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Proteolytic activity of *Oenococcus oeni* Enables the Increase in Antioxidant and Antihypertensive Activities from Wine

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Abstract: *Oenococcus oeni* is a lactic acid bacterium involved in winemaking where it generally carries out the malolactic fermentation converting the wine's *malic acid* into *lactic acid*. In this work were used the strain m of *Oenococcus oeni*. The culture was inoculated at 10^8 Log CFU/mL in a synthetic wine medium (SW) supplemented with a protein and polypeptide fraction (PPF) of high molecular weight (higher than 12,400 Da) obtained from Cabernet Sauvignon and Syrah wines from Colalao del Valle, Tucumán, Argentine. In presence of PPF, *O. oeni* maintains viability after 48 h incubation time and enables the increase in extracellular proteolytic activity. Therefore, a maximum release of low molecular weight peptides by 1.247 and 1.373 mg N/L at 48 h of incubation time was detected in SW supplemented with PPF from Cabernet Sauvignon and Syrah wines respectively. Concomitantly with the maximum peptide release, an increase in antioxidant and antihypertensive activities *"in vitro"* were detected. The released peptides from Cabernet Sauvignon wine enables the increase in the ferric reducing antioxidant power (FRAP) capacity, the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging activity, and the angiotensin I-converting enzyme inhibitory (ACEI) activity in 392.8 µmol $FeSO_4$/L, 9,7% and 63.9%, respectively. In presence of PPF of Syrah wine, the released peptides increases in 156.5 µmol $FeSO_4$/L, 5.5% and 13.8% the FRAP, DPPH and ACEI activities, respectively. The utilization of the strain m of *Oenococcus oeni* to carry out the malolactic fermentation during winemaking would provide additional benefits to wine such as an increase in bioactive peptides with multifunctional beneficial activities.

Keywords: Antihypertensive activity, Antioxidant activity, bioactive peptides, *Oenococcus oeni*, Proteolytic activity, Wine.

INTRODUCTION

Biologically active peptides or functional peptides are food-derived peptides that in addition to their nutritional value exert a physiological effect in the body. Numerous peptides exhibiting various activities have been reported [1]. 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 [2, 3]. 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 [4] 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 [5].

Regarding wine, the scientific reports published so far described the release of peptides with ACE inhibitory activity (ACEI activity) as well as oxygen radical scavenging capacity from autolyzed wine yeasts [6, 7]. Aredes Fernández, Stivala, Rodríguez Vaquero and Farías [7] found that the proteolytic X_2L strain of *Oenococcus oeni* increase peptide nitrogen concentration from proteins released after accelerated yeast autolysis 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 [7, 8].

O. oeni, the major species found in wine during malolactic fermentation, is able to hydrolyze wine proteins via extracellular enzyme activity [9-11]. *O. oeni* m was previously reported as a proteolytic strain with technological properties relevant to the vinification process [12- 13]. The role of 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 [14]. The current study examines the modification of the antihypertensive and antioxidant activities of peptides produced by the proteolytic

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activity of *O. oeni* m in a synthetic wine medium supplemented with the high molecular weight protein fraction of Cabernet Sauvignon and Syrah Argentinean wine varietals.

MATERIALS AND METHODS

Isolation of the Wine Protein-polypeptide Fraction

Commercial Cabernet Sauvignon and Syrah wines samples from Colalao del Valle (Tucumán, Argentine) were obtained directly from wine stores. To obtain the protein-polypeptide fraction (PPF), the wine samples 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.

Microorganism, Culture Medium and Cultivation Conditions

Oenococcus oeni m isolated from Argentine wine [12]. The microorganism was grown at 30°C in the grape juice broth described by Aredes Fernández, Farías and Manca de Nadra [15]. Cells were grown to the exponential growth phase (OD_{560} = 0.6), harvested by centrifugation at 5,000 *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), in SW supplemented with the PPF of Cabernet Sauvignon (SWca) and in SW supplemented with the PPF of Syrah (SWsy) 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 $MgSO_4.7H_2O$/L, pH 4.8. Protein concentration in SWca and SWsy mediums 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 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.

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, Shibata and Matoba [16] (method 1), using 1 mM L-leucine as standard. Results are expressed in mM of leucine.

Proteins

Proteins were quantified using the method described by Smith, Penner, Bennett and Bakalinsky [17]. Briefly, protein was precipitated by adding 1 mL of -20 C acetone containing 10% (w/v) freshly prepared trichloroacetic acid (TCA) to 0.5 mL 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.

Free Amino Acids and Peptides

Free amino acids concentration was determined according to Doi, Shibata and Matoba [16] (method 5). Determinations of free amino acids plus peptides were carried out with the conventional ninhydrin method [16] (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).

ACEI Activity

ACEI activity was determined by the method first described by Cushman and Cheung [18] and later modified by Hernández-Ledesma, Martín-Alvarez and Pueyo [19]. 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 (samples). Absorbance was measured at 228 nm and the activity is expressed as the percentage of ACE inhibition.

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 [20]. Absorbance was measured at 593 nm with a microplate reader. A standard curve was constructed using Fe(II) sulfate solution (100-3,500 μM) and the results are given in μmol $FeSO_4$/L.

Free Radical Scavenging Ability

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined with the method proposed by Von Gadow, Joubert and Hansmann [21]. 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.

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.

RESULTS

Viability of *Oenococcus Oeni*

In SW medium, *O. oeni* m was inoculated at a concentration of 8.0 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, suggesting that no bacterial lysis occurred and therefore no significant release of nitrogen compounds was evidenced. In SW supplemented with PPF of Cabernet Sauvignon and Syrah wines, bacterial viability decrease by 1 log cycle after 96 h (Fig. **1**).

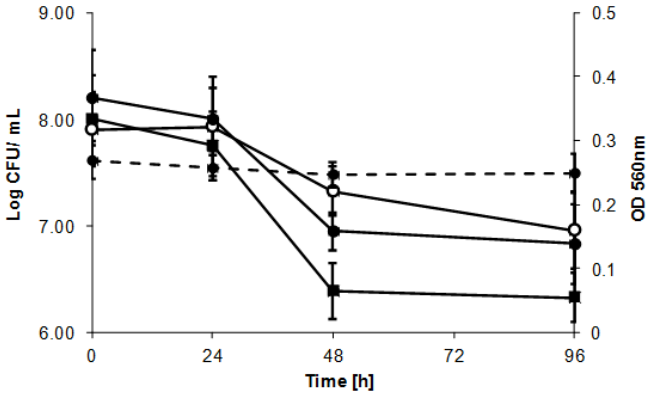

Fig. (1). Lines represent the modification of *Oenococcus oeni* m viability in SW (filled square), SWca (filled circle), SWsy (open circle). Dashed line represents the optical density determination in all assayed media. Values are means of three independent determinations.

Proteolytic Activity

Fig. (**2**) shows that low proteolytic activity (0.401 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).

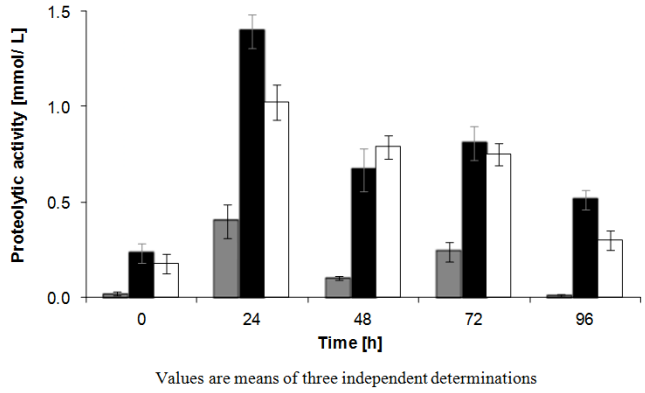

Fig. (2). Extracellular protease activity in synthetic wine (gray bars) and in synthetic wine supplemented with protein-polypeptide fraction of Cabernet Sauvignon wine (black bars) or Syrah wine (white bars). Values are means of three independent determinations.

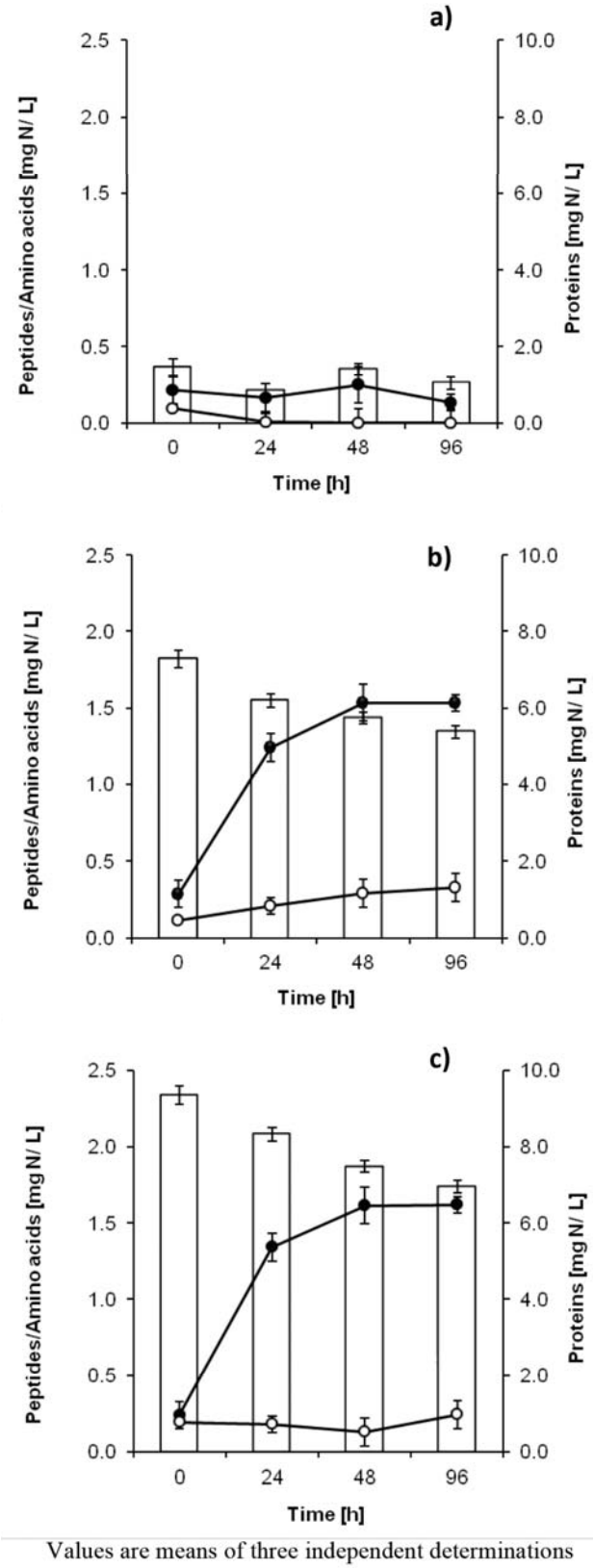

Fig. (3). Nitrogen compounds modification during incubation of *O. oeni* m in synthetic wine (**a**), and synthetic wine supplemented with the protein-polypeptide fraction of Cabernet Sauvignon wine (**b**) or Syrah wine (**c**). Bars represent protein modification and lines represent peptides (filled circle) and amino acids (open circle).

The proteolytic activity increased after 24 h incubation in SW supplemented with PPF of Cabernet Sauvignon and Syrah wines, reaching a maximum value of 1.395 mmol/L and 1.024 mmol/L respectively. From this time on, it decreased 63% and 71% in SWca and SWsy, respectively until 96 h incubation.

Modification of Nitrogen Organic Compounds

In SW, *O. oeni* metabolism did not significantly modify the nitrogen organic compounds during the incubation time (Fig. **3a**), 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 (Fig. **3b**), after 48 h incubation, a significant decrease of 2.037 mg N/L in protein nitrogen was detected. In this medium, peptide nitrogen concentration increased 1.247 mg N/L at 48 h. From this time on, peptide concentration did not modify significantly.

In the SWsy medium (Fig. **3c**), protein nitrogen concentration drop in 1.864 after 48 h incubation. The maximum consumption of protein nitrogen was detected after 96 h incubation (2.395 mg N/L). In this medium, the maximum increase in peptide concentration being observed after 48 h incubation (1.373 mg N/L). From this time, peptide nitrogen concentration did not modify significantly until 96 h incubation.

Modification of Biological Activities

Table **1** shows the modification of beneficial biological activities in the presence of *O. oeni* m in SW supplemented with PPF from Cabernet Sauvignon and Syrah wines. In SWca, results show that concomitantly with peptide release (Fig. **3b**) a maximum ACEI activity of 79.47 % was detected at 48 h incubation. The maximum ACEI activity in SWsy medium was also observed at 48 h incubation (32.45%).

A significant increase in ferric reducing power of 401.39 µmol $FeSO_4$/L was detected at 24 h incubation in SWca. In SWsy the maximum increase was observed at 48 h (156.53 µmol $FeSO_4$/L).

Radical scavenging activity reached 9.19% and 5.23% after 24 h incubation in SWca and SWsy respectively.

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 both wines, without bacterium inoculum, were similar to those found in SW (data not shown).

DISCUSSION

The cultivation of *O. oeni* m in synthetic medium supplemented with the PPF from Cabernet Sauvignon and Syrah wines produces an enhanced in extracellular bacterial proteolytic activity. This fact was evidenced concomitantly with the decrease in protein concentration 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. Consequently, these results confirm that the release of peptides with biological activities was caused by bacterial proteolytic activity against the wine proteins. The proteolytic activity detected during incubation of *O. oeni* m is related to protein consumption and release of peptide nitrogen from PPF of SWca and SWsy. No significant amino

Table 1. Modification of Biological Activities of Released Peptides by *Oenococcus oeni* Exoprotease in Synthetic Wine (SW), and Synthetic Wine Supplemented with PPF of Cabernet Sauvignon (SWca) and Syrah (SWsy) Wine Varietals.

Media	Incubation Time [h]	ACEI [%]	FRAP [µmol $FeSO_4$/L]	DPPH [%]
SWM	0	0.41[a]	25.17[a]	0.00[a]
	24	1.62[a]	38.18[a]	0.01[a]
	48	3.64[a]	26.91[a]	0.18[a]
	96	1.89[a]	13.70[a]	1.75[a]
SWca	0	15.54[b]	131.29[b]	0.25[a]
	24	74.81[e]	532.68[e]	9.19[c]
	48	79.47[e]	524.12[e]	9.91[c]
	96	66.52[e]	498.63[e]	11.17[c]
SWsy	0	18.67[b]	169.45[b]	0.50[a]
	24	26.14[b]	273.00[c]	5.23[b]
	48	32.45[c]	325.98[d]	5.97[b]
	96	27.56[b]	293.25[d]	6.94[b]

ACEI: angiotensin I-converting enzyme inhibitory activity; FRAP: Ferric reducing antioxidant power; DPPH scavenging: 2,2- diphenyl-1-picrylhydrazyl radical scavenging capacity. Values are the means of three independent determinations. Values with the same letter in the same column are not significantly different ($p < 0.05$).

acid modification was detected in any of the studied media. This observation agrees with the study of Aredes Fernández, Farías and Manca de Nadra [15], who reported that the strain X_2L of *O. oeni,* releases principally peptides from grape juice protein as a consequence of their extracellular proteolytic activity. These peptides released are important for bacterium growth because are efficiently used by *O. oeni* as nitrogen source for fulfill amino acid requirements and sustain viability [22-23-24]. In addition, peptide rather than amino acids utilization enables the bacterium to save a considerable amount of energy [25].

Peptides released from the protein and polypeptide fraction from Cabernet Sauvignon and Syrah wines by *O. oeni* m have antihypertensive, antioxidant and radical scavenging activities. The amount of released peptides from PPF of both wines are in the same order, but biological activities, particularly ACEI and Ferric reducing capacity, are significantly higher in SWca. These results are in agreement whit the publication of Apud, Rodríguez Vaquero, Rollan, Stivala and Aredes Fernández [26], who reported that, in the same experimental conditions, the proteolytic activity of *O. oeni* m enable the increase in peptides with biological activities from the PPF of four wine varietals from wine cellar of Cafayate, another region of Argentine. The released peptides from PPF of Cabernet Sauvignon wine from Colalao de Valle (this study) have higher ACEI activity than the released from PPF of same wine varietal from Cafayate [26].

The current study provides information about the role of the proteolytic activity of *O. oeni* m 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 wine. This study confirms that the biological activities of peptides released by *O. oeni* exoprotease vary according to different wine protein-polypeptide fraction utilized as substrate (varietal dependence), and also are dependent of wine region of production for a same wine varietal.

Ongoing experiments, using the purified protease of *O. oeni* m against the FPP of Cabernet Sauvignon and Syrah wines as a substrate, are being performed in order to isolate and characterize the bioactive peptides, and subsequently, testing them on a spontaneously hypertensive rats.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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