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Spectroscopy reveals that ethyl esters interact with proteins in wine

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ABSTRACT

Impairment of wine aroma after vinification is frequently associated to bentonite treatments and this can be the result of protein removal, as recently demonstrated for ethyl esters.

To evaluate the existence of an interaction between wine proteins and ethyl esters, the effects induced by these fermentative aroma compounds on the secondary structure and stability of VVTL1, a Thaumatin-like protein purified from wine, was analyzed by Synchrotron Radiation Circular Dichroism (SRCD) spectroscopy. The secondary structure of wine VVTL1 was not strongly affected by the presence of selected ethyl esters. In contrast, VVTL1 stability was slightly increased by the addition of ethyl-octanoate, -decanoate and -dodecanoate, but decreased by ethyl-hexanoate. This indicates the existence of an interaction between VVTL1 and at least some aroma compounds produced during fermentation. The data suggest that proteins removal from wine by bentonite can result in indirect removal of at least some aroma compounds associated with them.

1. Introduction

Protein haze formation is the main non-microbiological defect of white wines. Although wine is inhospitable solvent for proteins due to acidic pH, ethanol concentration and polyphenols, proteins of grape origin persist into the wine after fermentation (Waters et al., 2005). In particular, some grape pathogenesis-related (PR) proteins, including Chitinases and Thaumatin-like proteins (TLPs) have been shown to be the main components involved in haze formation (Waters, Shirley, & Williams, 1996). To remove the hazing proteins, white wines are treated with bentonite before being bottled (Waters et al., 2005). This treatment, however, while giving stability to the wine, has several drawbacks, including the impairment of aromatic compounds of wine (Van Sluyter et al., 2015). It has been demonstrated that this effect is mainly due to the direct adsorption of aromatic molecules by the bentonite clay (Lambri, Dordoni, Silva, & De Faveri, 2013; Lubbers, Charpentier, & Feuillat, 1996; Vincenzi, Panighel, Gazzola, Flamini, & Curioni, 2015). However, the removal of some ethyl esters by bentonite is increased in the presence of wine proteins, as recently demonstrated. This result suggests that these fermentative aromas could be in some way associated with those wine proteins that are removed by bentonite (Vincenzi et al., 2015) However, no direct evidence of this association has been reported to date.

Thaumatin-like proteins are the most abundant protein class present in white wines (Vincenzi, Marangon, Tolin, & Curioni, 2011). Recently, the X-ray structures of three grape TLPs have been resolved (Marangon, Van Sluyter, Waters, & Menz, 2014), displaying the presence of three domains and a cleft located between domains I and II. Domain I is formed by several β-strands; domain II is characterized by the presence of α-helix segments, while domain III comprises β-strands and small loops. The presence of disulfide bridges stabilizes these domains with a conserved spatial distribution throughout the protein. The cleft positioned between domains I and II is involved in interactions with different ligands. Ligand selectivity is due to the presence of different amino acids with acidic, neutral, or basic side-chains (Marangon et al., 2014).

To confirm the hypothesis of an interaction between esters and wine proteins (Vincenzi et al., 2015), the ability of ethyl esters to interact with a purified wine VVTL1 was investigated using fluorescence and circular dichroism spectroscopies. In particular, Synchrotron Radiation Circular Dichroism (SRCD) spectroscopy employing the Diamond B23 beamline has been used to obtain structural information about the interactions by analyzing the

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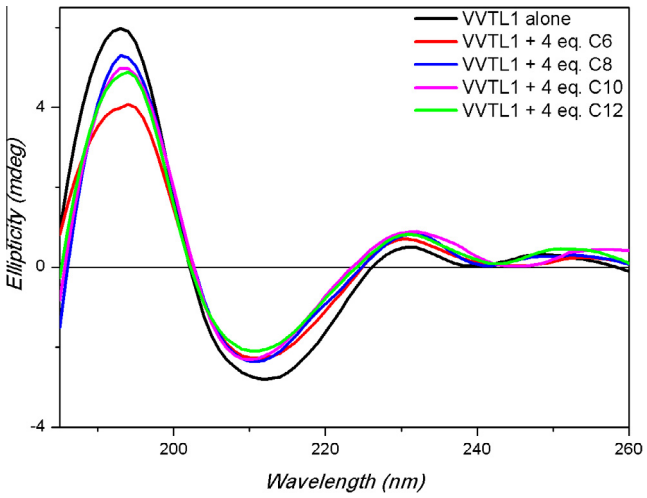

Fig. 1. Far-UV SRCD spectra of VVTL1 in model wine solution. 0.400 mg/mL of VVTL1 measured with B23 module B, in presence (4 eq.) and absence of ethyl esters. VVTL1 (black), VVTL1 + **C6** (red), VVTL1 + **C8** (blue), VVTL1 + **C10** (magenta) and VVTL1 + **C12** (green). Integration time 1 s, 0.02 cm cylindrical cell filled with 40 μL solution, monochromator slit widths 0.500 mm (1.2 nm bandwidth). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effect of ethyl esters with different chain lengths (from C6 to C12) on the VVTL1 UV-photo and thermal stability. The high performance of the B23 beamline to detect sample perturbations in terms of sensitivity, speed, ease, and small amount of material required (Hussain, Jávorfi, & Siligardi, 2012a), enabled the investigation of ligand-binding interactions otherwise unattainable using bench top CD and NMR instruments.

2. Materials and methods

2.1. Protein purification

VVTL1 was purified from the juice of Manzoni Bianco grape provided by commercial wineries in the Conegliano area (Italy) according to a modified procedure of Van Sluyter et al., 2009. Briefly, one hundred liters of grape juice were treated with 4.0 g/L of polyvinylpolypyrrolidone (Sigma-Aldrich, Milan, Italy), 1.5 g/L of charcoal (Sigma-Aldrich), 0.6 g/L of potassium metabisulfite (Everintec, Venice, Italy) and stored overnight at 0 °C. The mixture was filtered on 0.45 μm cellulose acetate regenerated membranes (Sartorius AG, Göttingen, Germany), and then adjusted to pH 3.0 with HCl 1 M. Aliquots of 10 L were loaded on a SP-Sepharose column (2.5 × 30 cm, GE healthcare, Milan, Italy) equilibrated with 30 mM sodium citrate buffer, pH 3.0, and eluted with a gradient from 0 to 40% of 30 mM MES buffer, pH 5.0, containing 1 M NaCl in 120 min. Fractions were collected on the basis of elution profiles at 280 nm using an Akta-purifier UPC-900 (GE healthcare) and subsequently analyzed by SDS-PAGE and reverse-phase HPLC. Ammonium sulfate was added to the pooled fractions containing the desired protein to achieve a final concentration of 1.25 M. The solution was loaded on a Phenyl-Sepharose (1.5 × 20 cm) column equilibrated by two volumes of 1.25 M ammonium sulfate and 50 mM sodium citrate buffer, pH 5.0. The column was eluted with a linear gradient from 0 to 100% of 50 mM sodium citrate buffer, pH 5.0, in 120 min. The fractions containing the desired protein were collected, dialyzed against MilliQ water (3.5 kDa MWCO) and freeze dried.

Ten mg of this protein batch was further purified by semipreparative HPLC using a Shimadzu LC-8 system (Shimazdu, Kyoto, Japan) on an Jupiter C18, 10 μm, 300 Å, (250 × 10 mm) column (Phenomenex, Torrance, CA). Protein was eluted by a linear gradient from 30 to 70% of eluent B (9:1 acetonitrile-water with 0.05% TFA) in 40 min against 0.05% TFA in water (eluent A), obtaining 6.08 mg of pure VVTL1 (>99% by analytical HPLC).

Peptide mass fingerprinting analysis on trypsin digestion products using a LC MS/MS Xevo G2-S Q-TOF (Waters) mass spectrometer and Mascot software (Berndt, Hobohm, & Langen, 1999) have been used to identify the isolated VVTL1.

2.2. Synchrotron radiation circular dichroism

Ethyl decanoate (**C10**) and ethyl dodecanoate (**C12**) were purchased from Fluka (Sigma-Aldrich), ethyl hexanoate (**C6**) from B. H.D. Laboratory Chemical Division (Poole, England) and ethyl octanoate (**C8**) from Eastman Organic Chemicals (Rochester, NY).

Protein sample was prepared by dissolving 0.4 mg/mL of lyophilized VVTL1 in a model wine solution (MWS, 5 g/L of *meso*-tartaric acid and 12% ethanol adjusted to pH 3.2). The optically inactive *meso* form of tartaric acid was used to minimize the interference in the CD spectrum of the chiral form. Stock solutions of ethyl esters (0.07 mM) were prepared by dissolving the appropriate amount of each ester in MWS. SRCD spectra from 180 to 260 nm were collected at the Diamond B23 beamline module end station B using integration time of 1 s, 1 nm digital resolution and 39 nm/min scan speed. Different bandwidths (1.2–1.8 nm) were used according the different experiments. Spectra were measured using Suprasil cell (Hellma Ltd.) with 0.02 cm pathlength. Thermal stability was monitored in the 5–70 °C temperature range at 5 °C increments with 5 min equilibration time using Quantum Peltier temperature controller. Protein UV photo-denaturation was investigated by measuring twenty consecutive repeated scans for each sample at 25 °C. SRCD spectra were processed and analyzed using the CDApps software (Hussain et al., 2015).

2.3. Fluorescence measurements

Fluorescence spectra were recorded from 285 to 385 nm using a Perkin Elmer LS50B spectrofluorimeter, with the excitation and emission slit widths set at 5 nm. The excitation wavelength was 280 nm. Briefly, small microliter amounts of ethyl ester stock solutions (3.95 μM) in MWS, were added to 900 μL of VVTL1 solution in MWS, (0.021 mg/mL) in a quartz cell (1.0 cm pathlength) to achieve a ethyl ester: protein molar ratios of about 4:1.

Table 1
Secondary structure content of VVTL1 (0.400 mg/mL) with and without ethyl esters calculated using CONTINLL (Siligardi & Hussain, 2015) of CDApps (Hussain et al., 2015).

Sample	% Secondary structure				T_M (K)
	β	Turns	Unordered	α	
VVTL1	41.4	22.3	31.9	4.4	270.35; 325.95
VVTL1 + C6	43.0	21.9	30.5	4.7	322.85
VVTL1 + C8	42.8	22.3	30.7	4.2	309.25; 327.65
VVTL1 + C10	43.3	22.3	30.2	4.3	293.45; 327.95
VVTL1 + C12	43.1	21.8	30.4	4.7	298.95; 328.55