin reagent according to Doi et al. (1981), method 1), using 1 mM L-leucine as standard. Results are expressed in mM of leucine.

2.6. Proteins

Proteins were quantified using the procedure described by Smith et al. (2011). Briefly, protein was precipitated by adding 2 volumes of −20 °C acetone containing 10% (w/v) freshly prepared trichloroacetic acid (TCA) to one volume of sample, which had been prefiltered through a 0.45 μm membrane. Samples were incubated for 45 min at −20 °C and centrifuged for 15 min at 21,500 ×*g* at 4 °C. The pellet was washed once with 20 °C acetone, air-dried, and solubilized in distilled water. Protein was measured in the solubilized wine precipitates with Bradford's method using a commercial kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions for the microassay procedure, with bovine serum albumin as a standard. Protein nitrogen concentrations are expressed as mg N/L eq BSA. For the calculation, the molecular weight of BSA (66,432 g/mol) and the number of nitrogen atoms present in the molecule (10,276 g/mol) were taken into account.

2.7. Free amino acids and peptides

Free amino acids concentration was determined according to Doi et al. (1981), method 5). Determinations of free amino acids plus peptides were carried out with the conventional ninhydrin method (Doi et al., 1981, method 1). Peptides were quantified by the difference between the results obtained with methods 1 and 5. Results are expressed in mg of nitrogen per liter (mg N/L) and referred to as amino acid or peptide concentrations. L-leucine was used as the standard (14 g N for every 131.17 g of leucine).

2.8. ACEI activity

ACEI activity was determined by the method first described by Cushman and Cheung (1971) and later 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 absence of an inhibitor. Absorbance was measured at 228 nm and activity is expressed as the percentage of ACE inhibition.

2.9. Ferric reducing antioxidant power (FRAP) assay

This assay measures the formation of a colored Fe(II)-tripyridyltriazine complex from colorless oxidized Fe(III) due to the action of electron-donating antioxidants (Benzie and Strain, 1996). Absorbance was measured at 593 nm with a microplate reader. A standard curve was constructed using Fe(II) sulfate solution (100–3500 μM) and the results are given in μmol $FeSO_4$/L.

2.10. Free radical scavenging ability

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined with the method proposed by Von Gadow et al. (1997). Ascorbic acid solution was used as positive control. Absorbance was measured with a microplate reader at 517 nm and the percentage of radical scavenging in the samples was calculated.

2.11. Statistical analysis

Data from analytical determinations are the means of three independent experiments carried out in duplicate. One-way analysis of variance was applied to experimental data. Variable means showing differences with statistical significance were compared using Tukey's test. Comparisons between viability data were made using Student's *t* test. All statements of significance are based on a probability of 0.05.

3. Results

3.1. Viability of Oenococcus oeni

In SW medium, *O. oeni* m1 was inoculated at a concentration of 8.1 Log CFU/mL. In this medium, microorganism viability was maintained after 24 h incubation. From this time up to 96 h, bacterial viability decreased by 1.8 log cycle without modification of optical density (data not shown), suggesting that no bacterial lysis occurred and therefore no significant release of nitrogen compounds was evidenced. In SW supplemented with different PPF wine varietals, bacterial viability was maintained after 48 h incubation and from this time up to 96 h it decreased by 1 log cycle (Table 1).

3.2. Proteolytic activity

Table 2 shows that low proteolytic activity (0.396 mmol/L) of *O. oeni* was detected in SW medium only at the beginning of incubation time (24 h). In SW control medium without bacterium inoculation no proteolytic activity was detected (data not shown).

Proteolytic activity increased after 24 h incubation in SWca and SWto media, reaching a maximum value of 1.372 and 1.054 mmol/L, respectively. From this time on, it decreased 65% in both trials until 96 h incubation. In SWma and SWtn media, proteolytic activity increased significantly at 48 h, reaching 0.992 and 1.536 mmol/L, respectively. From this time on, it decreased to 0.336 mmol/L in SWma and to an undetectable amount in the SWtn medium at the end of incubation time (Table 2).

3.3. Modification of nitrogen organic compounds

In SW, *O. oeni* metabolism did not significantly modify the nitrogen organic compounds during the incubation time (Table 2), confirming that autolysis did not occur during incubation of the microorganism. A similar behavior in nitrogen organic compounds was observed in SW without bacterium inoculation (data not shown).

In the SWca medium, after 48 h incubation, a significant decrease of 1.537 mg N/L in protein nitrogen was detected, maximum reduction being observed at 96 h incubation (1.907 mg N/L). In this medium, peptide nitrogen concentration increased 1.067 mg N/L at 48 h. At the final incubation time (96 h), peptide concentration decreased 0.263 mg N/L.

Protein nitrogen decreased significantly after 24 h incubation in SWma medium (1.145 mg N/L). At this time, 0.341 mg N/L of peptides was released. From this time on, neither protein nor peptide nitrogen concentrations showed significant modifications.

In SWtn medium, a decrease in protein nitrogen of 0.899 mg N/L was detected after 24 h incubation, with a release of 0.844 mg N/L of peptide nitrogen. From this time on, protein and peptide nitrogen concentration was not significantly altered until 96 h incubation.

In SWto medium, after 24 h incubation, the microorganism enabled the decrease of 0.755 mg N/L and the release of 0.427 mg N/L of protein and peptide nitrogen respectively. After 48 h incubation, protein nitrogen consumption increased to 1.324 mg N/L and peptide nitrogen release reached 0.705 mg N/L.

Table 1
Viable cell count of *O. oeni* m1 in synthetic wine medium supplemented with the PPF of different wine varietals.

Time [h]	*Oenococcus oeni* m1 viability [Log CFU/mL]				
	SW	SWca	SWma	SWtn	SWto
0	8.1^{a}	7.9^{a}	8.0^{a}	8.2^{a}	8.0^{a}
24	7.8^{a}	7.9^{a}	7.9^{a}	8.2^{a}	7.9^{a}
48	6.4^{b}	7.3^{a}	7.8^{a}	7.8^{a}	7.3^{a}
96	6.3^{b}	7.0^{b}	7.0^{b}	7.1^{b}	7.2^{b}

Values are the means of three independent experiments carried out in duplicate. Values with the same letter in the same column are not significantly different ($p<0.05$).

Table 2
Proteolytic activity and nitrogen compounds modification in synthetic wine supplemented with the PPF of different wine varietals during incubation of *O. oeni* m1.

Media	Incubation time [h]	Proteolytic activity [mmol/L]	Proteins [mg N/L]	Peptides [mg N/L]	Amino acids [mg N/L]
SW	0	0.019^{a}	1.459^{a}	0.212^{a}	0.094^{a}
	24	0.396^{b}	0.837^{a}	0.166^{a}	0.007^{a}
	48	0.102^{a}	1.395^{a}	0.249^{a}	0.000^{a}
	96	0.000^{a}	1.038^{a}	0.132^{a}	0.000^{a}
SWca	0	0.232^{a}	7.285^{c}	0.226^{a}	0.111^{a}
	24	1.372^{c}	6.194^{b}	1.242^{d}	0.207^{b}
	48	0.669^{d}	5.748^{b}	1.293^{d}	0.090^{a}
	96	0.482^{b}	5.378^{b}	1.030^{c}	0.229^{b}
SWma	0	0.000^{a}	7.326^{c}	0.161^{a}	0.161^{a}
	24	0.218^{b}	6.181^{b}	0.502^{b}	0.086^{a}
	48	0.992^{e}	6.139^{b}	0.558^{b}	0.024^{a}
	96	0.336^{b}	5.940^{b}	0.538^{b}	0.001^{a}
SWtn	0	0.000^{a}	8.609^{e}	0.252^{a}	0.093^{a}
	24	0.305^{b}	7.710^{c}	1.096^{c}	0.257^{b}
	48	1.536^{c}	7.225^{c}	1.168^{c}	0.174^{ab}
	96	0.000^{a}	7.200^{c}	1.055^{c}	0.141^{a}
SWto	0	0.000^{a}	6.517^{c}	0.195^{a}	0.109^{a}
	24	1.054^{e}	5.762^{db}	0.622^{b}	0.128^{a}
	48	0.714^{d}	5.193^{d}	0.900^{cd}	0.146^{a}
	96	0.348^{b}	5.378^{d}	0.812^{d}	0.148^{a}

Values are the means of three independent determinations. Values with the same letter in the same column are not significantly different ($p<0.05$).

3.4. Modification of biological activities

Table 3 shows the modification of beneficial biological activities in the presence of *O. oeni* m1 in media supplemented with each PPF obtained from different wine varietals. Results show that ACEI activity significantly increased after 24 h incubation in all media studied with respect to the SW medium. A higher inhibition of ACE was detected in SW supplemented with PPF from red wines. Concomitantly with peptide release (Table 2), a maximum ACEI activity of 63.8 and 70.0% was detected in SWca and SWtn media respectively at 24 h incubation. In SWma medium, ACEI activity increased by half with respect to those observed in SWca and SWtn. ACEI activity in SWto was 3.5- and 4-fold lower than in SWca and SWtn respectively after 24 h incubation.

Table 3
Modification of biological activities in synthetic wine supplemented with the PPF of different wine varietals during incubation of *O. oeni* m1.

Media	Incubation time [h]	ACEI [%]	FRAP [μmol $FeSO_4$/L]	DPPH [%]
SW	0	0.4^{a}	25.2^{a}	0.0^{a}
	24	1.6^{a}	38.2^{a}	0.0^{a}
	48	3.6^{a}	26.9^{a}	0.2^{a}
	96	1.9^{a}	13.7^{a}	1.7^{a}
SWca	0	18.5^{b}	167.9^{b}	0.3^{a}
	24	63.8^{d}	565.8^{d}	8.6^{c}
	48	65.8^{d}	534.0^{d}	9.2^{c}
	96	46.5^{c}	437.5^{d,c}	10.7^{c}
SWma	0	17.8^{b}	171.5^{b}	0.7^{a}
	24	36.4^{c}	288.0^{c}	5.3^{b}
	48	36.4^{c}	319.9^{c}	4.6^{b}
	96	28.0^{bc}	280.2^{c}	5.4^{b}
SWtn	0	19.8^{b}	181.2^{b}	0.5^{a}
	24	70.0^{d}	517.2^{d}	7.0^{b}
	48	76.0^{d}	480.0^{d}	7.7^{bc}
	96	56.4^{cd}	494.8^{d}	2.2^{ab}
SWto	0	2.2^{a}	74.0^{a}	0.5^{a}
	24	18.0^{b}	79.5^{a}	5.3^{b}
	48	15.8^{b}	104.5^{ab}	6.4^{b}
	96	6.6^{a}	86.5^{a}	5.7^{b}

Values are the means of three independent determinations. Values with the same letter in the same column are not significantly different ($p<0.05$).

A significant increase in ferric reducing power of 397.9 and 336.0 μmol $FeSO_4$/L was detected in SWca and SWtn at 24 h incubation, respectively. The maximum increase in SWma medium was detected at 48 h incubation (148.4 μmol $FeSO_4$/L). In SWto medium, no significant modification was detected in this parameter during incubation time.

In SWca and SWtn media, radical scavenging activity reached 8.6% and 7.0%, respectively, after 24 h incubation. In SWma, this activity was 1.6- and 1.4-fold lower than in SWca and SWtn, respectively. In SWto medium, DPPH activity was similar to the one found in SWma medium.

In SW, no significant modifications in biological activities were detected during *O. oeni* incubation. Biological activities determined during 96 h incubation in a control medium supplemented with PPF of different wines, without bacterium inoculum, were similar to those found in SW (data not shown).

4. Discussion

During *O. oeni* m1 cultivation in the presence of different protein substrates, bacterial proteolytic activity was evidenced concomitantly with the decrease in protein and peptide release. In addition, since no modification in optical density was detected during bacterial cultivation, the release of low molecular weight nitrogen compounds would not be coupled with bacterial lysis. Thus, these data could confirm that the release of biologically active peptides was caused by bacterial proteolytic activity against the proteins from different wine varietals. In a previous report, Manca de Nadra (2007) demonstrated that *O. oeni* X_2L isolated from Cafayate wines is able to hydrolyze wine proteins via extracellular enzyme activity. In the same way, other authors showed that the strain X_2L causes a decrease in the concentration of proteins released by yeast autolysis and an increase in the release of peptides with biological activities in synthetic wine medium (Aredes Fernández et al., 2011). The proteolytic activity detected during incubation of *O. oeni* m1 is related to protein consumption and to the release of peptide nitrogen after 24 h incubation. No significant amino acid modification was detected in any of the studied media. This observation agrees with the study of Aredes Fernández et al. (2010), who reported that the strain X_2L of *O. oeni* releases principally peptides from protein consumption in grape juice medium as a consequence of their extracellular proteolytic activity. Peptides are a more important source of nitrogen than amino acids and are efficiently used by *O. oeni* to fulfill its amino acid requirements to sustain viability (Aredes Fernández et al., 2004; Remize et al., 2006; Ritt et al., 2008). In addition, peptide rather than amino acid utilization enables the bacterium to save a considerable amount of energy (Konings, 2006).

Peptides have important tensoactive and bioactive functional properties in wines. Moreover, they play a role in foam stability and in the sensorial properties of wine (Pozo-Bayón et al., 2009). Peptides released from the protein and polypeptide fraction of different Argentinean wine varietals by *O. oeni* m1 have antihypertensive, antioxidant and radical scavenging activities. These activities are higher when the peptidic nitrogen source is derived from red wines, particularly Cabernet Sauvignon and Tannat varietals. Maximum increase in antioxidant activity was caused by the peptides released from PPF of Cabernet Sauvignon wine, while the peptides released from PPF Tannat wine varietal produced the highest increase in antihypertensive activity. In a previous publication, Aredes Fernández et al. (2011) demonstrated that the strain X_2L of *O. oeni* is able to release peptides with the same multifunctional biological activities from the proteins released after autolysis from *S. cerevisiae*. Other authors also reported peptides with multifunctional biological activities derived from fermented foods (Hernández-Ledesma et al., 2005) and yeast autolysis under wine conditions (Alcaide-Hidalgo et al., 2007).

In conclusion, the proteolytic activity of *O. oeni* m1 was able to release peptides with biological activities in all studied media, especially in media with PPF from Cabernet and Tannat wine varietals.

The current study for the first time provides information about the role of the proteolytic activity of *O. oeni* m1 under similar conditions to those in wine with regard to the release and/or production of peptides with biological activities derived from nitrogen compounds of high molecular weight (higher than 12.4 KDa) from different wine varietals. These peptides have multifunctional biological activities with beneficial health properties that could contribute to an improvement in wine quality. Ongoing studies on the subject include experiments using the purified protease of *O. oeni* m1 and the high molecular weight protein–polypeptide fraction of regional wines as a substrate in order to isolate the bioactive peptides released from wine proteins.

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