Biotechnol Lett (2011) 33:359–364
DOI 10.1007/s10529-010-0446-y

ORIGINAL RESEARCH PAPER

Increase in antioxidant and antihypertensive activity by *Oenococcus oeni* in a yeast autolysis wine model

Pedro A. Aredes Fernández · María G. Stivala · María J. Rodríguez Vaquero · Marta E. Farías

Received: 9 September 2010 / Accepted: 11 October 2010 / Published online: 23 October 2010

Abstract Accelerated autolysis of *Saccharomyces cerevisiae* mc2 in synthetic wine medium enabled the release of 3.7 mg peptide nitrogen/l, concomitantly with an increase in antioxidant properties (243 μmol $FeSO_4$/l in the case of ferric reducing antioxidant power and 0.5% in 2,2-diphenyl-1-picrylhydrazyl radical scavenging) and antihypertensive activity (22% in angiotensin I-converting enzyme inhibitory activity). Sequential inoculation of a proteolytic *Oenococcus oeni* strain in the synthetic medium after yeast autolysis produced an increase in peptide nitrogen concentration of 1.5 mg/l after 48 h of growth. After this incubation time an improvement in antihypertensive and antioxidant activities was detected. *Oenococcus oeni* X_2L could give additional value to wine because of the bioactive peptides with multifunctional beneficial activity released as consequence of its proteolytic activity.

Keywords Bioactive peptides · Yeast autolysis · *Oenococcus oeni* · Proteolysis · Wine

P. A. Aredes Fernández · M. G. Stivala · M. J. Rodríguez Vaquero · M. E. Farías (✉)
CERELA, Chacabuco 145, Tucumán, Argentina
e-mail: mfarias@cerela.org.ar

M. J. Rodríguez Vaquero · M. E. Farías
Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, Tucumán, Argentina

Introduction

Peptides with biological activity and health effects have been detected in different fermented and unfermented foods (Möller et al. 2008). Particularly regarding wine, scientific research is mainly focused on determination of the presence of bioactive peptides with an antihypertensive effect (Pozo-Bayón et al. 2007). In the winemaking process, alcoholic fermentation is generally carried out by *Saccharomyces cerevisiae* and occasionally followed by malolactic fermentation, carried out by lactic acid bacteria, mostly belonging to *Oenococcus oeni*. After alcoholic fermentation yeast autolysis occurs, and as a consequence, release of a variety of intracellular compounds takes place. Nitrogen compounds released due to yeast lysis are mainly peptides (Alexandre and Guilloux-Benatier 2006) and, to a lesser extent, amino acids and proteins. Some peptides are inactive but can be activated through the action of proteolytic enzymes from eukaryotic microorganisms (Korhonen and Pihlanto 2006). Several authors have found that antihypertensive peptides with inhibitory activity toward the angiotensin I-converting enzyme (ACEI activity) in fermented foods also presented radical scavenging (antioxidant) activity, suggesting the existence of multifunctional activity in these compounds (Hernández-Ledesma et al. 2005). Alcaide Hidalgo et al. (2007) found that peptides released during accelerated autolysis of *S. cerevisiae* in a wine model showed ACEI activity and oxygen radical scavenging capacity.

The role of lactic acid bacteria from wine such as *O. oeni* in the production of these bioactive peptides is unexplored at present. *O. oeni* X_2L involved in malolactic fermentation is able to hydrolyze wine proteins via extracellular enzyme activity (Manca de Nadra 2007).

The objective of the current study was to examine the modification of the ACEI and antioxidant activity by *O. oeni* X_2L inoculated in synthetic wine after autolysis of *S. cerevisiae*. The results will be of great relevance to obtain a better understanding of the role of *O. oeni* in biological activities in wine.

Materials and methods

Microorganisms, growth conditions and accelerated yeast autolysis

Saccharomyces cerevisiae mc2, isolated from Argentine wine (GenBank Data Library, accession number FJ800031), was grown in YPD (10 g yeast extract/l, 20 g peptone/l and 20 g glucose/l) at 30°C. The yeast cells were washed and resuspended in modified synthetic wine medium (SWM) according to Feuillat (1987), at 10^8 cells/ml. SWM contained: 10% (v/v) ethanol, 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 $MgSO_4 \cdot 7H_2O$/l, pH 3.0. Autolysis was obtained by incubation of the yeast suspension at 40°C under shaking conditions (60 rpm) during 24 h. Viable cell count and dry weight were determined before and after yeast autolysis. After centrifugation, the supernatant was adjusted to pH 4.0 and filter sterilized.

Oenococcus oeni X_2L, isolated from Argentine wine (Manca de Nadra and Strasser de Saad 1987) and grown at 30°C in MRS broth with 15% (v/v) tomato juice (pH 4.8), was resuspended (10^8 cells/ml) in sterile SWM before and after yeast autolysis. Bacterial growth was evaluated by viable cell counts on MRS agar (pH 4.8) at 30°C and dry weight measurements. SWM without yeast inoculation was used as control. The experiment was carried out in triplicate.

Analytical determinations

Proteolytic activity

Proteolytic activity was determined in supernatants with autoclaved grape juice as substrate. After 1 h of incubation at 30°C, the reaction was stopped by addition of 24% (w/v) trichloroacetic acid (TCA). In all cases controls were obtained by precipitation with TCA immediately before incubation. Total free amino acids were quantified in supernatants using Cd-ninhydrin reagent according to the method by Doi et al. (1981), with 1 mM L-leucine as standard. Results are expressed in mM of leucine.

Proteins

The protein concentration was determined with the Bradford method using bovine serum albumin as standard. Results are expressed in mg of nitrogen per liter (mg N/l). 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 were 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 method 1 and 5. Results are expressed in mg of nitrogen per liter (mg N/l) and referred to amino acid or peptide concentrations. L-leucine was used as standard (14 g N for every 131.17 g of leucine).

ACEI activity

ACEI activity was determined with slight modifications of 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. The absorbance was measured at 228 nm and 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 (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.

Free radical scavenging ability

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

Statistical analysis

All results are the means of three independent experiments carried out in duplicate. One-way analysis of variance was applied to all experimental data. Variable means showing differences with statistical significance were compared using Tukey's test. All statements of significance are based on a probability of 0.05.

Results and discussion

Table 1 shows that after 24 h of *S. cerevisiae* mc2 autolysis in SWM, the yeast viability decreased dramatically and a reduction of 38% was observed in dry weight. The autolysis process enabled the release of different types of nitrogenous molecules. Under these conditions, an increase in the concentration of proteins, peptides and amino acids of 7.92, 3.66 and 5.97 mg N/l, respectively, was detected. Similar results were evidenced in previous reports (Martínez-Rodríguez et al. 2001, 2002; Alexandre et al. 2001).

When *O. oeni* X_2L was inoculated in SWM after induction of yeast autolysis, the microorganism did not modify the total biomass, but a decrease in viability of approximately 0.5 log cycles was observed after 48 h of incubation in synthetic wine (Fig. 1).

Table 2 shows that *O. oeni*, grown during 24 h in SWM and sequentially inoculated after yeast autolysis, produced a decrease of 2.81 mg N/l in the protein concentration due to expression of the proteolytic system in the microorganism. Besides,

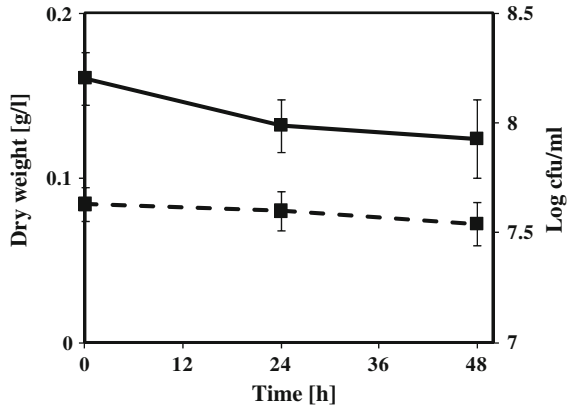

Fig. 1 Viable cell count (*solid line*) and optical density (*dashed line*) of *O. oeni* X_2L sequentially inoculated in a synthetic wine medium previously modified by autolysis of *S. cerevisiae* mc2

Table 1 Growth parameters, nitrogen compounds and proteolytic activity of *S. cerevisiae* mc2 in synthetic wine medium under accelerated autolysis conditions

Yeast incubation time (h)	Growth parameters		Concentration of nitrogen compounds‡ (mg N/l)			Proteolytic activity (mM)
	Dry weight (g/l)	Viability (log cfu/ml)	Proteins	Peptides	Amino acids	
0	1.52 ± 0.21[a†]	8.09 ± 0.09[a]	0.84 ± 0.03[a]	0.06 ± 0.02[a]	0.10 ± 0.02[a]	ND
24	0.93 ± 0.08[b]	ND	8.76 ± 0.35[b]	3.72 ± 0.17[b]	6.07 ± 0.32[b]	ND

ND not detected

Values are the means of three independent determinations carried out in duplicate

‡ Residual concentration in culture supernatant

† Mean values with different superscript letters within the same column are significantly different according to Tukey's test ($P \leq 0.05$)

Table 2 Change in nitrogen compounds and proteolytic activity due to sequential inoculation of *O. oeni* X_2L in synthetic wine medium after autolysis of *S. cerevisiae* mc2

O. oeni Incubation time (h)	Concentration of nitrogen compounds[‡] (mg N/l)			Proteolytic activity (mM)
	Proteins	Peptides	Amino acids	
0[§]	$8.66 \pm 0.22^{a\dagger}$	3.63 ± 0.12^{a}	5.53 ± 0.41^{a}	ND
24	5.85 ± 0.18^{b}	5.17 ± 0.45^{b}	4.71 ± 0.36^{b}	0.03 ± 0.00^{a}
48	5.06 ± 0.14^{c}	5.05 ± 0.34^{b}	5.32 ± 0.39^{ab}	0.06 ± 0.01^{b}

ND not detected

Values are the means of three independent determinations carried out in duplicate

[‡] Residual concentration in culture supernatant

[§] Values at time $T = 0$ correspond to 24 h of yeast autolysis in SWM

[†] Mean values with different superscript letters within the same column are significantly different according to Tukey's test ($P \leq 0.05$)

Table 3 Change in antihypertensive and antioxidant activity in synthetic wine medium after yeast autolysis during sequential inoculation of *O. oeni*

O. oeni incubation time (h)	Biological Activity		
	ACEI (%)	FRAP (μmol $FeSO_4$/l)	DPPH scavenging (%)
0[§]	$22.4 \pm 1.8^{a\dagger}$	243.33 ± 12.98^{a}	0.5 ± 0.1^{a}
24	20.4 ± 1.9^{a}	300.17 ± 18.65^{b}	1.6 ± 0.1^{b}
48	36.6 ± 2.0^{b}	430.67 ± 27.46^{c}	3.5 ± 0.2^{c}

Values are expressed as the difference between the absolute values and the respective controls and are the means of three independent determinations carried out in duplicate

ACEI angiotensin I-converting enzyme inhibitory activity; FRAP: ferric reducing antioxidant power

DPPH scavenging

2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity

[§] Values at time $T = 0$ correspond to values after 24 h of yeast autolysis in SWM

[†] Mean values with different superscript letters within the same column are significantly different according to Tukey's test ($P \leq 0.05$)

the peptide concentration increased with 1.54 mg N/l and consumption of free amino acids was 0.82 mg N/l. Consumption of amino acids after 24 h was probably stimulated to satisfy metabolic and energetic requirements of the bacterium to maintain cell viability (Konings 2006). The unaltered viability of *O. oeni* without reduction in biomass confirms that peptide release was only caused by proteolytic activity using proteins from yeast autolysis. From 24 h onward, the protein concentration decreased further but there was no significant change in the peptide and amino acid concentration.

Table 3 shows that induction of yeast autolysis yielded an increase in ACEI activity. This result is in agreement with that reported by Alcaide-Hidalgo et al. (2007) who demonstrated that a peptide fraction obtained from autolysates of a commercial *S. cerevisiae* strain played a principal role in antihypertensive activity. In addition, an increase in ferric reducing power and radical scavenging activity (antioxidant activities) was demonstrated in SWM after yeast autolysis. Other authors also reported ACEI and radical scavenging activity by peptides derived from fermented food (Hernández-Ledesma et al. 2005). Alcaide-Hidalgo et al. (2007) showed that peptides released by *S. cerevisiae* EC1118 during autolysis under wine conditions presented multifunctional antihypertensive and antioxidant activities.

Sequential inoculation of *O. oeni* in SWM after yeast autolysis produced a significant increase in

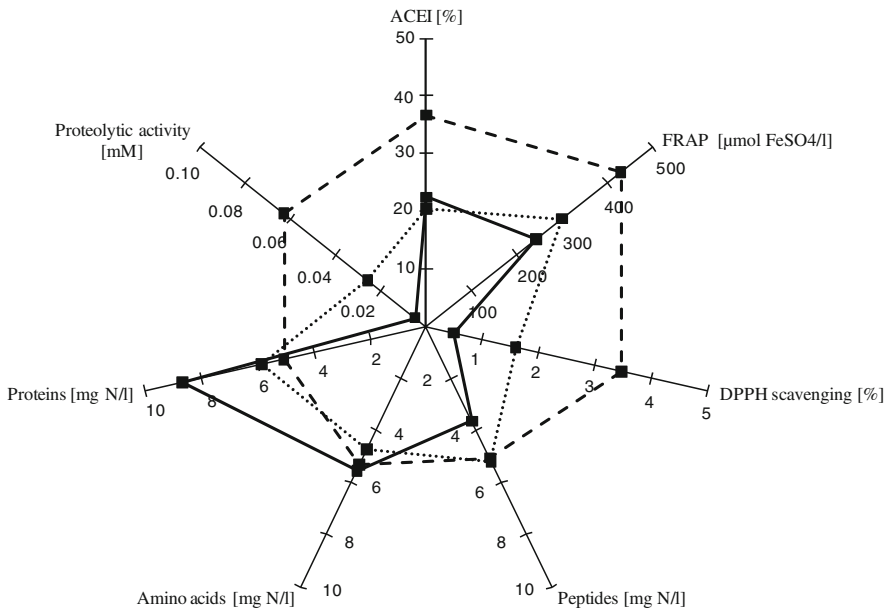

Fig. 2 Concentration of nitrogen compounds (peptides, amino acids and proteins) and biological activities (FRAP: Ferric reducing antioxidant power; DPPH scavenging: 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity; ACEI: angiotensin I-converting enzyme inhibitory activity) after 0 h (*solid line*), 24 h (*dotted line*) and 48 h (*dashed line*) of sequential inoculation of *O. oeni* X_2L in synthetic wine medium after *S. cerevisiae* mc2 autolysis. Time $T = 0$ corresponds to 24 h of yeast autolysis

ACEI (36.5%) and antioxidant activities (430.67 µmol $FeSO_4$/l and 3.5% for FRAP and DPPH scavenging, respectively) after 48 h of incubation. Figure 2 shows the relationship between the change in nitrogenous compounds and the two beneficial biological activities during growth of *O. oeni* in synthetic medium after yeast autolysis. The bacterium was able to express a proteolytic system under stressful conditions (Manca de Nadra 2007) and this activity produced a decrease in proteins released during yeast autolysis. Concomitantly, amino acid and peptide concentrations rose with increasing biological activity.

In conclusion, autolysis of *S. cerevisiae* mc2 in a synthetic wine model enabled the release of peptides with ACEI and FRAP activity. Sequential inoculation of *O. oeni* enhanced these activities, probably because of its proteolytic system. In addition, *O. oeni* was able to raise DPPH scavenging activity through nitrogen compounds released after yeast autolysis.

The current study provides new information about the role of proteolytic activity of *O. oeni* X_2L under conditions similar to those in wine, regarding the release and/or production of peptides with biological activity derived from nitrogen compounds after autolysis of *S. cerevisiae* mc2. These peptides have multifunctional biological activities with beneficial health properties, which could contribute to the improvement in wine quality.

Acknowledgments This research was supported by grants of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT).

References

Alcaide-Hidalgo JM, Pueyo E, Polo MC, Martínez-Rodríguez AJ (2007) Bioactive peptides released from *Saccharomyces cerevisiae* under accelerated autolysis in a wine model system. J Food Sci 72:276–279

Alexandre H, Guilloux-Benatier M (2006) Yeast autolysis in sparkling wine—a review. Aust J Grape Wine Res 12:119–127

Alexandre H, Heintz D, Chassagne D, Guilloux-Benatier M, Charpentier C, Feuillat M (2001) Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions. J Ind Microbiol Biotechnol 26:235–240

Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as measure of antioxidant power the FRAP assay. Anal Biochem 239:70–76

Cushman DW, Cheung HS (1971) Spectrophotometric assay and properties of the angiotensin I-converting enzyme of rabbit lung. Biochem Pharmacol 20:1637–1648

Doi E, Shibata D, Matoba T (1981) Modified colorimetric ninhydrin methods for peptidase assay. Anal Biochem 118:173–184

Feuillat M (1987) Préparation d'autolysats de levures et addition dans les vins effervescents élaborés selon la méthode champanoise. Rev Fr Oenol 109:17–27

Hernández-Ledesma B, Martín-Alvarez PJ, Pueyo E (2003) Assessment of the spectrophotometric method for determination of angiotensin-converting-enzyme activity: influence of the inhibition type. J Agric Food Chem 51:4175–4179

Hernández-Ledesma B, Miralles B, Amigo L, Ramos M, Recio I (2005) Identification of antioxidant and ACE-inhibitory peptides in fermented milk. J Sci Food Agric 85: 1041–1048

Konings WN (2006) Microbial transport: adaptations to natural environments. Antonie Van Leeuwenhoek 90:325–342

Korhonen H, Pihlanto A (2006) Bioactive peptides: production and functionality. Int Dairy J 16:945–960

Manca de Nadra MC (2007) Nitrogen metabolism in lactic acid bacteria from fruits: a review. In: Méndez-Vilas A (ed) Communicating current research and educational topics and trends in applied microbiology. Formatex, Spain, pp 500–510

Manca de Nadra MC, Strasser de Saad AM (1987) Evolution of lactic acid bacteria during the different stages of vinification of Cafayate (Argentine) wines. Micr Alim Nutr 5:235–240

Martínez-Rodríguez AJ, Carrascosa AV, Polo MC (2001) Release of nitrogen compounds to the extracellular medium by three strains of *Saccharomyces cerevisiae* during induced autolysis in a model wine system. Int J Food Microbiol 68:155–160

Martínez-Rodríguez AJ, Carrascosa AV, Martín-Alvarez PJ, Moreno-Arribas V, Polo MC (2002) Influence of the yeast strain on the changes of the amino acids, peptides and proteins during sparkling wine production by the traditional method. J Ind Microbiol Biotechnol 29:314–322

Möller NP, Scholz-Ahrens KE, Roos N, Schrezenmeir J (2008) Bioactive peptides and proteins from foods: indication for health effects. Eur J Nutr 4:171–182

Pozo-Bayón MA, Alcaide JM, Polo MC, Pueyo E (2007) Angiotensin I-converting enzyme inhibitory compounds in white and red wines. Food Chem 100:43–47

Von Gadow A, Joubert E, Hansmann CF (1997) Comparison of antioxidant activity of aspalathin with that of other plant phenols of Rooibosed tea (*Aspalathon linearis*), α-tocopherol, BHT, and BHA. J Agric Food Chem 45:632–638