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Increase of *trans*-resveratrol in typical Sicilian wine using β-Glucosidase from various sources

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Abstract

β-Glucosidase (EC 3.2.1.21) (β-G) from different sources were tested to increase the *trans*-resveratrol in some Sicilian wines by hydrolysing resveratrol glucoside. β-G from *Aspergillus niger* mould was tested as a crude and purified enzyme, and compared with the same enzyme from *Saccharomyces cerevisiae* yeast. Specific purification served to eliminate collateral enzyme activities so that β-G could be used simply and economically. *Aspergillus niger* β-G produced *trans*-resveratrol increases of up to 75%, with no change in physico-chemical properties and bouquet, and an increase in health and nutritional properties. *S. cerevisiae* β-G raised free-terpenol levels, but impaired wine colour due to anthocyanase activity.

Keywords: Resveratrol; β-Glucosidase; Enzyme; Purification; Anthocyanins

1. Introduction

Trans-resveratrol is a stilbene phenol and precursor of a large group of hydroxylated stilbenes which can be found in many plant families including *Vitaceae* and specifically in *Vitis vinifera* (Gorham, 1980; Sotheeswaran & Pasupaty, 1993). Many of these stilbenes are dimers (ε-viniferin), trimers (α-viniferin and gnetin-H) and tetramers (r-viniferin) (Langcake & Pryce, 1976; Mattivi & Nicolini, 1997).

In wine, stilbene synthesis starts with phenylalanine, the same route used in chalcone synthesis, which is a flavanoid precursor; for this reason some authors have suggested a negative correlation between resveratrol and anthocyanin which is not significant given the quantity of resveratrol present in wine (Becker et al., 2003; Soleas, Diamandis, & Goldberg, 1997).

Trans-resveratrol, together with the "*cis*" isomer and glucoside, is found in vine leaves and grape skin acting as a fungicide (*Botrytis cinerea, Plasmopara viticola*) and behaving like a phytoalexin as do other polydatins, these latter compounds are less pharmacological than *trans*-resveratrol but this needs further investigation (Commun et al., 2003). Plant–parasite interaction is not the only example of stilbene synthesis: even abiotic factors like UV rays, heavy metals, detergents and injury induce it (Darvill & Albersheim, 1984). Several studies have highlighted various properties of resveratrol such us antiatheromatosic (Belguendouz, Fremont, & Gozzelino, 1998; Kopp, 1998; Teissedre et al., 1996), potential oncogene tumour inhibitor, antioxidant against the peroxidation of low density lipoprotein (LDL) in the prevention of many cardiovascular diseases (Siemann & Creasy, 1992).

This study makes the use of commercial β-Glucosidase (EC 3.2.1.21, β-G) in winemaking to increase quantities of biologically active *trans*-resveratrol in typical Sicilian wine *Nocera*, beginning with the precursor glucoside. This

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process can enhance the health properties of wines which could eventually be labelled "high resveratrol" or as nutriceuticals, without modifying the wine's original physico-chemical and sensory properties. Currently, the β-Gs used in oenology contain other glucosidases such as α-L-arabinosidase and α-L-rhamnosidase which together with β-G, and by means of a sequential mechanism, increase the level of free terpenols in wine, modifying its distinctive bouquet and this effect is not always desirable particularly for DOC and DOCG wines. Other activities could also change the sensory properties of wine, e.g. esterase (Est) and polyphenoloxidase (PPO). Furthermore, β-G (anthocyanin-β-glucosidase) anthocyanase could partially hydrolyse the anthocyanins – the pigments in red wine – and therefore causing a reduction in hue intensity. Thus, the β-Gs need to be very effective against stilbenes but ineffective against anthocyanins at least as far as the eye can see. So, using purified β-G to obviate these undesirable effects could be a solution worth testing.

Current literature shows various researches into β-G activity: (a) selected yeasts with uninhibited glucosidase activity in wine and/or must. These micro-organisms must be GRAS certified and approved by the Office International de la Vigne et du Vin (OIV), and must not have negative side-effects on the bouquet as esterase and decarboxylase do forming negative aromatic compounds like ethylphenols, vinylphenols and guaiacols (Coghe, Benoot, Delvaux, Vanderhaegen, & Delvaux, 2004); (b) yeasts which have been genetically modified (GMO) by introducing genes from other micro-organisms (GRAS) and which produce high levels of β-G activity (Gonzáles-Candelas, Gil, Lamuela-Raventós, & Ramón, 2000). However, this solution should not only take into account the science but also the ethics of numerous consumer groups regarding GMO in foods; (c) UV use in wine (Threlfall, Morris, & Mauromoustakos, 1999; Tomás Barberán, Espín, & Cantos Villar, 2002); (d) improvement in the enzymatic hydrolysis of resveratrol glucosides in wine using expensive β-G preparations from almond (La Torre et al., 2004).

Consequently, the last but not least aim of this work was to provide a practical and inexpensive procedure for the simple use of β-G in oenology.

2. Materials and methods

2.1. Materials

Nocera and *Nero d'Avola* and widely available 2005 vintage Sicilian wines were used for this research; all samples were stored in the dark and analysed immediately after opening. Fungal β-Glucosidase (β-G) from commercial *Aspergillus niger* mould and β-G from commercial *Saccharomyces cerevisiae* yeast was obtained from Zymo (Turin, Italy). Prior to use, the yeast preparation was reconstituted in a 10% glucose solution, incubated at 30 °C for 3 h, centrifuged and the pellet diluted with citrate-phosphate buffer to pH 4.0 in order to get the same enzymatic activity of fungal β-G. *Trans*-resveratrol and *trans*-4-hydroxystilbene used as an internal standard were obtained from Sigma Chemical Co. (Milan, Italy), and *cis*-resveratrol by UV radiation from a *trans*-resveratrol solution by means of a wood lamp (La Torre et al., 2004; Threlfall et al., 1999).

2.2. Chemicals

HPLC grade solvents were obtained from Baker (Deventer, Holland). All the other reagents were analytical grade and supplied by Carlo Erba (Milan, Italy).

3. Physico-chemical determination

Titrable and volatile acidities, pH, alcohol level and sulphur dioxide (free and total) were determined according to the Official OIV Methods of Analysis (1990).

Total polyphenol analysis was carried out by Folin–Ciocalteu reagent and the polyphenols were expressed as mg/L of gallic acid according to Singleton (1974). Proanthocyanidins were quantified by transforming them into anthocyanins in hot acidic media according to Margheri and Falcieri (1972) and reported as cyanidin chloride. Flavans reactive to vanillin were determined according to Di Stefano, Cravero, and Gentilizi (1989) and the results expressed as mg/L of catechin. For anthocyanin determination, all samples were diluted with a solution of Ethanol:H_2O:HCl (70:30:1, by volume) and content was quantified on the basis of their maximum absorption in the visible range (536–540 nm) against a blank, Ethanol:H_2O:HCl (70:30:1, by volume). The extract pigment was calculated in mg/L assuming an average anthocyanin (average MW = 500) mix absorption for Cabernet Sauvignon grape to be 18,800 M^{-1} cm^{-1} (Di Stefano et al., 1989; Glories, 1984).

All determinations were performed in triplicate.

4. Trichromatic values

Wine colour variations before and after treatment with β-G from different sources were evaluated by the standard method of the Commission Internationale de l'Eclairage (CTE) updated in 1986, in which the purity and brightness of the dominant wavelength is measured, and appropriate software calculates lightness (L^*), green–red (a^*) and blue–yellow (b^*). Colour analysis was carried out using a Varian Cary 1*E* spectrophotometer (Australia), together with an optically coupled glass cuvette of focal length 1 mm. On the basis of these values, ΔE, defined as $[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, was calculated first. Values greater than at least 2, indicated two colours distinguishable by the human eye (Spagna et al., 1994).

5. β-G purification and enzymatic assay

Commercial β-G was purified according to Barbagallo, Spagna, Abbate, Azzaro, and Palmeri (2002) using an inexpensive method. The procedure comprises precipitation of

extraneous substances by adding ethanol and $CaCl_2$, ultrafiltration and adsorption, first on bentonite and then on chitosan. A sample of commercial enzyme from *A. niger* was dissolved in 0.01 M citrate–citrate (C–C) buffer at pH 4.0, to which cold ethanol 30% (v/v) was added and left for 2.5 h at 4 °C. After centrifuging (4000*g*, 4 °C, 10 min), $CaCl_2$ was added to the supernatant. Subsequently it was again centrifuged and ultrafiltred by membrane (Pellicon XL, Millipore) with a cut-off at 50 kDa. The enzyme was then suspended in 0.01 M C–C buffer, pH 5.6, and the resulting solution exposed first to bentonite and then to chitosan for 3 h. The solution was centrifuged at 4000*g* for 20 min at 25 °C and the resulting supernatant added directly to the wine. Every bottle was corked in nitrogen and kept at 16 ± 1 °C for 96 h.

β-Glucosidase (β-G), α-arabinosidase (Ara), α-L-rhamnosidase, (Rha) and esterase (Est) activities were carried out according to Barbagallo et al. (2002). The anthocyanase activity of β-G was determined using the Mian, Pifferi, and Spagna (1992) method. Protein content was determined according to Bradford's method (1976) using bovine serum albumin as standard.

6. Resveratrol determination

The quantitative evaluation of resveratrol was carried out by stilbene liquid–liquid extraction. A 0.5 mL ethanol solution (*trans*-4-hydroxystilbene 200 mg/L in ethanol) was added to 50.0 mL of wine and neutralised to pH 7.0 with 1 M NaOH. The sample was further diluted with bidistilled water in a 100 mL flask. Ten milliliters of diluted neutral wine were put through a separating funnel and mixed with 20.0 mL of a mixture of dichloromethane and ethyl acetate (1:1, v/v), the whole vigourously shaken for 5 min and left to separate. Then, in the organic phase, an aliquot was placed in a 100 mL flask and vacuum evaporated to solvent loss. The concentrated extract was mixed with 4.0 mL 1 mM phosphoric acid together with acetonitrile (1:1) and then analysed by HPLC (Shimadzu, Japan) using two pumps (LC-10A), a control system (SCL-10A), an injector (Rheodyne with 5 μL loop), a photodiode detector (SPD-M10A), a C18 Alltima ODS Hypersil column 250 × 4.6 mm I.D. (Milan, Italy) and a similarly packed pre-column. The mobile phase consisted of 1 mM phosphoric acid (A phase) and acetonitrile (B phase). Sample elution used the time gradient programme: 0 min 20%B, 0.01–25.00 min 60%B, 25.01 35.00 min 100%B, 35.01–40.00 min 100%B, 40.01–45.00 min 20%B, 45.01–55.00 min 20%B. All chromatographic tests were carried out at 25 °C with 1 mL/min flow. The samples were filtered with PTFE 0.45 μm filters before HPLC injection. The peaks were identified by comparison with retention times and the UV–VIS spectra between 190 and 400 nm of the *trans* and *cis*-resveratrol standards. The purity of each stilbene was controlled by HPLC and the identity was confirmed according to Mattivi (1993). Samples were quantified by the internal standard method. The calibration graph obtained using pure *trans*- and *cis*-resveratrol, and the 4-hydroxystilbene as internal standard. The concentration range was from 0.0 to 5.0 μg/mL for all standards, using seven points in triplicate.

7. Sensory analysis

Twenty judges were recruited from students of the Department of Food Science and Technology, Catania University. The candidates were submitted to preliminary tests to determine their sensory performance on basic tastes and aromas associated with wines. The sensory profile was constructed using a selected panel trained over five sessions. A list of descriptors was selected on the basis of the frequency (%) of the terms used by the judges over several sessions. Reference standards (Noble et al., 1987) were available to define descriptors. The final set consisted of six descriptors, one referring to appearance (colour), one referring to aroma and four referring to oral perception (acid, astringent, sweetness, bitter). The different descriptors were quantified using a height point intensity scale (ISO, 2003a,b). The four wines were tested in triplicate. All evaluations were conducted from 10.00 to 12.00 pm in individual booths (ISO, 1988). Thirty milliliters of each wine was served at 22 ± 1 °C (room temperature) in glasses (ISO, 1977) labeled with a 3-digit code and covered to prevent volatile loss. The order of presentation was randomized among judges and sessions. Water was provided for rinsing between wines.

8. Results and discussion

Table 1 shows the principal physico-chemical properties of *Nero d'Avola* and *Nocera*. With regard to acidity (titrable and volatile, as tartaric and acetic acid, respectively), pH, alcohol degree and sulphur dioxide (free and total)

Table 1
Physico-chemical properties of *Nero d'Avola* and *Nocera* wines

Analysis[a]	Wine *Nero d'Avola*	*Nocera*
Acidity (g/L)		
Titrable (tartaric acid)	5.44 ± 0.26	1.98 ± 0.19
Volatile (acetic acid)	0.74 ± 0.03	0.47 ± 0.02
pH	3.36 ± 0.17	3.63 ± 0.18
Alcohol	12.20 ± 0.56	12.40 ± 0.62
SO_2 (mg/L)		
Free	6.22 ± 0.26	5.89 ± 0.16
Total	33.89 ± 1.96	27.71 ± 1.26
Total Polyphenol (g/L)	1.26 ± 0.06	1.54 ± 0.09
Proanthocyanindins (g/L)	1.14 ± 0.05	1.60 ± 0.09
Flavans (mg/L)	502.31 ± 18.92	593.15 ± 29.68
Anthocyanins (mg/L)	317.42 ± 14.98	358.34 ± 17.62
trans-resveratrol (mg/L)	1.31 ± 0.07	1.63 ± 0.11
Trichromatic values		
L^*	77.39 ± 3.81	77.09 ± 3.73
a^*	23.99 ± 0.86	30.80 ± 1.50
b^*	7.37 ± 0.73	2.54 ± 0.42

[a] The determinations were carried out in triplicate and results are means ± standard deviation (SD).

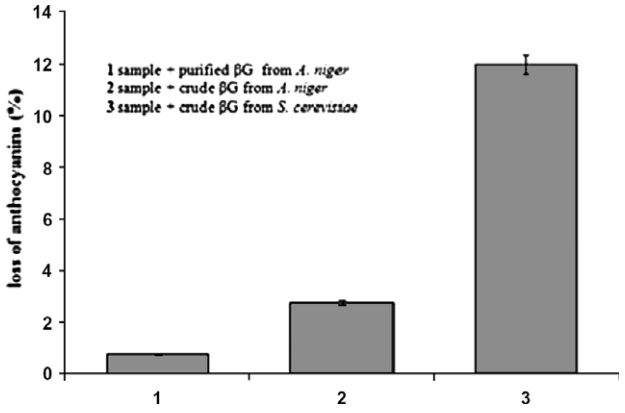

Fig. 1. Loss of anthocyanins content after treatment with β-G from various sources.

there were no differences. The results are the average of three replications. Not even the phenol profile showed any variations.

Since *Nocera* showed higher phenol content than *Nero d'Avola* (proanthocyanidin, +40%; flavans, +18%; anthocyanins, +13%; and resveratrol, +25%), this research was continued by studying β-G in the first wine alone.

To hydrolyse resveratrol, β-G was used in non-cellular form from an extract of *Aspergillus niger* (crude and purified) as well as in a cellular form from commercial *Saccharomyces cerevisiae* yeast. The Barbagallo et al. (2002) purification had the advantage of isolating β-G from any collateral activities which might have affected wine bouquet, such as the glycosidases, anthocyanase (anthocyanin-β-glucosidase), α-L-arabinosidase (Ara) and α-L-rhamnosidase (Rha), as well as esterase (Est) and polyphenoloxidase (PPO). After treatment, β-G showed no collateral activity, whereas anthocyanase proved weak (about 1/10,000 of β-G).

Nocera wine was then inoculated 12 h at 20 °C, with 5 U/l of β-G from *A. niger* (crude or purified) or *S. cerevisiae* (4.1×10^2 U/g of cells) in order to get the same enzymatic activity for each bottle. *S. cerevisiae* showed no collateral activity except for anthocyanase which was relatively high at 1/10 of β-G. After inoculation with the different β-Gs, the bottles were opened and tested.

Fig. 1 shows how anthocyanin loss was negligible using crude (2.8%) or purified (0.8%) βG from *A. niger* compared to start levels (358.34 ± 17.62 mg/L). The yeast strain showed a 12% drop in anthocyanins. Anthocyanin loss is probably due to the very active anthocyanase (anthocyanin-β-glucosidase) in commercial *S. cerevisiae* yeast: this makes it less useful in red wines given there should be no change in bouquet. The main anthocyanin sugar residue is glucose, so decolorization is due to the breaking of the bond between the sugar residue and the anthocyanidin which degrades spontaneously into colourless compounds. The same result was obtained through trichromatic analyses (Table 2), which shows the negative effect of *S. cerevisiae* above as regards channel a^* between yellow and red, with ΔE values above those normally perceptible. Furthermore, colour variation was perceived by the test panel of judges, showing an average of three appraisals by each judge (Fig. 2).

Fig. 3 shows both cellular and non-cellular β-G causing an increase in free resveratrol in wine with better results (75%) using purified *A. niger*.

Table 3 shows the effect on primary aroma content of adding various glycosidases and the variation in the aroma of treated wines was verified by sensory tests.

The wine treated with crude β-G from *A. niger* showed differences compared to the untreated wine, particularly a greater specificity towards aromatic compounds linked to the sugar moiety primary carbon (geraniol), rather than the tertiary carbons (linalool) probably due to other enzymatic action. The test panel noticed no other significant

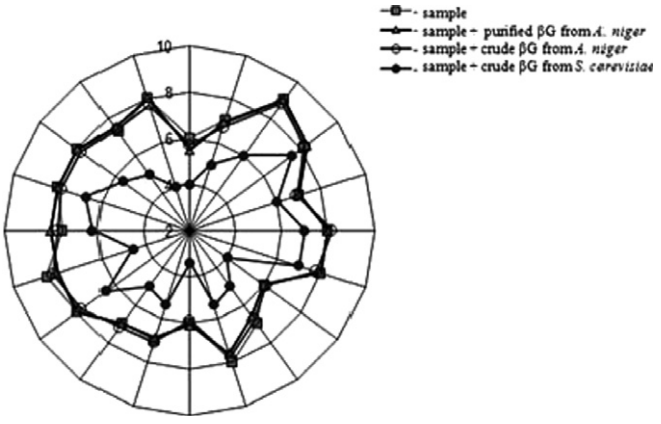

Fig. 2. Colour variation in wine *Nocera* according sensory analysis.

Table 2
Variation of lightness (L^*), green–red (a^*) and blue–yellow (b^*) parameters for each enzymatic treatment; $[\Delta E = (\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$

	Colour parameters[a]							
	Nero d'Avola				*Nocera*			
	L^*	a^*	b^*	ΔE	L^*	a^*	b^*	ΔE
Sample	78.63	25.70	5.53		74.52	30.05	1.39	
+crude βG from *A. niger*	78.12	25.78	5.73	0.55	74.98	29.76	1.51	0.55
+purified βG from *A. niger*	78.56	25.94	5.23	0.39	74.45	29.26	1.62	0.82
+crude βG from *S. cerevisiae*	77.20	19.54	5.99	6.34	78.25	27.54	1.91	4.53

[a] The determinations were carried out in triplicate.

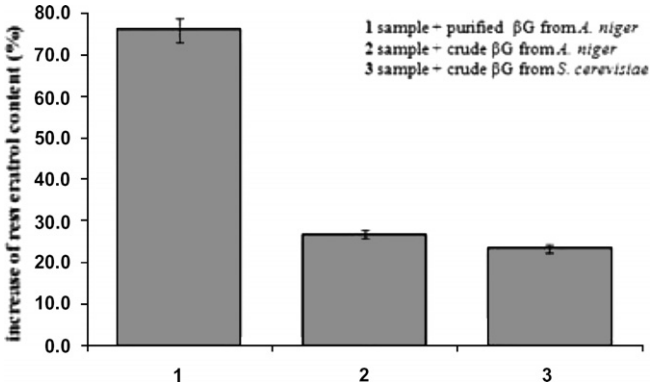

Fig. 3. Increase of *trans*-resveratrol from resveratrol glucoside in wine *Nocera* after different enzymatic treatments.

Table 3
Variation of aromatic compounds in *Nocera* wine treated with β-G from different source

Free aromatic compounds (μg/L)	Sample	*Nocera*		
		Sample + purified β-G from A. *niger*	Sample + crude β-G from *A. niger*	Sample + crude β-G from *S. cerevisiae*
Linalool	14.0	13.9	15.8	14.2
Citronellol	1.1	0.9	1.9	1.2
Geraniol	5.6	5.4	12.0	5.5
HO diol (I)	12.0	11.9	32.0	12.2
HO diol (II)	6.2	6.2	25.0	6.0
Benzyl alcohol	188.0	186.8	215.0	190.1
Phenylethyl alcohol	20364.0	20354.2	20498.0	20368.7
trans-3-hexenol	44.0	44.3	50.0	43.8
cis-3-hexenol	12.7	12.5	18.9	12.6
trans-2-hexenol	2.8	2.5	6.3	2.7
Bound aromatic compounds (μg/L)				
Linalool	1.9	2.0	1.6	2.1
Citronellol	3.1	2.9	1.9	3.0
Geraniol	18.3	18.5	11.2	18.4
HO diol (I)	0.9	0.9	0.2	0.8
HO diol (II)	0.6	0.7	0.2	0.6
Benzyl alcohol	1134.0	1128.6	1231.0	1136.5
Phenylethyl alcohol	656.0	651.8	673.0	653.2
trans-3-hexenol	2.5	2.4	1.9	2.5
cis-3-hexenol	13.0	12.8	14.9	12.9
trans-2-hexenol	26.0	26.3	24.1	25.9

The determinations were carried out in triplicate.

differences in bouquet, while aromatic differences were not noted in wines treated with purified β-G. Wines inoculated with β-G from *S. cerevisiae* showed significant differences in anthocyanins and colour but, as noted earlier, without variation in original aroma.

9. Conclusions

Purified commercial β-G from *A. niger* was capable of raising *trans*-resveratrol levels (by as much as 75%) with high specificity, and did not significantly influence physico-chemical properties or bouquet. Furthermore, commercial *S. cerevisiae* yeast strains need to be carefully selected in order not to affect anthocyanins or alternatively used only in white wines.

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