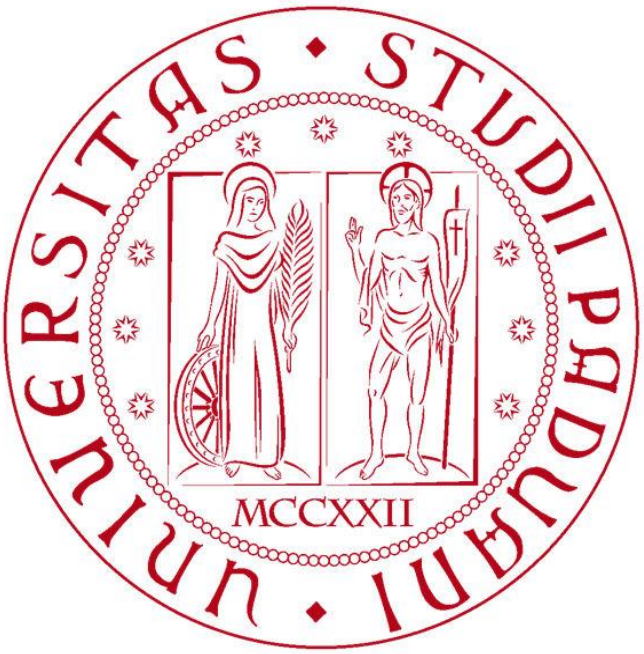

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Proteins In White Wines: Their Interaction With Tannins And Aroma Compounds

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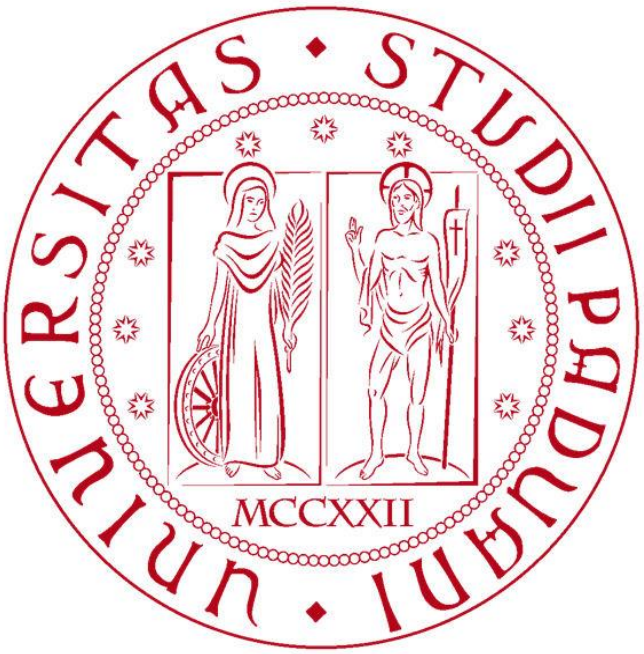

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Proteins In White Wines: Their Interaction With Tannins And Aroma Compounds

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Anno accademico

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Questa tesi è il risultato di tre lunghi ed intensi anni, progettato e realizzato dal team di ricerca del Centro Interdipartimentale per la Ricerca in Viticoltura ed Enologia dell'Università di Padova.

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I have no particular talent. I am merely inquisitive

“Albert Einstein “

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LIST OF ABBREVIATION

B1: procyanidin B1

B2: procyanidin B2

BCA: bicinchoninic Acid

C6: ethyl hexanoate

C8: ethyl octanoate

C10: ethyl decanoate

C12: ethyl dodecanoate

CD: circular dichroism

CEC: cation exchange chromatography

CGE: capillary gel electrophoresis

C_P: thermal capacity

D: diffusion coefficient

D_h: hydrodynamic diameter

DLS: dynamic light scattering

DSC: differential scanning calorimetry

EPE: electroendosmotic Preparative Electrophoresis

FAEE: fatty acid ethyl esters

HIC: hydrophobic interaction chromatography

IAA: Indole-3-acetic acid

IEC: ion-exchange chromatography

IEP: Isoelectric Point

I_S: intensity of scattering

KDS: potassium dodecyl sulphate

LTP: lipid transfer protein

MB 1-7: Manzoni Bianco explant 1-7

MFL: malolactic fermentation

MPs: methoxypyrazines

MS: mass spectrometry

MSM: Murashige and Skoog culture medium

MWCO: molecular weight cut-off

MWS: model wine system

N: native

N-PAGE: native - polyacrylamide gel electrophoresis

NAA: 1-Naphthaleneacetamide

NMR: nuclear magnetic resonance

N_N: non-native

PI: polydisperse index

PRP: Pathogenesis-Related proteins

PVPP: polyvinylpolypyrrolidone

Q: quercetin

R: rutin

RP-HPLC: reverse phase – high performance liquid chromatography

SAXS: Small-angle X-ray scattering

SDS-PAGE: sodium dodecyl sulphate - polyacrylamide gel electrophoresis

SEC: size exclusion chromatography

SRCD: synchtron radiation circular dichroism

T0: tannins purified few days after bottling

T8: tannins purified after eight months from full-filled bottle

TA: tannic acid

TLP: thaumatin-like protein

T_m: melting temperature

U: thermal treated

VVTL1: *Vitis Vinifera* thaumatin-like 1

WTE: wine tannins extract

SUMMARY

The presence of protein in white wines represents a major problem for the wine industry mainly due to the fact that proteins generate haze in the bottled white wines. Protein instability, which results in wine haze formation, is due to some grape PR-Proteins that thanks to their intrinsic resistance survive the vinification process, pass into the wine where cause the appearance of undesirable haze and deposits, leading to rejection by consumers. Protein hazing of white wines is considered to be a three-step process, involving protein denaturation followed by aggregation into colloidal particles able to scatter the visible light and make the wine turbid. Because of the complexity and the variability of the wine matrix, the factors and mechanisms involved in this process are still largely unknown. Commonly, winemakers prevent haze formation by removing the proteins through the use of bentonite. However, this treatment causes loss of wine and, being unspecific, also the removal of some aroma compounds. It has been calculated that the total cost deriving from bentonite treatments corresponds to a worldwide total amount of 1 billion dollars per year. Therefore, basic and applied research is still needed to solve the problem of protein haze formation in white wines.

Firstly, the present thesis faces the problem of the impairment of aroma due to bentonite fining. In particular, the study arises from a previous investigation which suggested the existence of an interaction between proteins and aroma compounds. In this context, the interaction of the main wine protein VVTL1 with some fatty acid ethyl esters (FAEE), which are important fermentative aroma compounds has been investigated. Due to the difficulty to determine this interaction at the molecular level, Synchrotron Radiation Circular Dichroism (SRCD) has been used to study the secondary structure of the wine protein as affected by the interactions with FAEE having different chain lengths. Subsequently, the research continued with the investigation of the role played by tannins in the phenomena leading to protein instability of white wines. To this purpose, the effects of several polyphenols (deriving from wine and not) on the stability of VVTL1 has been investigated using SRCD. In parallel, the capability of tannins to react with the proteins over time in bottled wine has been evaluated by Dynamic Light Scattering (DLS) studies in a model wine system. In addition, the thermal stability of two purified proteins, which are representative of the major classes of proteins in white wine (i.e. a class IV Chitinase and the VVTL1), has been investigated by Differential Scanning Calorimetry (DSC) in the presence of the tannins purified from wine at different times after bottling. Finally, the last

part of the research focuses on the possibility to produce good quantities of grape proteins in pure form starting from the *in vitro* culture of berry pulp tissues. These proteins can be used for molecular and functional characterisation. In particular, with this technique it is possible to label the proteins by cultivating the cellular tissues in the presence of N^{15} which allows the study of their fine structure and interactions by spectroscopic methods.

RIASSUNTO

La presenza di proteine nei vini bianchi rappresenta un problema di grande importanza per l'industria del vino, principalmente dovuto alla formazione di torbidità nei vini bianchi in bottiglia. L'instabilità proteica, nonché formazione di torbidità, è associata ad alcune proteine di difesa della pianta che per mezzo della loro intrinseca resistenza e stabilità sopravvivono al processo di vinificazione, passando nel vino dove causano la comparsa dell'indesiderata torbidità e di depositi in bottiglia, la quale non incontra le aspettative del consumatore e viene quindi scartata. La torbidità proteica nei vini bianchi è considerata come un processo a tre stadi, che coinvolge la denaturazione delle proteine seguita dall'aggregazione in particelle colloidali capaci di disperdere la luce visibile e far divenire il vino torbido. Data la complessità e la variabilità della matrice vino, i fattori e meccanismi coinvolti in questo fenomeno sono ancora largamente sconosciuti.

Normalmente gli enologi prevengono la formazione di torbidità rimuovendo le proteine attraverso l'uso della bentonite. Tuttavia questo trattamento causa la perdita di vino, ed essendo aspecifico, causa anche la rimozione di alcuni composti aromatici. È stato calcolato che il costo totale derivante dall'uso di bentonite corrispondi a 1 miliardo di dollari l'anno. Pertanto è ancora necessaria della ricerca di base e applicata per risolvere il problema della formazione di torbidità proteica nei vini bianchi.

La prima parte di questa tesi tratta il tema dell'impoverimento aromatico causato dal trattamento con bentonite. In particolare questo studio prende ispirazione da un precedente lavoro, il quale suggerisce l'esistenza di un'interazione tra le proteine e i composti aromatici. In questo contesto è stata studiata l'interazione della principale proteina dei vini bianchi, la VVTL1, e alcuni esteri etilici degli acidi grassi, i quali sono importanti aromi fermentativi del vino. Data la difficoltà di determinare a livello molecolare questo tipo d'interazione è stata sfruttata la luce di sincrotrone applicata al dicroismo circolare (SRCD) per studiare l'effetto di alcuni esteri etilici di acidi grassi a media catena sulla struttura secondaria della proteina del vino. In seguito, la ricerca è proseguita con lo studio mirato a comprendere l'esatto ruolo dei tannini nel fenomeno che conduce all'instabilità proteica dei vini bianchi. A questo scopo, sono stati studiati tramite sincrotrone gli effetti di diversi polifenoli (derivati o no dal vino) sulla stabilità della VVTL1. In parallelo la capacità nel tempo dei tannini di reagire con le proteine in bottiglia è stata valutata svolgendo degli studi di diffusione dinamica della luce (DLS) in vino modello. Con l'ausilio della Calorimetria differenziale a scansione (DSC), è stato inoltre studiata, la stabilità termica di

due tra le più rappresentative proteine del vino (la VVTL1 e la chitinasi classe IV) in presenza di tannini purificati a tempi diversi di evoluzione da vino imbottigliato. Infine, l'ultima parte della ricerca si focalizza nella possibilità di produrre proteine dell'uva in quantità accettabili in forma pura partendo dalla coltivazione *in vitro* di tessuti di polpa della bacca. Queste proteine possono essere utilizzate per la caratterizzazione strutturale e funzionale. In particolare, con questa tecnica si rende possibile la marcatura delle proteine facendo crescere i tessuti cellulari d'uva in presenza di N^{15}, il quale consentirebbe, per mezzo di metodi spettroscopici, lo studio in dettaglio della loro struttura e delle loro interazioni.

BACKGROUND AND JUSTIFICATION

Introduction

Wine is a beverage having the most important history and tradition in the world (Varriano, 2010). Talk about wine quality both aesthetic characteristics and organoleptic experiences are considered (Charters et al., 2003; 2005). Beyond the cultural tradition, wine assumes a key role in world economy contributing with U.S.$ 277.5 billion to world economy (Wine: Global Industry Almanac; 2012). What is required especially for white wines are the clarity of the final product. Therefore, securing wine stability prior to bottling is an essential step in the winemaking process, being also a harsh challenge for winemakers. There are mainly three defects normally encountered that affects the haziness of white wines: the microbial instability, tartrate instability and protein instability (Ribéreau-Gayon et al., 2006). While the first two ones are relatively easy to solve, the protein instability still needs other investigation to be fully understood. Proteins are one of the main family of macromolecules in wine, along with polysaccharides and polyphenols (Waters et al., 2005). Waters and colleagues found that the most abundant proteins in white wines are Pathogenesis-Related (PR) proteins deriving from the grape berry, particularly chitinases and thaumatin-like proteins (Waters et al., 1992, 1996), although also the presence of good quantities of vacuolar invertase was observed (Marangon et al., 2009). Despite their relative low concentration in wine (Waters et al., 2009; Vincenzi et al., 2005; Mesquita et al., 2001; Ferreira et al., 2002), they are implicated in numerous different phenomena in wine. The proteins exhibit positive effects to the foamability of sparkling wines (Liger-Belair et al., 2008; Coelho et al., 2011; Vincenzi et al. 2014a), strongly influence the tartrate stability in wine (Moine-Ledoux et al., 2002; Lambri et al., 2014) and their presence influences the perception and composition of aroma compounds in wine (Jones et al., 2008; Pozon-Bayon et al., 2009; Vincenzi et al., 2015). Thus, proteins play a key role in almost all processes occur in wine. The following text introduces the topics of this doctoral research that focuses on the study of proteins and their interaction with aroma and polyphenols in white wines.

Aroma compounds

The presence of aroma compounds is obviously fundamental for the sensorial quality of wine. The presence of volatile compounds is strongly dependent on grape variety, fermentation processes and wine storage conditions.

During winemaking, several phenomena occur which can lead to modification of the wine aroma composition. In addition, practices such as ageing of wine in barrels or on the yeast lees further modify the composition of wine aroma.

According to these processes the wine aroma compounds are divided in pre-fermentative (primary aroma), fermentative aroma (secondary aroma) and post-fermentative aroma compounds (tertiary aroma). Among the large family of aroma compounds there are some C6-compounds (i.e. hexanal, 1-hexanol, etc.). They are released by lipoxygenase process during the crushing of grape. These molecules are responsible for herbaceous odour (*Ribéreau-Gayon et al., 2006)*

Primary aroma compounds (Varietal Aroma)

The main varietal aroma compounds found in wine belongs to the family of Monoterpenes, which is typically associated to Muscat wines. Among the wide range of monoterpens it has been found linalool, geraniol, citronellol and nerol (Iriti and Faoro 2006). Most part of these compounds are present in grape as odorless glycosylated precursors, which are released during alcoholic fermentation by chemical and enzymatic processes.

Another class of varietal aroma found in wines are the thiol compounds. Among the best known are 4-methyl-4-mercaptopentan-2-one and 3-mercapto-1-hexanol (Swiegers and Pretorius 2007).

The carotenoids also play a role in varietal aroma. From their oxidation are generated some odour-fragments known as C13-norisoprenoids including β-ionone (violet aroma), β-damascenone (exotic fruits), β-damascone (rose), and β-ionol (fruits and flowers) (Iriti and Faoro, 2006). Recently another varietal aroma has been discovered for Shiraz wines variety. It is associated to pepper aromas and corresponds to the sesquiterpene rutondone (Siebert et al., 2008). Methoxypyrazines (MPs) are another varietal aroma compounds found in some Vitis vinifera grapes and their wines. often associated with “unripe” character or also green character (i.e. 3-iso- butyl-2-methoxypyrazines, 2-isopropyl-3-methoxypyrazine…) (Pickering et al., 2007).

Secondary aroma compounds (Fermentative Aroma)

Quantitevely, the compounds formed during fermentation represent the most important flavour component of wine. The fermentative aroma include all the volatile molecules produced by yeast (alcoholic fermentation) and bacteria (malolactic fermentation).

It is well documented that the yeast strain specifically modulates the production of these compounds (Lambrecht and Pretorius 2000). Certainly, ethanol constitutes an important volatile factor because influence not only the volatility of aroma compounds, but also the global perception of wine.

The most important family of fermentative aroma compounds are the higher alcohols (fusel alcohols) and the corresponding esters. Most part of higher alcohols such as isoamyl alcohol, active amyl alcohol and isobutanol deriving from the degradation of amminoacids in the yeast cells through the Enrlich pathway (Ehrlich, 1904). It is documented that high concentration (above 400 mg/L) of these compounds have a negative impact on perceived wine aroma, whereas when they are present in concentration below 300 mg/L the complexity of wine is improved (Rapp and Versini, 1995).

Generally, the main esters found in wine are ethyl esters with fatty acid. The most significant esters are ethyl acetate (fruity, solvent-like), isoamyl acetate (isopentyl acetate, pear-drops aromas), isobutyl acetate (banana aroma), ethyl caproate (ethyl hexanoate, apple aroma) and 2-phenylethyl acetate (honey, fruity, flowery aromas) (Thurston and Tubb, 1981). On the other hand, fatty acid esters, which are produced in wine, result in lower volatility and unpleasant smell when the length of the carbon chain increase (Francis and Newton, 2005).

Other than esters and higher alcohols there are some compounds associated to yeast metabolism that contribute to the complexity of wine fermentative aroma. One of these is ethanal (i.e. acetaldehyde). This compound is produced from pyruvate during vinification and constitutes almost the totality of aldehydes of wine. The acetaldehyde is a highly volatile compounds with reminiscence to fruity aroma at low concentration, but becomes unpleasant with green grass and apple odour at high concentration (Liu and Pilone, 2000).

After alcoholic fermentation, some wines can undergo a secondary fermentation known as malolactic fermentation (MLF). Generally, MLF can enhance wine fruity aroma and the buttery notes. (Liu, 2002).

Tertiary aroma compounds (Post-fermentative aroma)

As described above the wine aroma is formed by numerous processes related to the varietal aroma composition, and occurring during pre-fermentation and fermentation. In addition, the wine undergoes periods of ageing or maturation in the bottle or oak barrel. In general, during ageing the wine losses the characteristic varietal aroma, and form novel typical compounds or atypical aromas associated to wine deterioration (Hernanz et al., 2009). Moreover, ageing of the wine in contact with the yeast lees causes the impairment of fruity aroma due to the loss some ethyl esters and the increase of long-chain alcohols and volatile fatty acids (Pérez-Serradilla, and De Castro, 2008).

On the other hand, the storage or fermentation of wines in oak barrels promote the extraction of aromatic compounds from the wood such as lactones and furfural aldehydes (Spillman et al., 2004). Lactones are the compounds released by the wood of the barrel by self-complexation between an alcoholic and acid function present in the same molecule. The 3- methyl-γ -octalactone, also known as "oak lactone" or "whisky lactone" is one of the most important compound extracted from wood (Spillman et al., 2004).

Furfural aldehydes are released within the wine by pyrolysis of hexose and pentose sugars of wood. The most abundant are 5-hydroxymethylfurfural, 5-methylfurfural (from C6), and furfural (C5). They have positive impact in aged wine with characteristic toasted almond odours (Bohre et al., 2015). Also oxidative reactions can make significant contributions to the flavour of aged wines as a result of formation of compounds such as acetaldehyde (nutty, sherry-like aroma) and acetic acid (vinegar aroma). However, above certain concentrations they can negative impact the organoleptic profile of wines (Ribéreau-Gayon et al., 2006).

Table 1. *Summary of the main class of varietal and fermentative aroma compounds find in wine (Ribéreau-Gayon et al., 2006).*

Aroma family	Compounds	Odor
Monoterpenes	Linalol	Flowery, rose
	Geraniol	Flowery, geranium
	citronellol	Citrus fruits
Norisoprenoids	β-damascone	Exotic fruits
	β-ionone	violet
Methoxypyrazines	3-isobutyl-2-methoxypyrazine	Green pepper
	3-isopropyl-2-methoxypyrazine	Cooked legumes
Mercaptans (Thiols)	4-mercapto-4-methylpentan-2-one	Tomato leaf
	3-mercaptohexan-1-ol	Grapefruit, passion fruit
Benzoids	4-hydroxy-3-methoxybenzaldehyde	Vanilla
	benzyl alcohol	Dry fruits, almond
Fusel alchols	Isoamyl alcohol	Alcohol
	Isobutanol	Fruity, solvent-like
Esters	Ethyl acetate	Fruity, solvent-like
	Isoamyl acetate	Banana, pear
	ethyl hexanoate	apple
	2-Phenyl acetate	Flowery, honey fruits

Polyphenols

Polyphenols are extracted from grape berries during vinification. Tannins are found in higher amount in red wines because the contact with the skin during maceration. They are composed by a benzene ring with one or more hydroxyl groups and they are divided in non-flavonoid compounds (i.e. phenolic acids) and flavonoid compounds (i.e. flavonols, flavanols, anthocyanins, flavanones).
The general distribution of polyphenols in the white grape berry is reported in figure 1.

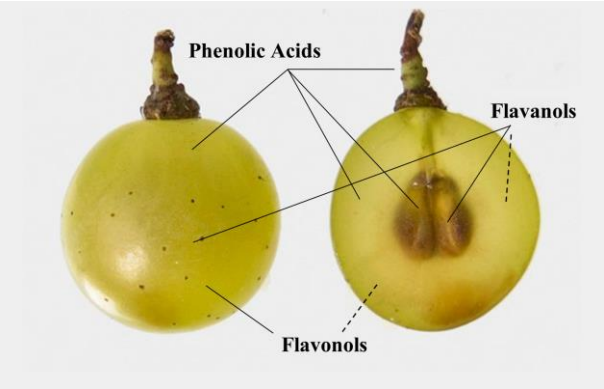

Fig.1 Distribution of Polyphenols in white grape berries.

Non-flavonoid compounds

This class of polyphenols is composed by phenolic acids, including benzoic acids, hydrocinnamates and their derivates such as stilbenes. Benzoic acids are generally found as gallic, syringic and p-hydroxybenzoic acids, the first one being the most abundant (Pozo-Bayón et al., 2003). Hydroxycinnamic acids are characterized by C3-C6 skeleton and they are mostly found as ester of tartaric acid. The caftaric acid, which structure as reported in figure 2, is the main hydroxycinnamic acid found in white grape. Ferulic acid and p-coumaric acid are the other hydroxycinnamic acids. Generally, the phenolic acids are colourless, but the esterification with the tartaric
acid make these compound extremely susceptible to oxidation contributing to the browning of white wines (Rigaud et al., 1991).

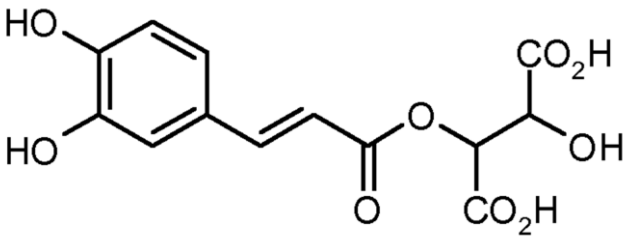

Fig. 2 Structure of caftaric acid

Stilbenes are a subclass of phenolic compound, synthetized in response to exogenous stress such as UV-irradiation and pathogen infection (Wang et al., 2010; Timperio et al., 2012). Production of the phytoalexins trans-resveratrol and delta-viniferin in two economy-relevant grape cultivars upon infection with Botrytis cinerea in field conditions. Plant Physiology and Biochemistry, 50, 65-71). Among the stilbenes the trans-resveratrol and its glycosylated derivative compound (i.e. piceid) are the most abundant in grape and they are mainly located in the grape skin (Gerogiannaki-Christopoulou et al., 2006; Romero-Pérez et al., 1999).

Flavonoid Compounds

Flavonoid compounds are present in grape as flavonols, anthocyanins and flavan-3-ols. The anthocyanins are responsible for the red pigments and they are not found in white varieties. These compounds are formed by two phenolic ring (A and B), linked together by heterocyclic pyran ring (c-ring) (Fig. 3). The oxidation degree of the pyran ring determine their flavonoid nature.

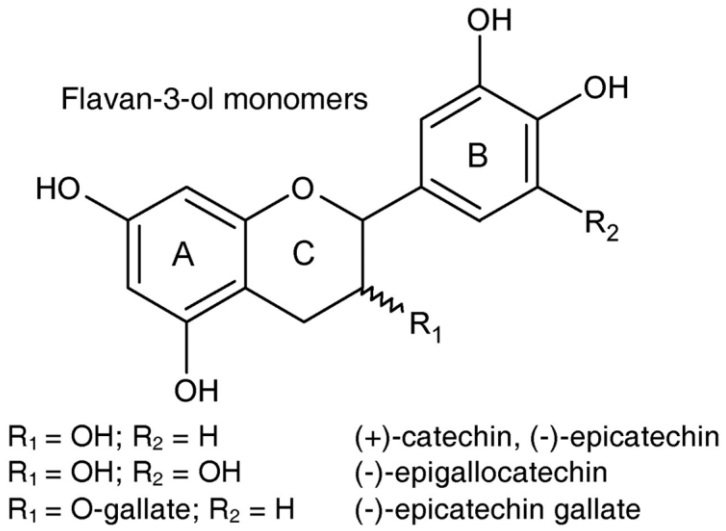

Fig. 3 Structure of the main flavon-3-ol monomers of grapes

The flavonols are also called flavan-3-ols because hydroxylated on the third position (fig. 3). They are found as monomers, oligomers and polymers. Along with the hydroxycinnamic acids are involved in the browning and hazes phenomena of wine (Rigaud et al., 1991, Siebert, 2006). They are mainly located in the skin and seeds of the grape berries (Kennedy et al., 2001; Souquet et al., 1996). They differ by the presence of galloyl substitute on the carbon C2 of the C-ring and by the hydroxylation degree of the ring B (Souquet 1996). The oligomeric and polymeric forms of flavan-3-ols are also called proanthocyanidins (i.e. condensed tannins) because they are able to generate anthocyanidins upon acid-catalysed cleavage at high temperature of interflavanic bonds. In particular, the procyanidins made by catechin and epicatechin units are converted in cyanidins, whereas prodelphinidins composed by galloylated monomers (i.e. gallocatechin and epigallocatechin) produce delphinidins (Bate-Smith, 1954; Cheynier et al., 1998). The polymers can be arranged in different manner depending on the type of monomers linkage (i.e. C4-C8 or C4-C6). Generally, the tannins are predominant formed by C4-C8 bonds, even if C4-C6 bonds can occur. Tannins in grape skins are made of catechin, epicatechin and epigallocatechin units whereas grape seed tannins contain catechin, epicatechin and epicatechin gallate units. In general, white wines contain less than 50 mg/L of procyanidins (Gorinstein et al., 2000).

Finally, flavonols are yellow pigments mainly located in the skin of grape berries. Some have been detected in the pulp and they are not found in seeds. Although the majority of them are present in the glycosylated form, some aglycones are released by acid hydrolysis

in wine. The main aglycone forms detected in grape are quercetin and myricetin (Castillo-Muñoz et al., 2008)

The origin of wines proteins

Grape proteins concentration in the grape berry is influenced by climate, variety and drought stress (Lee 1985, Hsu and hearthebell 1987a; Meier et al., 2016). However, their amount in wines are always largely lower than that found in grapes (Mequita et al., 2001; Ferreira et al., 2002; Vincenzi et al., 2005; Waters et al., 2009). The fermentation is considered primarily responsible for the difference between grapes and wines in term of proteins content. This is attributed to degradation and denaturation of the grape proteins caused by proteases and pH change occurring when the juice is extracted (Bayly & Berg 1967; Feuillat et al., 1980; Murphey et al.1989), but also to the presence of polyphenols and the increase content of ethanol occurring during fermentation. (Somers and Ziemelis, 1973). It has been demonstrated that wine is mainly composed by proteins having a defence role in the grape berry, which are the most resistant to vinification, the PR-proteins (Murphey et al.1989; Waters et al., 1992; Marangon et al., 2009; Sauvage et al., 2010). Generally, in white wines, proteins amount ranges from 15 to 230 mg/L (Ferreira et al., 2002), but sometimes they can reach up to 700 mg/L depending on the oenological practices and the methods of quantification (Vincenzi et al. 2005).

Role and characteristics of wine proteins

As mentioned above the main proteins in wines are invertases (60-72 kDa) and PR-proteins (9-41 kDa). In particular, among the PR-proteins chitinases and thaumatin like proteins (TLPs) has been demonstrated to be the most abundant protein classes in the white wines (Waters et al., 1992; Sauvage et al., 2010). Also β-1,3-glucanases (Esteruelas et al., 2009; Sauvage et al., 2010) and the Lipid Transfer Proteins (LTPs) (Marangon et al., 2009; Wigand et al., 2009) were described as wine protein components (Fig. 4). Most of these proteins are positively charged at the wine pH. Unlike invertase and the other PR-proteins (pI = 4-6), β-glucanase and LTP have higher isoelectric point (about 9) (Hsu and hearthebell, 1987b; Brissonet et al., 1993; Dawes et al., 1994; Ferreira et al., 2002; Gasteiger et al., 2005; Wigand et al., 2009). Although there are only few numbers of

protein families in wine, the existence of a wide range of structurally similar but distinct proteins has been demonstrated. On the basis of 2D electrophoresis and cation exchange Monteiro et al. (2003) demonstrated the presence of distinct protein patterns between the same grapes harvested in two consecutive years. Marangon and colleagues (2009), confirmed these results observing different spots for TL-protein and chitinase family, which differ for hydrophobic characteristics. Moreover, even if in minor extent they observed similar trend in the correspondent unfined wines. (Monteiro et al., 2003).

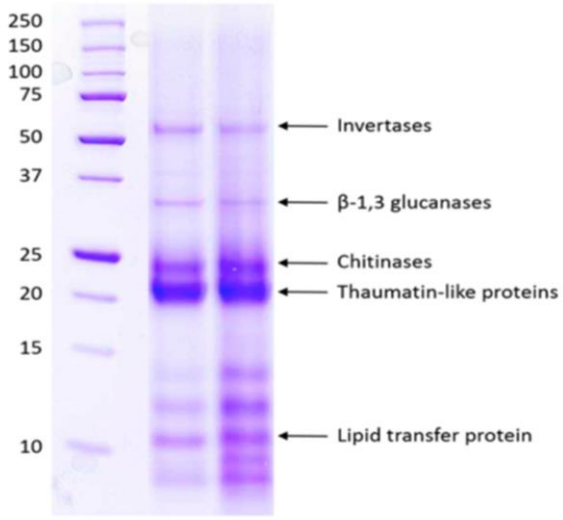

Fig. 4 *Classic SDS-PAGE protein profile of two white wines. The identity of proteins was assigned in according to proteomic analysis (Adapted Marangon et al., 2012a).*

Invertase

The grape vacuolar invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) is the grape protein with the highest molecular mass found in wine. It is a high glycosylated protein involved in the conversion of sucrose in the two simple sugar glucose and fructose with theoretical molecular weight of 71.5 kDa and pI of 4,6 (Jégou et al., 2009). The estimated secondary structure of this glycoprotein is mainly characterized by random coil and antiparallel β-sheet (Dufrechou et al., 2012). It demonstrated very high resistance to heat with a melting temperature above 80°C, however irreversible denaturation process in a model wine at pH 3 was observed (Falconer et al. 2010). It plays an important role in the foam quality and stability of sparkling wines (Marchal et al.,1996; Dambrouck et al., 2005). In addition, a fragment of yeast invertase showed protective effect against protein instability (Moine-Ledoux et al., 1999).

β-1-3-Glucanases

β-glucanases are the second grape proteins in term of size found in wine (37.5-41 kDa). Their biological role in grapes is associated to the hydrolysis of the glucans of the fungal cell walls (Ferreira et al., 2004; Feuillat et al., 1998), belonging to the PR-2 family (Datta et al., 1999). This protein is not always found in white wines, where its presence was observed only in few cases (Cilindre et al., 2007; Esteruelas et al., 2009, Sauvage et al., 2010). In particular, Esteruelas and co-authors (2009) detected the β-1,3-glucanase in the natural haze aggregates (Esteruleas et al., 2009), suggesting an involvement of this class of proteins in haze formation. β-1,3-glucanase exhibits much higher sensitive to heat compared to the other wine proteins. This has been attributed to the different charge of this protein at wine pH (Sauvage et al. 2010), which influence the heat-precipitation process of proteins (Chi et al., 2003). Progress in the investigation suggested that the protein is able to penetrate in the interstitial space of the clay due to their flexible structure. Indeed, the disulphide bridges, which generally stabilize the structure of proteins, result absent in β-Glucosidase. (Jaeckels et al., 2015). Unfortunately, the structure of grape β-glucanase is not solved yet. However, a homology model was predicted using as template the crystal structure of banana β-Glucosidase. The modelled structure of grape glucanase contains α-helices and β-sheets which form a β/α-TIM-barrel motif. Moreover, the distribution of charged amino acid confer a positive charge on the surface of protein (Jaeckels et al., 2015).

Chitinases

Another main class of PR-proteins always found in white wines are the chitinases. These proteins manifest antifungal activity catalysing the hydrolysis of the chitin present in the cell wall of fungi (Colligine et al., 1993; Saltzmann et al., 1998; Vincenzi et al., 2014b). Along with thaumatin like proteins, chitinases are the main class of proteins in white wines and always considered the most involved in haze formation (Falconer et al., 2010; Sauvage et al., 2010). Although showing a mobility of about 31 kDa in SDS-PAGE (Manteau et al. 2003; Vincenzi et al., 2014b), actually they have molecular mass ranging between 24-28 kDa, as demonstrated by mass spectrometry (Van Sluyter et al., 2009; Sauvage et al., 2010; Waters et al., 1996). On the basis of their fractionation by hydrophobic interaction chromatography (HIC) these proteins has been characterized by their low surface hydrophobicity (Marangon et al., 2010). The main chitinase found in white wines belong to

the class IV (PR-3) (Datta et al., 1999). This protein is characterised by having mostly an α-helix conformation and globular structure with melting temperature at 55/56 °C and showing irreversible thermal denaturation (Falconer et al., 2010). Vincenzi and colleagues (2014b) have recently characterized the functionality of the main wine chitinase and they have individuated the optimum of activity at pH 6 and 30°C.

Thaumatin like proteins

The class of proteins most represented in white wines belong to the Thaumatin like proteins (TLP) family (PR-5). They even represent the ~ 50% of proteins in some chardonnay wines (Sauvage et al., 2010). Their antifungal activity is associated to the capacity of forming transmembrane pores on the cell wall of fungi as demonstrated for their homologous proteins, osmotins (Datta et al., 1999). TLP are the widest family of proteins in wine with MW ranging between 19 – 24 kDa (Tattersal et al., 1997; Waters et al., 1996; Van sluyter et al., 2009; Sauvage et al., 2010; Marangon et al., 2014). Tattersal and co-authors characterized a 24 kDa TLP isoform in grapes, which was named *Vitis Vinifera* Thaumatin-Like 1 (VVTL1), and found that the accumulation of this protein in the berry paralleled that of sugars (Tattersal et al., 1997). More recently VVTL1 has been identified as the most common proteins in white wines (Vincenzi et al., 2011). They are characterized by higher surface hydrophobicity compared to chitinase (Marangon et al., 2010), although it has been demonstrated to differ among the TL-proteins form (Marangon et al., 2009; 2014). Experiments with Differential Scanning Calorimetry (DSC) showed thermal stability between 56 to 62 °C (Falconer et al., 2010; Marangon et al., 2014). In addition, in contrast to chitinases and invertases some isomers of VVTL1 manifested reversibility after thermal unfolding (Falconer et al., 2010). The crystal structures of three different TL-proteins has been recently solved revealing the predominance of β-sheet conformation distributed in three different domains. Only few segments of α-helix in the II domain was observed. The presence of disulphide 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.

Lipid transfers proteins

Although in minor extent, also the presence of lipid transfers proteins (LTPs) was detected in white wines (Marangon et al., 2009; Okuda et al., 2006). LTPs are able to transfer lipids

from mitochondria to artificial liposomes and for this reason they were called lipid transfer proteins (Kader et al., 1996). These proteins have shown antifungal activity, affecting the permeability of fungal spores even if the mechanism of action is still not fully clarified (Cammuet et al., 1995; Cheng et al., 2004). Recently, the antifungal mechanisms of LTPs were associated to the inhibition of α-amylases (Zottich et al., 2011; Diz et al., 2011). The wine LTPs possess high isoelectric point (around 9) and a molecular weight ranging from 9-12 kDa (Jaeckels et al., 2013). The crystal structure of numerous LTPs were resolved revealing typical α-helice bundles and eight conserved cysteine that form four disulphide bonds (Guerbette et al., 1999; Salcedo et al., 2004; Jaeckels et al., 2013). These proteins have been indicated as responsible for allergic reactions in humans (Pastorello et al., 2003; Salcedo et al., 2004).

Table 2. *The characteristics of the main white wine proteins.*

	Invertases	**β-glucanases**	**TLPs**	**Chitinases**	**LTPs**
Theoretical mass (kDa)	71.5	37.5	20-25	24-28	9-12
Theoretical IEP	4.6	7.06	4.76	5.38	9
Melting temperature (°C)	81	N.A.	56-62	55	N.A.
Class	Transferase	PRP 2	PRP 5	PRP 3	PRP 12

Protein instability in white wines

The aggregation of proteins in wines lead to the formation of colloidal suspensions that scatter the light causing undesirable turbid appearance (fig.5). Such development of haze and sediments in the wine is seen as visual defect for consumer, although it has no effect on the organoleptic properties of wine. The haze formation is primarily due to protein unfolding in the bottled wines under bad conditions of storage or transport (Pocock et al., 2006; Waters et al., 2005). Among the wine proteins chitinases and TLPs, are primary involved in this phenomenon (Waters et al., 1992; 1996), although other proteins such as β-glucanases have been associated to haze formation (Esteruelas et al., 2009; Sauvage et al., 2010). However, these latter are much less abundant compared to chitinases and TLPs

and for this reason they have been not extensively studied. In addition, although it is still not demonstrated yet for wine, also some LTPs seemed to be involved in the haze formation in beer (Limure et al., 2009).

Fig. 5 *Unclouded white wine and turbid white wine (Van Sluyter et al. 2015)*

All these proteins, being devoted to the plant defence, are very resistant to proteases, low pH, ethanol and ionic strength, thus persisting into the wine after vinification (Bayly & Berg 1967; Feuillat et al., 1980; Murphey et al.1989).

In the recent past, haze formation was reported to be a two-stages process. The first stage includes protein denaturation and unfolding caused by temperature fluctuations occurring during storage of the bottled wine. The second stage seems to involve the formation of colloidal aggregates, which remain in suspension forming visible haze (Dufrechou et al., 2010). However, some experimental evidences led to hypothesise of the occurrence of three different stages: (i) protein unfolding and exposition of hydrophobic sites, (ii) self-aggregation of unfolded proteins by hydrophobic attraction and (iii) growth of protein aggregates due to cross-linking processes with the other wine molecules (Van sluyter et al. 2015). Although the presence of proteins is essential, it seems that they alone cannot fully explain haze formation in wines. Indeed, this problem is considered as a complex phenomenon, which involves also non-proteinaceous compounds (Batista et al., 2010; Pocock et al., 2007; Marangon et al., 2010; Waters et al., 1993, 1994,1995; Esteruelas et al., 2009, 2011; Dufrechou et al., 2015).

The first candidates are the phenolic compounds given their well-known ability to bind the proteins. Since the beginning of the research on wine hazing, many authors have suggested the existence of an interaction between grape proteins and phenolic compounds (Koch and Sajak, 1959; Bayly et al., 1967; Somers et al., 1973). In particular, Bayly and Berg (1967) ascribed to the presence of phenolic compounds the different protein instability results obtained from wines with similar total protein concentration. Waters and colleagues (1995); and more recently Esteruelas and colleagues (2011), found that natural wine haze aggregates contained polyphenolic compounds, suggesting an involvement in the haze formation of these compounds. Among them it has been identified a relative good amount of caffeic acid (0.052 % w/w). Chagas and colleagues (2016 – *in press*), confirmed that caffeic acid as major no-protein compound in some Moscatel wine precipitates, suggesting an involvement in the haze formation. Therefore, they studied the contribution of this phenolic compound and its ester of tartaric acid (caftaric acid) on haze formation. However, they concluded that these phenolic compounds are not correlated to the formation of turbidity in wine. Notwithstanding controversial results regarding the role of polyphenols in wine haze formation, the interaction of these compounds with wine proteins has been largely demonstrated (Waters et al., 1995; Siebert et al., 1996, 1999; Batista et al., 2010; Marangon et al., 2010; Gazzola et al., 2012). Usually, reversible tannin-protein interactions involve non-covalent forces such as hydrogen bonding, Van der Waals forces and, especially, hydrophobic interactions (Poncet-Legrand et al., 2006; Siebert et al., 2006; Richard et al., 2006; Prigent et al., 2009). Conversely irreversible interactions are possible when covalent bonding are involved trough oxidation and quinone formation (Haslam et al., 1996). Non covalent interactions are however suggested as the main driving-forces of protein-tannin interaction in wine, because this generally occurs under non-oxidative conditions.

Sulphate has been indicated as a key factor needed for the protein haze formation (Pocock et al., 2007; Marangon et al., 2011a). Not only sulphate influences the electrostatic repulsion of proteins as modulator of ionic strength, but also promotes the protein salting-out because able to cleavage the hydrogen bonding between proteins and water molecules (Israelachvili, 1991; Marangon 2011a). Marangon and colleagues (2010) observed that a TL-proteins fraction purified from wine increased their propensity to form visible haze in the presence of reducing agents. They suggested that one of the factors influencing protein hazing could be the decrease, over time in the bottle, of the wine red-ox potential. More

recently it has been supposed that the sulphur dioxide added to wines could cleave a specific disulphide bridge between Cys140 and Cys 213 of a TLP isoform, thus allowing hydrophobic aggregation to occur (Marangon et al., 2014). The effect of SO2 on haze formation has been in some way confirmed by Chagas et al. (2016). They suggested that upon heating sulphur dioxide causes the formation of new disulphide bonds and that this novel assembly of proteins make them more sensitive to heat, leading to haze formation. However, to date, further studies are required to provide an integrated view of the role of SO_2 (and the redox potential) in the stability of wine proteins.

Along with proteins and polyphenols there is another colloidal family of compounds has been reported to influence haze formation, the polysaccharides. These compounds are constituted by the pectic polysaccharides deriving from the grape berry (i.e arabino galactani, rhamnogalacturonan) and compounds released by the autolysis of yeast (i.e. mannoproteins, invertase). There is conflicting documentation regarding the effect of these molecules on wine haze formation. Several papers reported that polysaccharides, especially those derived from the yeast cell wall (mannoproteins) have stabilizing effects toward protein aggregation (Waters et al., 1994; Moine-ledoux et al. 1999; Brown et al., 2007), although also a role as promoters of instability has been reported for these compounds (Mesquita et al., 2001). It is documented that polysaccharides carry negative charges at the wine pH and that they are able to interact by electrostatically with the other components present in the wine, including proteins (Vernhet et al., 1996). Such statement seems to be confirm by Esteruelas et al., (2009), which found together to proteins and polyphenols a small amounts of polysaccharides in the natural precipitates of Sauvignon Blanc (about 4%). A proteoglycan typical of the yeast cell walls (a 420kDa mannoprotein) has been reported to decrease the intensity of heat induced haze in wine as did also a fragment of yeast invertase (Moine-ledoux et al., 1999). To explain this fact, the inhibition of the growth of haze particles was proposed (Waters et al., 1993, 1994). Gazzola and co-authors (2012) found different behaviour of interaction with polysaccharides on wine proteins, demonstrating that peculiar TLP isoforms show distinct interaction with polysaccharides whereas no significant interaction occurs between polysaccharides and wine chitinase. Furthermore, Dufrechou et al. (2015) tested the effect of two different fractions of natural wine polysaccharides on wine protein aggregation at room temperature. They identified the two fractions as neutral mannoprotein and arabinogalactan and negatively charged rhamnogalacturonan II dimer (RG-II) and arabinogalactan and demonstrated that those

polysaccharides are able to interfere with the kinetic of protein aggregation, but not to prevent it. Pellerin and colleagues (1994) suggested also steric repulsion between polysaccharides and proteins. They investigated the effect of two proteoglycans, Arabic gum (from Acacia Senegal) and an arabinogalactan from apple, on BSA aggregation. The amount of Arabic gum necessary to inhibit protein aggregation was ten times higher than that needed for the apple arabinogalactan (Pellerin et al., 1994).

Finally, other minor factors have been showed as possibly involved in protein haze formation in wine. To my knowledge exist only one work reported organic acids as important contributors to reduce haze potential of proteins in wine (Batista et al., 2010). They studied L(+)tartaric, L(-)malic, citric, succinic and gluconic acids effect on a purified pool of Portuguese wine proteins (280 mg/L) at different pH values (from 2.8 to 3.8). They investigated the wines by heat stability test revealing that organic acids have always positive impact toward the haze formation. The mechanism proposed is related to the net negative charge of the acids at wine pH, which allows them to interact with proteins preventing their combination with the (X) factor (Batista et al., 2010). The same authors (Batista et al., 2009) evaluated the effect of different pH on the protein stability in model wine, highlighting two distinct behaviours. Haze formation increased at the highest pH value (pH 3,8), whereas at low pH value (pH 2,8) the visible haze was drastically lower, evidencing a strong pH-dependent correlation. Also the effect of ethanol concentration on wine protein stability was studied, but changes in its concentration within the wine range had no significant impact on haze formation (Sarmento et al., 2000; Pocock et al., 2006; Batista et al., 2010)

Solutions to protein instability in white wines

The main solution to face the problem of haze formation is the removal of proteins before bottling. To do it, generally the winemakers treated the wine with bentonite after fermentation. The bentonite it's a natural aluminium silicate mainly composed by montmorillonite (Gougeon et al., 2003). The substitution of aluminium with magnesium or iron provides the negative charge necessary for protein adsorption. The negative charge is balanced by different exchangeable cations, mostly sodium and calcium. In particular, their ratio distinguishes three different kind of bentonite: sodium, calcium and activated calcium bentonite. The first one are the main used in enology (Marchal et al., 1995). Although

bentonite is an efficient tool to stabilize the wine, it has been shown to not be specific for proteins (Lambri et al., 2010; Vincenzi et al., 2015). Furthermore, an enrichment in the concentration of ions and heavy metals was observed after the treatment of wine with the montmorillonite, and sometimes this content was not in compliance with the OIV normative (Catarino et al., 2008). The bentonite represents also an economical damage for the wineries. Indeed, beyond the low cost of bentonite (0.6 to 1€/kg), its usage causes the loss of wine volume due to the formation of sediments, which lead even a loss of 10% of total wine (Tattersal et al., 2001). Moreover, it is not recycled and doses employed to stabilize wines have been in constant augmentation during the last decade, increasing its negative effects. Therefore, basic and applied research are necessary to find valid alternative to bentonite fining.

In this context, the use of enzymes (i.e. proteases) to degrade the hazing proteins is finding great interest in the scientific community. It represents a potential efficient method to avoid haze formation with the preservation of the sensory proprieties and a minimum wine volume loss. Indeed, it can be an advantage because the products formed by the degradation of proteins can constitute a valid alternative of organic nitrogen for yeast, decreasing the exogenous contribution of this element and improving wine aroma. The difficulty is find an efficient protease able to work at wine pH and possibly without increasing the temperature. Marangon and colleagues developed an efficiently new method to obtain wine free from haze, which involves the use of a specific protease (Aspergilloglutamic peptidase). The enzyme requires the treatment of juice with high temperature (flash pasteurization 75°C per 1 minute) in such way the proteins were unfolded and available for the proteolytic activity (Marangon et a., 2012a). However, this method implies the consumption of energy and possible negative sensory effects due to the grape juice heating (Heatherbell et al., 1984; Lloyd et al., 2005). More recently Bennucci et al., 2014 tested a proteolytic enzyme (stem bromelain) extracted from the stem of pineapple plant as clarifying agent for white wine. They tested the enzyme at room temperature in laboratory scale both in free form and immobilized form (commercial chitosan beads). The method was able to reduce the potential haze by 70% after 24h of treatment.

Salazar and colleagues in the 2006, 2007 investigated the application of zirconia (i.e. zirconium oxide) for the removal of proteins in white wines. The zirconia is a metal oxide characterized by low corrosion potential, low thermal conductivity, hardness, and high

thermal and mechanical resistances (Manicone et al. 2007; Marangon et al., 2011b). To this reason it found applications as support material in many different fields, including medical sector (Chevalier 2006, Manicone et al., 2007). Its capacity to reduce haze coupled with the possibility to easily regenerate the material and reduce the acidity and metal ions in wine make it a very interesting candidate to replace bentonite for the stabilization of wine (Marangon et al., 2011b).

An interesting alternative can be related to the use of polysaccharides, which should interfere with hydrophobic driven interactions of proteins (Dufrechou et al., 2012). Some researchers have tested the capability of carrageen and pectin on the protein stability in wine (Marangon et al., 2012b), others have investigated the ability of yeast mannoproteins to stabilize wine proteins (Schmidt et al., 2009).

Carrageenan is extracted from red seaweed and is currently used in the clarification of beer (pasini 2005). It has been tested in wine with good success, however its removal from wine could represent a problem (Marangon et al., 2013; Cabello-Pasini et al., 2005).

Another interesting adjuvant with potential implication in reduce the haze formation is the chitosan. The chitosan is a copolymer of the glucosamine obtained by the deacetylation of chitin (Shahidi et al., 1999.; Kasaai et al., 2009). In particular, the fungi chitosan of *Asperigillus Niger* has been approved from OIV for the oenological use (OIV-OENO 336B-2009). At the begging the chitosan has been applied for the clarification of fruit juice, including grape juice, with optimum effects both in term of clarification effects (Chartterjee et al., 2004) and organoleptic sensory properties of the treated fruit juices (Soto-Peralta et al., 1989). Recently the chitosan has been successfully applied in the stabilization of beer (Gassara et al., 2015), but scarce information is known as potential application in wine.

Separation and Characterisation of grape and wine proteins

An in depth study of the mechanisms of haze formation implies the isolation of single proteins in order to investigate their behaviour, stability and functionality. Currently, chromatography techniques are the commonly employed to separate and isolate single components, and this is obviously true also for the grape and wine proteins (Van Sluyter et al., 2009; Marangon et al., 2009; la Bourse et al., 2010). Before protein purification it is important the preparation of the sample. Grape juice and wine have low amount of proteins and contain numerous substances (i.e. polyphenols, carbohydrates, salts, and so on) that may interfere during the separation/purification steps (Curioni et al., 2008). Moreover, during extraction of the protein fraction from the medium (i.e. grape juice or wine), they can undergo possible structural alteration of their native state, affecting their behaviour. Therefore, the correct application of purification methods that do not affect protein structure is needed when their functionality has to be studied.

Dialysis against water is a well-known method to remove small compounds without the denaturation of proteins (Le Bourse et al., 2010) and coupled with ultrafiltration allows also the concentration of the sample in a single step.

Currently, ion exchange (IEC) and size-exclusion chromatography (SEC) are much used as pre-fractionation steps for the isolation of grape and wine proteins because these techniques normally do not result in protein denaturation (Van sluyter et al., 2009). While SEC separate the molecules on the basis of their molecular weight, IEC is based on the reversible electrostatic interaction of proteins with the stationary phase employed for the separation. The electrostatically bound proteins are usually eluted from the matrix by a continuous or stepwise salt gradient such as NaCl. Both strong and weak cation and anion exchange can be used. Cation exchange allows the separation of proteins at a pH close to the that of the grape juice or wine (pH 3) (Ferreira et al., 2002). Hydrophobic Interaction chromatography (HIC) allows to separate the grape proteins that differs in hydrophobicity and was developed as a second step for purification after IEC to obtain several grape isoforms of chitinases and Thaumatin Like Proteins, achieving high degree of purity (Marangon et al., 2009). Affinity chromatography (AC) is also a used as a second step after ion exchange chromatography to isolate proteins with the ability to be bound by a specific ligand present in the matrix. Wine mannoproteins can be isolated using this technique with a concanavalin A (ConA, a lectin with affinity for mannose) column (Waters et al., 1993;

Marchal, 1996). Gel filtration chromatography was also used as second step to isolate an arabinogalactan-protein from grape berries (Saulnier et al., 1989). However, it was also set up as single purification step by Esteruelas et al. (2009) to isolate the natural haze protein in white wine.

Sodium Dodecyl Sulfate-Polyacrilamide Gel Elettrophoresis (SDS-PAGE) is widely used to assessed protein composition of several matrices and to check the purity of the components separated by chromatography (Marangon et al., 2009; Van Sluyter et al., 2009). However, electrophoresis can be used also as a preparative method to collect grape and wine proteins. Vincenzi and co-authors (2003), proposed a one-step purification of wine proteins by electroendosmotic preparative electrophoresis (EPE) using native (N-PAGE) and SDS-PAGE systems. With this system, the proteins were electrophoretically separated in a cylindrical gel, then eluted through a capillary by exploiting the electroendosmotic flow generated during electrophoresis. Finally, capillary electrophoresis (CE) has been applied to the grape proteins (Luguera et al., 1998; Dizy and Bisson, 1999). CE can separate proteins according to their isoelectric point, molecular mass, or charge/mass ratio. An evolution of this method is represented by capillary gel electrophoresis (CGE). Rodríguez-Delgado et al. (2002). This technique applies the same separation principle of SDS-PAGE in which the largest proteins were retarded, but with the advantaged to enhance the speed of separation, employ low sample volumes and automatic qualitative and quantitative evaluation of protein peaks. (Guttman, 1996).

In vitro plant cell cultures

In vitro cell cultures consist in a series of methodologies aimed to retain and develop part of plants (cells, tissues and organs) on growth media with a well-defined nutrient composition and under sterile conditions. In particular, plant cultures rely on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, or (less commonly) roots can often be used to generate a new plant on culture media by giving the required nutrients and plant hormones (Vidyasagar, 2006).

The induction of plant cell culture is a simple procedure. The basic protocols were well established by the 1960s when some researchers proposed a universal medium for *in vitro* plant cultivation (Murashige and Skoog, 1962) and the numerous modifications that have followed (Gamborg et al., 1968). These media are aqueous solutions in which macroelements (Ca^{2+}, Mg^{2+}, K^{+}, $NO3^{-}$, $NH4^{+}$, $PO4^{2-}$, $SO4^{2-}$, Cl^{-}, chelated iron), microelements: (B, Cu, Mn, Mo, I, Zn, Co), sugar (glucose or sucrose), vitamins and hormones are included (Schenk and Hildebrandt, 1972). In addition, to obtain a correct development of cultures other factors are important such as the pH, which is generally adjusted to sub-acids values (5.6-5.8), the temperature (25±1°C), the photoperiod and the agitation (for cellular suspensions) (Dörnenburg and Knorr, 1995). In the reality, these protocols have been far less than universally successful. Indeed, many species and cultivars do not respond to existing protocols. Too often the published protocols for particular species are not reproducible by others. This indicates that every cultivar needs specific composition of the medium and/or the correct set-up to be propagated. Normally, the explant used for the *in vitro* propagation is naturally contaminated on its surface (and sometimes also inside) with microorganisms. Thus the selection of healthy explants and a superficial treatment with antimicrobial chemicals (i.e. ethanol) or bleaching are always required (Akin-Idowu et al., 2009). The tissues are then usually placed on solid or in liquid culture media. The composition of the media, particularly the nitrogen and hormones content, have a key role in the explant growth. An excess of auxin often results in a proliferation of roots, while an excess of cytokinin induces the development of shoots. An equilibrium of both usually produce an unorganised cell growth, commonly named *callus* 6 (Akin-Idowu et al., 2009). In order to allow the growth or to alter the morphology of the culture the *calli* are sliced off and moved to new fresh media (sub-cultured). While in the

early days of tissue culture application it was assumed that the plants produced would be clonal (that is, genetically uniform), it is now well recognised that this is not always the case (Sahijram et al., 2003). Somaclonal variation has perhaps been the greatest threat to the widespread application of micropropagation and other tissue culture techniques to agricultural crops (Peschke and Phillips, 1992).

Grape Cell Cultures

Numerous grapevine cultures were successfully developed in the recent years. The best results were obtained with MS and B5 culture media (Murashige and Skoog, 1962; Gamborg et al., 1968) with low content or absence of kinetin as preferred by dicotyledonous species. The use of cell cultures allows to investigate at the laboratory scale the metabolic processes taking place within the plant.

The accumulation of secondary metabolites in grape such as polyphenols, especially the phytoalexin (i.e. stilbenes), were strongly associated to the plant defence (Hammerschmidt et al., 1999; Schnee et al., 2008), and human health (Siemann et al., 1992). Therefore, many researchers employed *in vitro* grapevine cultures with the aim to study the pathway of formation and accumulation of these metabolites in the plant (Franceschetti et al., 2005; Zamboni et al., 2015; Ferri et al., 2009; Laura et al., 2007; Liu et al., 2010; Escoriaza et al., 2013). Moreover, some researchers evaluated also the effect of elicitors such as chitosan (Laura et al., 2007; Ferri et al., 2009), and pathogens (Escoriaza et al., 2013), highlighting an increase in stilbene production as a defensive response of the grapevine against pathogen and exogenous stresses.

Grape cell cultures were also used to investigate the expression of plant genes during ripening (Sharathchandra et al., 2011). In particular, the authors applied the proteomic approach to demonstrate the advantage of *in vitro* culture to study the intricate regulatory and signalling networks responsible for berry development and ripening. As described in the previous paragraph, *in vitro* cultivation of plant tissues allow to produce a whole plant from the cell culture and this can be also adopted for grapevine cell culture. The interest in this application concerning the possibility to produce free-pathogen plant material (Das et al., 2006).

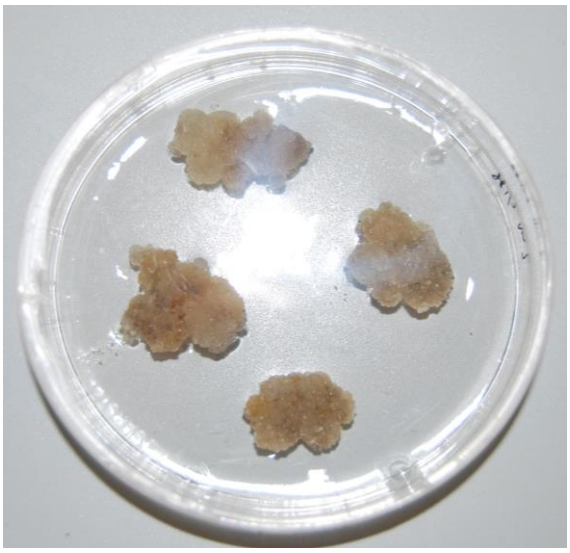

Fig. 6 *Callus of Manzoni Bianco grape pulp*

Recent history of haze-forming proteins

The research interest in the investigation of wine proteins has increased in the recent years because these components are the main responsible for haze formation.

It was established that the protein responsible for haze formation are the grape PR-proteins and in particular chitinases and Thaumatin-Like Protein (TLPs). However, their isolation and characterization has been always a difficult task because the complexity of the proteins mixture in wine (Monteiro et al., 2001; Vincenzi et al., 2011). Proteins haze formation in white wines is a very complex phenomenon, which basically was explained as a phenomenon occurring in two step: (i) protein unfolding and denaturation and (ii) aggregation of the denatured proteins into particles (Van Sluyter et al., 2015). Both the steps are poorly understood and the reason of their occurrence are also unknown. Therefore, it is fundamental to study the behaviour and the structural characteristic of these haze-forming proteins in order to clarify the mechanisms of wine haze formation. Temperature is considered a main factor for haze development due to its effects on thermal denaturation of the wine proteins. When heated in solution TLPs and chitinases present different aptitude to unfold. Studies by Differential Scanning Calorimetry showed that chitinase are generally less resistant (unfolding at around 56°C) than TLPs (unfolding at around 61°C) Moreover, once folded chitinase did not return to its original structure, whereas the TLPs showed reversible unfolding (Falconer et al., 2010).

This irreversible denaturation promotes chitinase aggregation and subsequent precipitation. Dufrechou et al., (2013) evaluated the stability of four grape proteins, including invertase, TLP and two chitinases at different condition of pH using spectroscopy techniques and Small Angle X-ray Scattering (SAXS). The authors concluded that only the chitinases showed significant modification in their conformation associated to a significant exposure of the hydrophobic sites. This conformational variation is enough to destabilize their native state. Most studies seem to indicate that TLPs are not implicated in phenomenon of aggregation. However, recent research showed that TLPs are present in the haze precipitates of wine (Vincenzi et al., 2011; Esteruelas et al., 2009). This contrasting results are most likely explained by the presence of different isoform of TLPs. It has been recently demonstrated the existence of TLP isoforms characterized by unfold/refold aptitude after heating and cooling, whereas other isoforms showed irreversible denaturation and consequent aggregation (Marangon et al., 2014). The X-ray structure of three TLP isoforms has been solved revealing two stable isoforms and one (called 4JRU) more ready to unfold. Al the molecular level, these differences are attributable to the conformation of a single loop and to the amino acid composition of its flanking regions.

TLPs are characterized by three domains with a predominant β-sheet conformation, and a cleft between domain I and II. The disulphide bridges stabilize the structure with a conserved spatial distribution throughout the protein (Marangon et al., 2014). Sulphate has been individuated as possible candidate needed for the onset of protein instability in all the stages (Pocock et al., 2007; Marangon et al., 2011a). Haze-forming proteins have a net positive charge at wine pH, and this can prevent protein aggregation due to electrostatic repulsion. The presence of other charged ions in solution such as salts or metals (ionic strength) decrease the repulsion forces among proteins, increasing hydrophobic interaction-driven aggregation (Israelachvili et al., 1991). Moreover, the sulphate act also as ionic kosmotrope that interacts with water suppressing the hydrogen bonding between the molecules of water and the proteins. This fact promotes the salting out of protein from an aqueous solution and induces protein aggregation (Marangon et al., 2011a).

As suggested by the recent advance in the research, the unfolding (or partial unfolding) of wine proteins due to high temperature, ionic strength, sulphates and pH can lead to the exposure of the hydrophobic sites, generally buried inside the protein (Marangon et al., 2011a, Dufrechou et al., 2010, 2012, 2013). Once exposed, these sites promote hydrophobic interactions which may occur among proteins (self-aggregation), but also

among proteins and other hydrophobic compounds such as tannins. As a matter of fact, the tannin-proteins reactivity in wine has been observed (Marangon et al., 2010). For TLPs this mechanism has recently been elucidated (Marangon et al., 2014). The unstable TLP has a hydrophobic region in the exposed loop protected by a disulphide bridge. The cleavage of this S-S bridge expose that hydrophobic region. On the other hand, stable TLP does not precipitate because a more hydrophilic region.

The presence of salts or sulphates can promote the aggregation of proteins neutralizing the natural forces of repulsion between proteins with similar charge (Marangon et al., 2011a). However, this is conceivable for the chitinase, which form typically large metastable aggregates (> 1µm) at the wine condition, but not for TLPs that tend to produce small metastable aggregates (<150 nm), which are too small to generate visible haze (Sauvage et al., 2010; Marangon et al., 2011a). Therefore, the current model of haze formation includes a third step for haze formation related to the growth of protein aggregates. This imply the involvement of other (X) factor/s that interacts with proteins leading to an enlargement of natural protein aggregates initially formed.

Beyond sulphate, polyphenols are good candidates to interact with proteins, most likely promoting cross-linkage of protein pre-existing aggregates to modulate the final haze formed. This is then the stage 3 of the last proposed model of interaction (Fig. 7) (Marangon et al., 2011a).

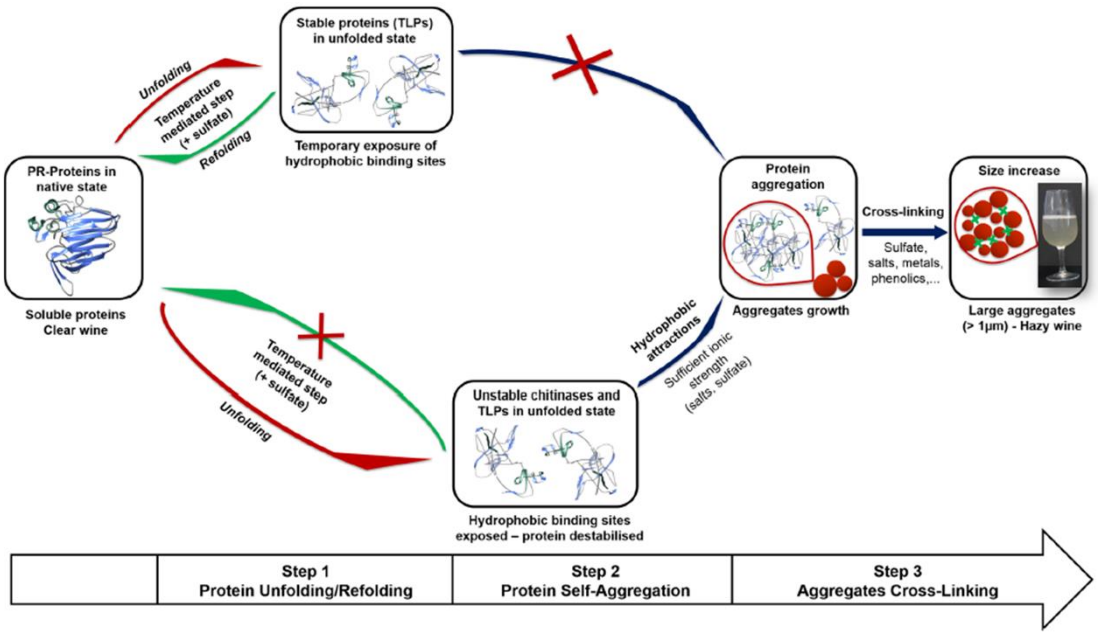

Fig. 7 Three-step revised mechanism of haze formation in white wines (Van Sluyter et al., 2015)

Recently, sulphur dioxide has been indicated as factor that should trigger the onset of haze formation in wines. Sulphur dioxide is almost always employed by winemakers to prevent oxidation and microbiological spoilage. This antioxidant compound is a chemical that is able to affect the red-ox potential of the wine and then to strongly contribute to the onset of the reducing conditions occurring in the bottled wine. This low potential can therefore affect the disulphide bonds stability of the wine proteins. In this context, the possibility of protein modification due to the S-S bond reduction by sulphur dioxide upon heating has been reported (Chagas et al., 2016). However, to date, further studies are required to provide an integrated view of the role of SO_2 (and the redox potential) in the stability of wine proteins.

In conclusion, understanding the mechanisms of haze formation and the behaviour of haze-forming proteins are still far from being fully understood this imply a deep investigation of these points.

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RESEARCH OBJECTIVES

Research Objectives

The present work aims to progress in the understanding of protein interactions in white wine. It is well known that the proteins, especially chitinases and TLPs, undergo modifications in wine that lead to the formation of floccules able to scatter the light and precipitate. The most suitable strategy to solve this problem is the removal of haze-forming protein before bottling by bentonite treatment.

However, this treatment has several drawbacks, including the impairment of aroma.

It has been reported that the effect of bentonite on the aroma compounds is less when the proteins are absent. This would mean that some aroma compounds are removed by bentonite as result of their complexation with proteins, suggesting also an indirect effect of bentonite. However, this point is still not demonstrated at molecular level. In order to better clarify wine proteins and aroma compounds complexation, the first aim here presented was to investigate interaction between the main white wine protein (VVTL1) and some important fermentative aroma compounds (FAEE) (Chapter I).

The second objective of the thesis was to gain new information on the mechanisms involved in haze formation in white wines, this being a complex phenomenon that involves many factors which are still to be studied in depth. Hydrophobicity of proteins is a key factor for their interaction with other compounds that are characterized by hydrophobic character. For this reason, due to their hydrophobic character, tannins are important candidates in this phenomenon, but their role in the haze formation is not clearly understood. Since haze formation normally occurs after some time of permanence of the white wine in the bottle, it is conceivable that, in addition to possible changes in the structure and functionality of the wine proteins, also changes in tannins structure is involved. Actually, polyphenols are known to change in wine, mainly because they undergo polymerisation, and this polymerisation is affected by the presence of oxygen. Therefore, the question is: are changes in tannins involved in producing molecular species with an increased reactivity towards the proteins present in wine? To clarify this, point the second aim was to study via reconstitution experiments the reactivity of the tannins purified from wine at increasing times after bottling and to determine the effect of their oxidation on the reactivity with the proteins (Chapter II). The aim of the research was then to understand whether the tannins are able to affect the stability and reactivity of proteins

over time in bottled wine and which is the role of oxygen in this phenomenon (Chapter III).

The last objective of the research was that to find a systems enable to obtain grape proteins to be studied and in an immediate future to be labelled for spectroscopic analysis aimed to understand the fine mechanisms of interactions with different ligands. The possibility to gain information on the structure and the behaviour of wine proteins implies a very high purity of these proteins. It is well known that proteins undergo structural modifications after crushing grape berries due to the presence of interfering substances (polyphenols, heavy metals, proteases…). Thus, find a suitable system to isolate the proteins in a form as pure as possible is crucial. The aim was thus to develop an in vitro system to obtain pure grape proteins by the induction of cell callus from the pulp of white grape berries in which labelling of the proteins by suppling labelled N is possible. The expression and production of these labelled proteins is expected to be a very useful tool for their characterisation at the molecular level and mainly to study protein-ligand interactions by spectroscopy (Chapter IV).

In conclusion, at the end of the research it is expected to gain new information on (i) the extent of a possible interaction between wine proteins and aroma compounds, (ii) the role of tannins in the haze formation and their effects on the protein structure also at molecular level, (iii) the potential application of in vitro grape cell culture in the study of grape proteins.

CHAPTER I

SPECTROSCOPY REVEALS THAT ETHYL ESTERS INTERACT WITH PROTEINS IN WINE

Publication 1

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 analysed 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.

KEYWORDS

Aroma, Ethyl esters, Proteins, Spectroscopy, SRCD, VVTL1, Wine

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 (Lubbers, Charpentier & Feuillat, 1996; Lambri, Dordoni, Silva & de Faveri, 2013; 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 protein (TLP) 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 disulphide 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

analysing the 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.

Materials and methods

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 litres 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 metabisulphite (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 1M. 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 analysed by SDS-PAGE and reverse-phase HPLC. Ammonium sulphate 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 sulphate 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 a Jupiter C18, 10μm, 300Å, (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.

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 analysed using the CDApps software (Hussain et al., 2015).

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.

Results and discussion

Among the protein that "survive" the fermentation of white musts, grape TLPs are the most abundant class and this is confirmed also for the Manzoni Bianco variety here used (Berndt et al., 1999). The TLP purified from Manzoni Bianco juice was identified as the grape VVTL1 (gi: 520729528, UniProtKB: o04708), whose X-ray structure (PDB: 4L5H) and some physio-chemical parameters have been recently determined (Marangon et al., 2014). Therefore, the relative abundance in wine and the knowledge of the molecular structure of this protein make it a good candidate for the study of the interaction between wine proteins and aroma compounds. Among the huge number of wine aromatic molecules, ethyl esters, which are produced by yeast during fermentation (Polášková, P., Herszage, J., & Ebeler, E. (2008) have been recently identified as those most affected by the presence of proteins during bentonite treatments to wines, suggesting that they are potentially largely involved in the interactions with protein (Vincenzi et al., 2015). As the ethyl esters are spectroscopically transparent in the far-UV region compared to proteins, their association with VVTL1 is not easy to detect. For this reason, the effect induced by the addition of these potential ligands on the circular dichroism (CD) spectrum of VVTL1 was analysed. CD is widely used to identify the secondary structure of protein as well as to evaluate the binding of ligands and their effects on proteins stability and structure (Siligardi & Hussain, 2015). To mimic the conditions found in wine, experiments were performed in a model wine solution at pH 3.2 containing tartaric acid and ethanol. The far-UV SRCD spectrum of VVTL1 at 25°C showed two positive peaks at about 195 and 230 nm attributed to the amide bond (π-π^*) and to the aromatic side-chains (La Tyr and Bb Trp bands according to Platt notation (Platt, 1949), respectively, and a negative peak at 210 nm due to the n-π^* transition of the amide bond (Fig. 1). The addition of aroma esters slightly modified the two amide bands at 195 and 210 nm, but not the positive bands at 230 nm likely associated to the aromatic side-chain π-π^* transitions (Fig. 1).

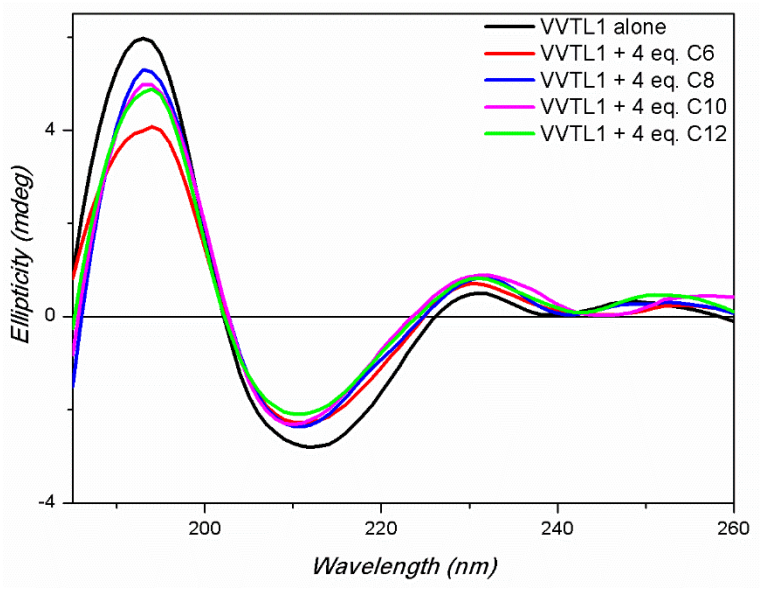

Figure 1. *Far-UV SRCD spectra of VVTL1 in model wine solution. 0.400 mg/mL of VVTL1 measured with B23 module B, in presence (4 eq.) and absence of ethyl esters. VVTL1 (black), VVTL1+**C6** (red), VVTL1+**C8** (blue), VVTL1+**C10** (magenta) and VVTL1+**C12** (green). Integration time 1s, 0.02cm cylindrical cell filled with 40 µL solution, monochromator slit widths 0.500 mm (1.2 nm bandwidth).*

From CD data, the estimation of the protein secondary structure content for VVTL1 alone and in presence of ethyl esters, was conducted using the Diamond CDApps software (Hussainet al., 2015) containing CONTIN algorithm (Sreerama, & Woody, 2000). The results revealed that the apo form of VVTL1 at 25°C was characterized by a dominating content of β-sheet, in accordance with the X-ray structure (Marangon et al., 2014). The addition of esters slightly increased the content of ordered structure (Table 1). These data suggest that the secondary structure of VVTL1 was barely affected by the presence of the ethyl esters and that the protein-aroma interaction did not involve aromatic residues as no spectral changes upon addition of ethyl esters were observed at 230 nm. To confirm this hypothesis, the fluorescence spectrum of VVTL1 in the 300-420 nm region was measured in the absence and presence of four molar equivalents of ethyl esters. As shown in Fig. 2, the addition of ligands did not significantly alter the emission spectrum of the tryptophan, indicating that ethyl esters did not interact with the side-chain of Trp residues or modify the protein structure around these residues

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

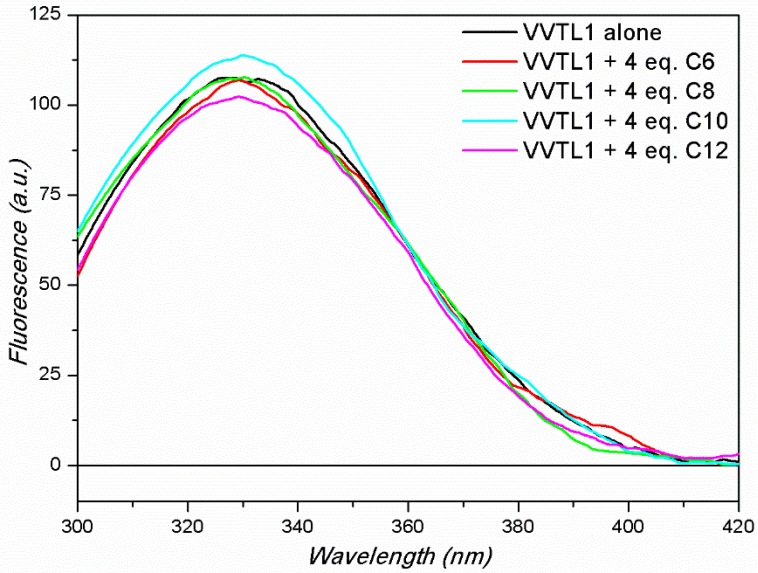

Figure 2. *Fluorescence emission spectra of VVTL1 with and without 4 molar equivalents of ethyl esters. VVTL1 (black), VVTL1+**C6** (red), VVTL1+**C8** (light blue), VVTL1+**C10** (magenta) and VVTL1+**C12** (green). TLP concentration was 0.021 mg/mL in model wine, pH 3.2; esters were 3.95 µM in the same buffer. Spectra were recorded at 25 °C.*

To verify that the ethyl esters are able to bind to VVTL1, both UV-photo and thermal denaturation assays were carried out. It has been reported that the high UV photon flux of B23 beamline (at Diamond Light Source, a synchrotron of third generation) can denature proteins (Hussain et al, 2012a). Indeed, a significant decrease of the amount of secondary structure upon light irradiation using the intense far-UV radiation has been observed for proteins with a significant content of α-helical and/or β-sheet structure (Clarke & Jones, 2004). Like thermal denaturation, UV denaturation varies from protein to protein, being also affected by the presence of ligands. The mechanism of photo-denaturation is likely to include free radical damage to the photosensitive tryptophan and tyrosine residues (Grosvenor, Morton, & Dyer, 2010). Experiments were conducted to check the sensitivity of the studied protein to photo-denaturation, as such phenomenon is likely to occur. This can also be used to assess the effect of both environmental parameters (solvent, detergent, pH, …) and interactions with molecules on protein stability and to qualitatively evidence these interactions (Hussain, Jàvorfi & Siligardi 2012b; Longo; Hussain & Siligardi, 2015).

The UV-denaturation assay was performed by measuring twenty consecutive repeated scans in the 185-260 nm far-UV region of VVTL1 in WMS in the presence of ethyl esters. An example is given figure 3 for ethyl-hexanoate. By keeping constant protein concentration, volume of the solutions and instrument parameters (cell pathlength and photon flux of the irradiating incident light beam), the observed SRCD spectral changes were indicative of protein photo-denaturation effects. This was better illustrated by the plot of the SRCD intensity at single wavelength versus the number of scans. The fitting of the experimental data using 1st or 2nd order exponential equations (CDApps) can be seen as the relative different rates of UV-denaturation between VVTL1 with and without ligands (Fig. 4A). The rates of UV-denaturation of the VVTL1 in the presence of ethyl esters are different from that of VVTL1 alone and this result is unambiguously indicative of an interaction between the protein and the esters. Ethyl octanoate (**C8**) appeared to increase the VVTL1 photo-stability more than the other esters (**C6, C10** and **C12**) with the following order: **C8**>**C10**>**C12** ≈ **C6**.

This was also consistent with the estimated amount of secondary structure (β-sheet and unordered) content from each of the 20 repeated consecutive SRCD scans that were different for VVTL1-**C8** and VVTL1-**C10** complex compared to that of VVTL1 (Fig. 4B).

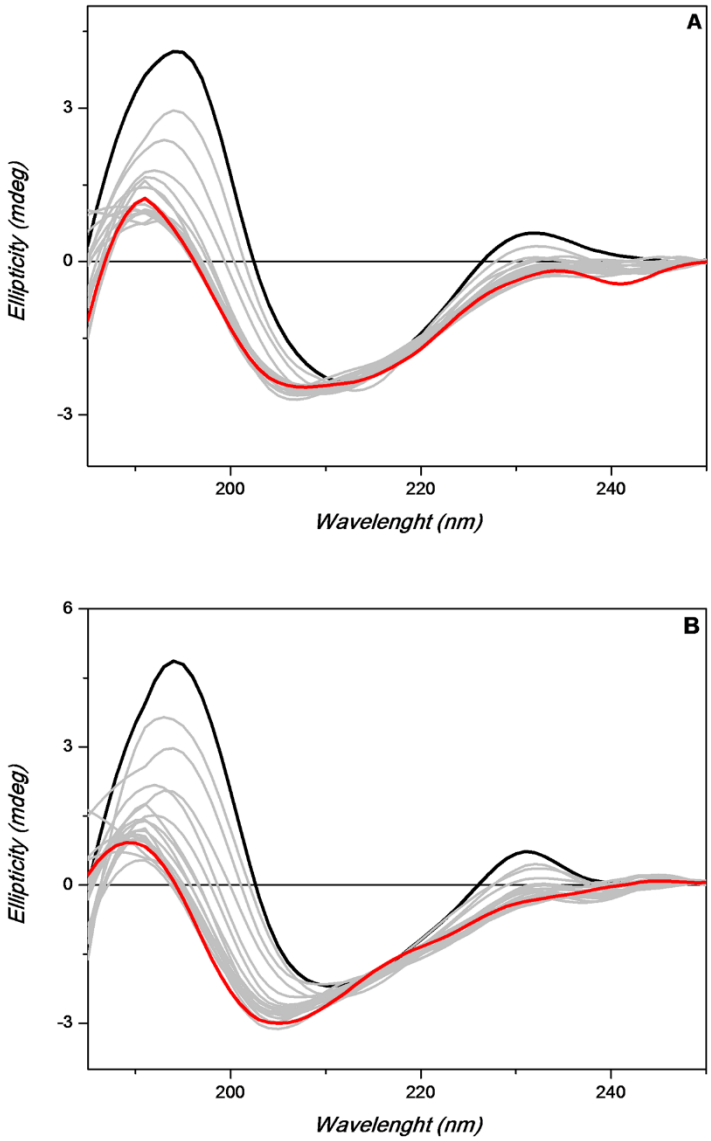

Figure 3. (**A**) *Twenty repeated consecutive SRCD scans of VVTL1 (0.400 mg/mL) in model wine, pH 3.2, measured with B23 module B. The solid black line indicates the first scan and the solid red line the 20th scan. Integration time 1s, 0.02 cm cylindrical cell filled with 30 µl solution, monochromator slit widths 1.000 mm (2nm bandwidth), and synchrotron ring current 268 mA.* (***B***) *Twenty repeated consecutive SRCD scans of VVTL1-ethyl hexanoate (0.400 mg/mL, molar ratio 1:4) in model wine, pH 3.2, measured with B23 module B under the same experimental conditions described for* (***A***).

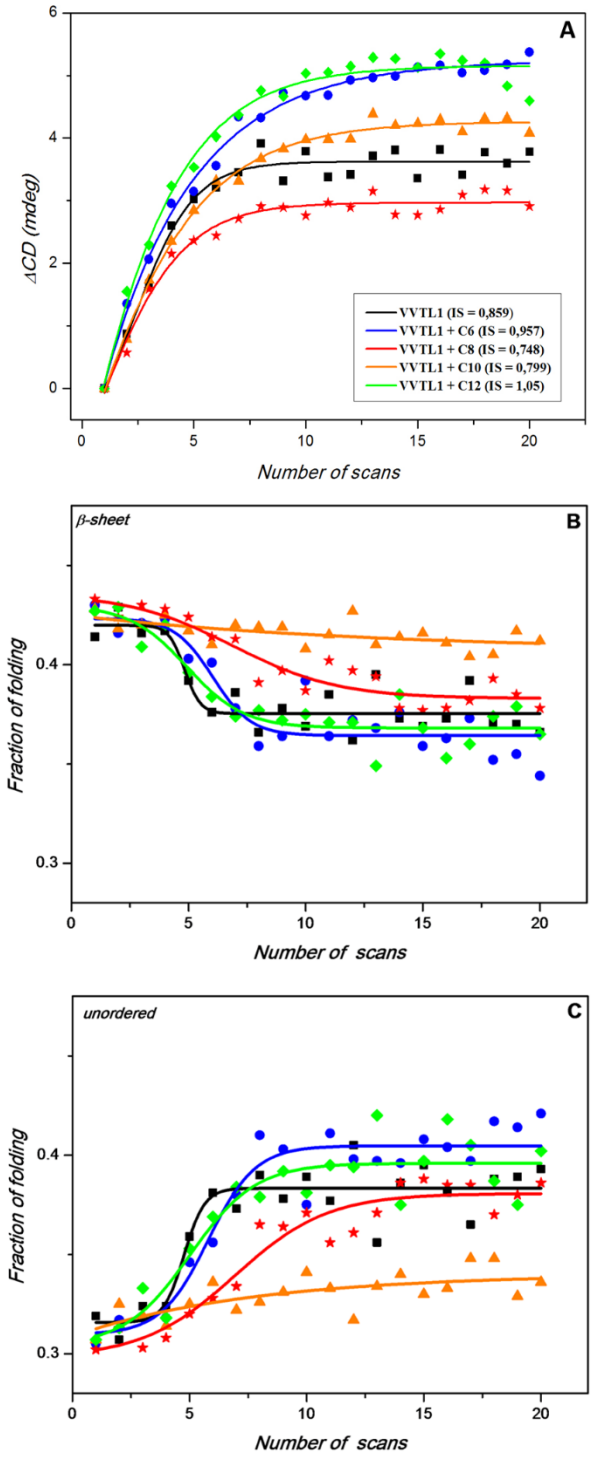

Figure 4. *(**A**) Plot of the SRCD signal at 195 nm for VVTL1 (black), VVTL1+**C6** (blue); VVTL1+**C8** (orange); VVTL1+**C10** (red) and VVTL1+**C12** (green) versus number of scans. For each sample IS is the value of the initial slope of the curve. (**B**) and (**C**) Plot of secondary structure content for VVTL1 (black), VVTL1+**C6** (blue); VVTL1+**C8** (orange); VVTL1+**C10** (red) and VVTL1+**C12** (green) determined with CONTINLL (Siligardi & Hussain, 2015) of CDApps (Hussain et al., 2015) from SRCD data versus number of scans. For all measurements VVTL1 concentration was 0.400 mg/mL, ethyl ester concentration was 0.07 mM, protein/ester molar ratio was 1:4. The SRCD spectra (unsmoothed) were measured using B23 module B, integration time 1s, 1.000 mm (2nm bandwidth) using a cylindrical Suprasil cell of 0.02 cm pathlength (Hellma) filled with 40 µl of solution.*

In particular, in presence of ethyl octanoate (**C8**) a very slight UV-denaturation of VVTL1 was observed. On the other hand, in presence of **C6** ester the highest change in the secondary structure of VVTL1 was detected (Fig. 4A-B), suggesting that this ester may act as a negative effector for protein stability.

The temperature study of VVTL1 in the 5-70°C temperature interval (Fig. 5A) revealed a drastic change in the SRCD spectrum in the 55-60°C range. In particular, the positive band at 230 nm disappeared whilst a negative band at about 203 nm appeared. This corresponds to a strong decrease of the β-sheet structure and subsequent increase of unordered conformation as shown in Fig. 6A. Analysis of the SRCD spectra recorded at 20°C before and after the temperature study showed that the native VVTL1 (N) thermally treated at 70°C (U) did not return to its native conformation N, but to a non-native conformation (N_N) with less CD intensity than that of the N protein. These data confirm previous results, which indicated partial reversibility for the (thermal) denaturation of grape TLPs (Falconer et al., 2010).

The behaviour of the SRCD spectra of the VVTL1 after heating and cooling was strongly affected by the addition of ethyl ester (such as protein spectra with ethyl hexanoate reported in Fig. 5B).

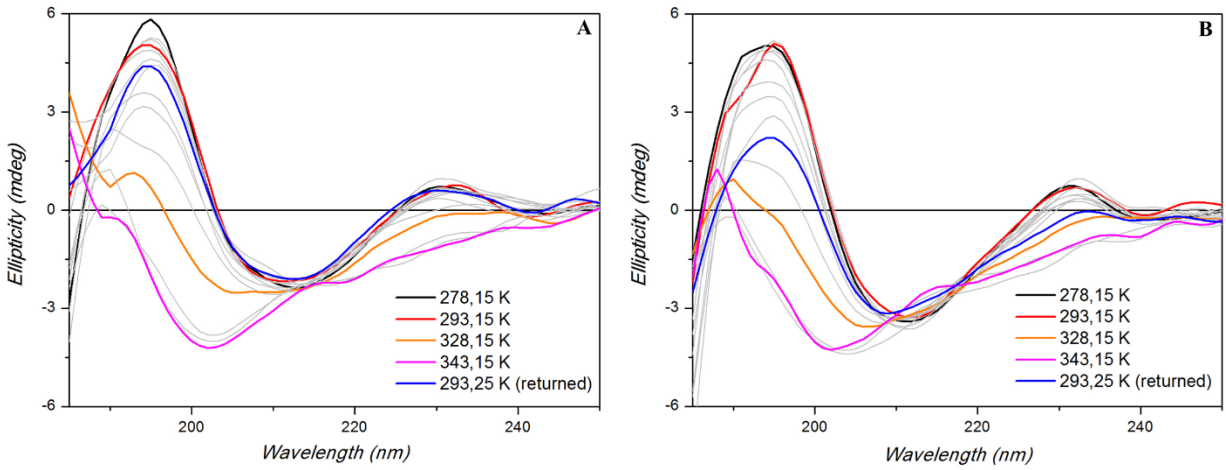

Fig. 5. **(A)** *Far-UV SRCD spectra of VVTL1 in model wine measured with B23 module B at different temperatures. VVTL1 concentration was 0.400 mg/mL; integration time 1 s, 0.02 cm cylindrical cell (40 µl), monochromator slit widths 0.500 mm (1.1nm band width (bw)) to eliminate the effect of UV denaturation.* ***(B)*** *Far-UV SRCD spectra of VVTL1 in model wine in presence of four molar equivalents of* ***C6*** *measured with B23 module B at different temperatures. VVTL1 concentration was 0.400 mg/mL; integration time 1 s, 0.02 cm cylindrical cell (40 µl), monochromator slit widths 0.500 mm (1.1 nm bw).*

In the presence of **C10**, **C12** and **C6** ethyl esters, the CD signal of the non-native conformation (N_N) further decreased in the following order: **C10** < **C12** < **C6** confirming the negative contribution of ethyl hexanoate to protein stability. On the contrary, the addition of **C8** ester increased the reversibility of the thermal denaturation process, which was consistent with the reduced susceptibility to photo denaturation observed in the UV-photo induced denaturation experiments in the presence of ethyl octanoate. As show in Figure 5B, the melting curve of VVTL1 alone can be fitted by a double Boltzmann equation, suggesting the presence of two equilibria, with melting temperatures (T_m) of 270.35 and 325.95 K. The addition of ethyl esters modified both equilibria. In particular, the presence of the **C6** ester caused the disappearance of the first equilibrium and the melting curve was fitted by a single Boltzmann equation with T_m of 322.85 K. On the other hand, in the presence of **C8**, **C10** and **C12** esters both equilibria were retained but with an increased T_m for the first equilibrium (Table 1). These results confirm the interaction of ethyl esters with grape VVTL1, although a different effect of each ester in terms of protein-stability is highlighted.

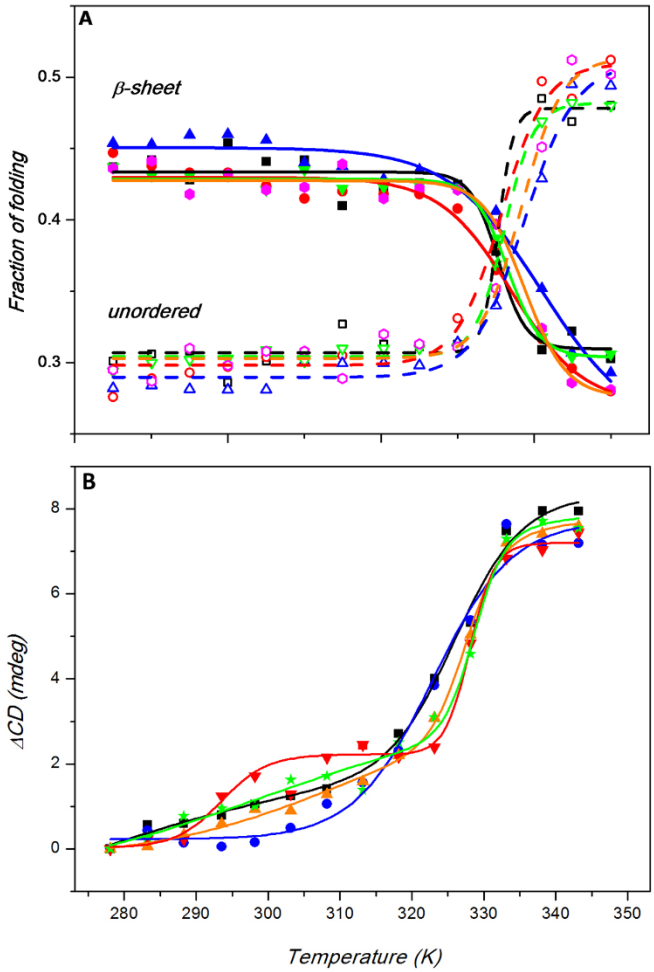

Figure 5. (***A***) *Plot of secondary structure content for VVTL1 (black), VVTL1+**C6** (blue); VVTL1+**C8** (orange); VVTL1+**C10** (red) and VVTL1+**C12** (green) determined with CONTINLL (Siligardi & Hussain , 2015) of CDApps (Hussain et al., 2015) from SRCD data versus temperature.* ***(B)*** *SRCD melting curves for VVTL1 (black), VVTL1+**C6** (blue); VVTL1+**C8** (orange); VVTL1+**C10** (red) and VVTL1+**C12** (green) obtained plotting the ellipticity at 195 nm versus temperature. For all measurements VVTL1 concentration was 0.400 mg/mL, ethyl ester concentration was 0.07 mM, protein/ester molar ratio was 1:4. The SRCD spectra (unsmoothed) were measured using B23 module B, integration time 1s, 0.500 mm (1.2 nm bw) using a cylindrical Suprasil cell of 0.02 cm pathlength (Hellma) filled with 40 µl of solution.*

Conclusions

The bentonite used for wine stabilization can directly remove some aroma compounds, thus affecting the organoleptic properties of wine (Lambri et al., 2013; Lubbers et al. 1996). However, also an indirect effect of bentonite treatments due to the removal of wine proteins, which may be complexed with aroma compounds, has been recently proposed (Vincenzi et al., 2015). The present work confirms this idea, by showing that a main wine protein, the Thaumatin-like Protein VVTL1, is able to bind ethyl esters of different chain length in a model system. Since fermentative ethyl esters substantially contribute to wine aroma, if the wine proteins interact with them, it is likely that removal of these latter by bentonite can negatively affect wine aroma. Therefore, the treatment with bentonite before fermentation (i.e. on the must, in which the aroma compounds produced during fermentation are absent) would be preferred in order to preserve wine quality.

Acknowledgements

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CHAPTER II

EVOLUTION OF TANNINS IN BOTTLED WINE AND THEIR REACTIVITY WITH PROTEINS

Publication 2

Evolution of tannins in bottled wine and their reactivity with proteins

Mattia Di Gaspero, Diana Gazzola, Simone Vincenzi, Céline Poncet-Legrand, Andrea Curioni and Aude Vernhet

In preparation

Abstract

The effects of oxygen on tannins ability to react with wine proteins were studied via reconstitution experiments in model wine. Tannins were purified eight months after bottling from full-filled bottles and half-filled bottles. In the last case the amount of air in the headspace was higher. In theory, this process allowed to speed up the evolution of tannins, or favour other mechanisms, and thus different chemical structures would have formed within the wine. Contrarily to that purified few days after bottling, the tannins obtained after eight months showed interaction with proteins already at room temperature, especially the ones from half-filled bottles. These data suggest an evolution of tannins reactivity with proteins in bottle, highlighting a fundamental role of oxygen. The study allowed to clarify the mechanisms of protein precipitation in bottled white wines.

Keywords

White Wine Tannins, Wine Proteins, Oxygen, Room Temperature, Protein Precipitation, DLS, DSC

Introduction

Residual grape proteins persist in the wine after bottling causing the rejected appearance of haze and sediments in bottled wine. Among them some grape pathogenesis-related (PR) proteins, particularly Chitinases and Thaumatin-like proteins (TLP), have been shown to be the main components involved in haze formation (Waters et al., 1992). Currently, to avoid haze formation, these proteins are removed from wine before being bottled (Waters et al., 2005). Although, many studies claim that the protein instability depends only on the total protein content (Waters et al., 1991), more recently the involvement of other compounds present in wine has been demonstrated. One of them was individuated, from the beginning, as the polyphenols (Mesquita et al., 2001). In particular, the tannins because they have, as main ability, the capacity to bind and precipitate proteins. However, the specific mechanism of wine proteins/tannins interaction in white wines is not fully understood. It has been shown that hydrophobicity is the main driving force for the interactions, probably enhanced by hydrogen bonding (Haslam et al., 1996). Other interactions such as ionic bonds between positively charged groups of proteins and negatively charged hydroxyl groups of polyphenols could play a minor role (Asano et al., 1982). As a matter of fact, phenolic compounds were found in the natural wine protein precipitate demonstrating their involvement in the haze formation (Esteruelas et al., 2009), but how they evolve over the time in the bottle and how this process could affect wine stability is still not well known. To this purpose, we have investigated the interactions of total and some purified wine proteins (i.e. VVTL1 and chitinase) with white wine tannins purified at different stages and conditions. The experiments were performed using Dynamic Light Scattering (DLS) and Differential Scanning Calorimetry (DSC). While DLS is able to monitor the kinetics of aggregation occurring into the wine at room temperature, the DSC allow to observe the effects of these pool of tannins on the thermal stability of some pure haze-forming proteins.

Materials and methods

Tannins and proteins purification

The proanthocyanidins were purified from fined Sauvignon Blanc few days after bottling (control, T0), eight months after bottling from full-filled bottles (T8) and half-filled bottles (T8 OX) with a modified procedure previously reported by Marangon et al., (2010). Briefly, batches of 1.5 litres of Sauvignon Blanc were loaded on glass column packed with 40 g Sephadex LH-20 previously equilibrated with 20% ethanol. The column was washed with 1050 mL of 20% ethanol in order to remove all non-bounded molecules. The tannins were eluted using 600 ml of ethanol 95% to remove low-molecular weight tannins and finally with 600 mL of 60% acetone. Fractions were evaporated at 30°C by rotavapor and freeze-dried. Before starting the experiment, the proteins have been removed from wine by 100 g/L of bentonite with the aim to hinder possible interactions between tannins and proteins.

Total wine proteins were purified from an unfined Pinot Gris wine produced in Conegliano (Italy). The wine was filtered at 0.2 µm, concentrated by ultrafiltration (3kDa) and finally dialyzed against distilled water (3). Once freeze-dried the sample was dissolved in citrated buffer pH 3 and loaded on SP-sepharose column. The flow through was removed in order to eliminate all uncharged molecules. The composition was checked by SDS-Page (Laemmli, 1970).

The VVTL1 and chitinase were purified from Manzoni Bianco grape juice in according to the modified procedure previously described with some modifications (Van Sluyter et al., 2009). Ten litres of wine were treated with 4 g/L PVPP (Sigma-Aldrich, Milan, Italy) and 1.5 g/L charcoal (Sigma-Aldrich, Milan, Italy) for 24 h at 15 °C in order to remove most of the polyphenols. The wine was then filtered with GF/A filters (Whatman, Kent, U.K.) and finally at 0.45 µm with cellulose acetate membranes (Sartorius AG, Göttingen, Germany). The pH was adjusted to pH 3 with HCl before loading on a S-Sepharose column (5 × 14 cm) (Pharmacia, Uppsala, Sweden), which was equilibrated in trisodium citrate buffer 30 mM pH 3.0. The column was washed with two volume of the same buffer and eluted with a gradient of NaCl from 0 to 1 M. The eluted fractions were checked by SDS-Page and the fractions containing the desired proteins (i.e. VVTL1 and chitinase) were separately pooled together. The protein pool obtained were diluted in 1.25 M ammonium sulphate and further purified by hydrophobic interaction chromatography (HIC) on a phenyl-sepharose HP

column (GE-Healthcare). The column was equilibrated with 1.25 M ammonium sulphate in 50 mM

sodium citrate, pH 5.0, and the proteins eluted with a 120 min linear gradient up to 100% 50 mM sodium citrate, pH 5.0. The peaks obtained with this second step were dialyzed against water (3.5 kDa MWCO) and freeze-dried. The purity and identity of the fractions were assessed by RP-HPLC and SDS-PAGE.

Dynamic Light Scattering (DLS) measurements

Analyses were performed via reconstitution experiments in model wine (5g/L of tartaric acid, 40 mM NaCl, 12% Ethanol) adjusted to pH 3,2 with NaOH 1N. A tannin/protein ratio of 0.3125, i.e. 320 mg/L of total wine proteins and 100 mg/L of wine tannins, was used. The samples were separately dissolved in model wine, centrifuged 15000g for 15 minutes and measured for 30 minutes at room temperature by Dynamic Light Scattering using a Malvern Autosizer 4700 (40mW He−Ne laser, λ = 633 nm, APD detection, Malvern Instruments, UK). Then the components were mixed and measured for 24 h at room temperature. The incident beam laser was arranged to an angle of 90°C to perform the experiments. Each value obtained from the experiments are the result of 10 subruns. Finally, the assays were performed in triplicate on independently prepared solutions. The hydrodynamic diameter of particles (Dh) was calculated applying the Stokes-Einstein equation to the diffusion coefficient (D) and assuming spherical shapes. It is expressed as a function of the temperature and viscosity of solvent:

$$D = kT/(3\pi\eta Dh)$$

where k is the Boltzmann constant, T the absolute temperature, and η the solvent viscosity. In addition, another important parameter coupled to Dh was calculated, the Polydispersity Index (PI). This index provides information in term of size distribution of particles in the sample. In this case the PI (ranging from 0 to 1) was calculated as the variance of the distribution of particle size (CUMULANT method). It is important to keep in mind that, for polydisperse suspensions (PI > 0.3), cumulant analysis gives a Dh value weighted according to the scattering intensity, which is in favour of the largest particles.

Differential Scanning Calorimetry (DSC) and potential haze formation

DSC experiments were performed with 200 mg/L of purified proteins and 62.5 mg/L of the pool of tannins. The blank was prepared with the tannins solution alone in a tartaric buffer (5 $g \cdot L^{-1}$) at pH 3.2, adjusted with NaOH 1N and 40 mM NaCl as ionic strength. After several preliminary trials, DSC assays were adapted to a heating rate of 1°C/min from 5°C to 80°C with a MicroCal VP-DSC (GE Healthcare, UK). Software Origin 7.0 was used for results analysis. The samples and the buffer were degassed by stirring under vacuum before injection. All the experiments were done twice.

The potential haze generated upon DSC experiments were estimated measuring the absorbance before and after thermal assay at 540 nm by UV-VIS spectrophotometer (Ultrospec 2100 pro, GE Healthcare, UK).

Results and discussions

Wine proteins

The proteins used in the present work represent the two most important grape proteins involved in the protein instability of white wines. In particular, they are an isomer of VVTL1 (I/4L5H) and a chitinase members of class IV of PR-proteins (Marangon et al., 2014; Vincenzi et al., 2014). VVTL1 showed higher resistance to heat, displaying higher melting temperature (62°C) and a poor ability to form haze (Marangon et al., 2014). Conversely, chitinase is one of the most unstable wine proteins (T_m = 55°C) and more implicated in the haze formation (Falconer et al., 2010). The SDS-PAGE profile reported in Fig.1 shows the purity of the two proteins and a pool of total wine proteins used for the DLS experiment. The latter showed the classic protein profile for a white wine (Sauvage et al., 2010; Dufrechou et al., 2012), with a majority of Thaumatin Like Proteins (TLPs) and chitinases in the range of 18-30 kDa (Fig.1). It was also present a noticeable band around 66 kDa likely corresponding probably to grape invertase (Sauvage et al., 2010; Dufrechou et al., 2012).

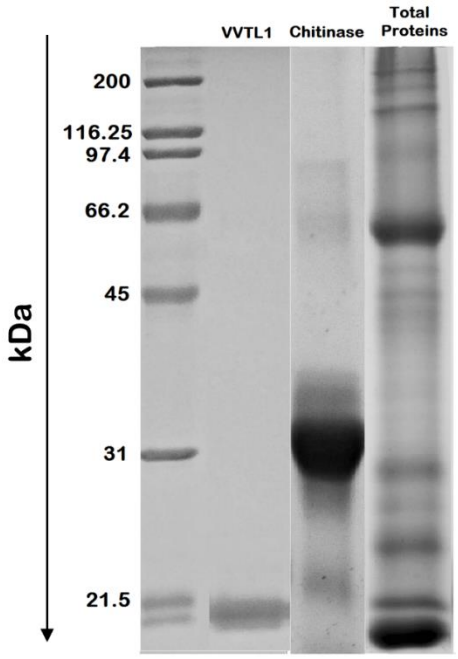

Fig. 1 *SDS-Page protein profile under reducing conditions for the protein band around 20 kDa (VVTL1), the protein band around 31 kDa (chitinase) and the pool of total wine proteins. The molecular weight standards are on the left.*

Kinetics of aggregation at room temperature (25 °C)

It is supposed that procyanidins could naturally evolve in the bottled wines by means of several agents that pull together (i.e oxygen, light, heavy metals…) (Monagas et al., 2005; Gambuti et al., 2013). The scope of the present work was to evaluate the evolution of tannins in bottled white wines and their capability to react with proteins, particularly the incidence of a major concentration of air in bottles, which should speed up the oxygen driven evolution of them over time. The hypothesis is that a major concentration of air in the headspace (obtained filling the bottles just to a half) can enhance the processes able to modify the chemical-physical properties of tannins.

Before to test proteins and tannins together, some preliminary test with these wine components were separately performed. A protein solution about three time more concentrated than that used for the experiments (900 mg/L) was tested at room temperature for few days. In parallel, 100 mg/L of more evolved tannins (T8 OX), which were those showing the major reactivity with proteins, were monitored overnight at 60°C. These trials were performed in order to assess whether the wine components alone were able to aggregate and scatter the light. The results showed that both the proteins and the tannins

did not show increments in term of light scattering and particles size (data not reported), suggesting that these macromolecules were not able to interact and aggregate alone at these conditions.

DLS is a non-destructive method to measure the aggregation of colloids in aqueous media (Pecora, 1985). In the case of wine protein instability, DLS has been employed to investigate the protein aggregation since the early stage of the phenomena (Marangon et al., 2011). Therefore, to better understand the role of tannin evolution over time in bottled wines, the effects of the tannins purified at three different stages on wine proteins were studied via reconstitution experiments in model wine. To mimic the real condition existing in wine, the experiment was initially performed with 160 mg/L of total proteins and 50 mg/L of tannins. However, the detected intensity of light scattering was really too low, particularly for the control (T0). For this reason, the concentrations of both tannins and proteins were doubled, leaving all the other parameters constant. Thus, 320 mg/l of total wine proteins and 100mg/L of tannins were monitored by DLS (T/P 0.3125) at room temperature in model wine composed by 5 g/L tartaric acid, 12% ethanol and 40 mM NaCl, adjusted to pH 3.2.

Surprisingly, observing the figure 2A we can note that the tannins after 8 months showed an increase in light scattering already at the begging of the experiment. Indeed, upon the mixing of protein and tannins solutions the value of light scattering was higher than those expected and increase over time. These data suggest that both the tannins T8 and T8 OX immediately interact with wine proteins, especially the latter ones, highlighting a very rapid interaction. These findings were even more consistent observing the trend over the 24h as showed in figure 1.

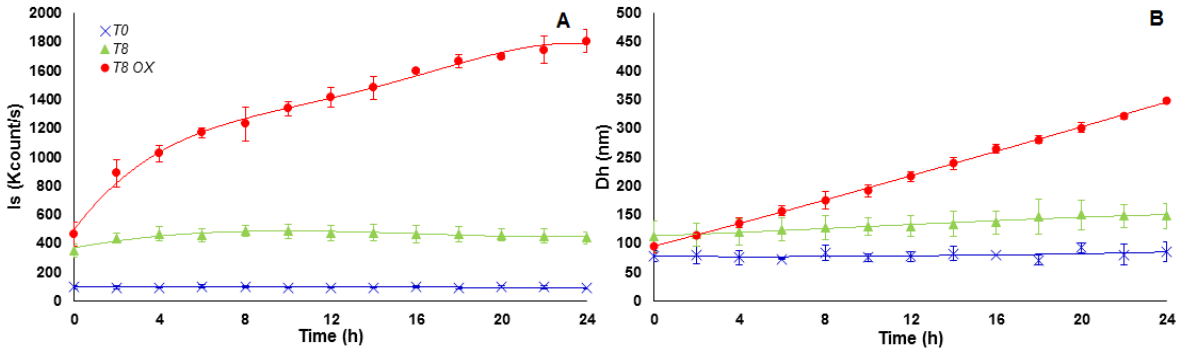

Fig. 2 ***A)*** *the Intensity of light scattering (Is) and* ***B)*** *the hydrodynamic diameter of particles (Dh) for total wine proteins in presence of tannins T8OX (red line), tannins T8 (green line), tannins T0 (blue line) in model wine monitored by DLS for 24 h at room temperature (25°C). The concentration of protein was 320 mg/L*

and tannins 100 mg/L with 0.3125 T/P ratio. The curves were illustrated plotting the mean of the measured values (three repetitions) every two hours.

The tannins purified few days after bottling showed a stable intensity around 90 Kcount·s^{-1} and a particle size lower than that of limit of detection of the instrument (in any case the measured values were included in the graph in order to compare the results). Tannins T8 after the mixing with proteins gradually increased the *Is* until 8 hours (~ 490 Kcount·s^{-1}) and then decreased, whereas the tannins purified from half bottles significantly increased the light scattering (~ 1800 Kcount·s^{-1}) and it suggested to continue over the measurement period (Fig. 2A). Also the calculated mean diameter followed the same trend. However, while the T8 tannins produced particles whose size remained almost constant during the 24 h of the experiment (70 nm), a significant increasing diameter was noted for the particles formed with the tannins of the half-filled bottle (fig. 2B). These result indicate that the reactivity of tannins with protein increases over time and that it is enhanced by the presence of a greater air content in the bottle.

Differential Scanning Calorimetry experiments

In order to better understand the effect of these tannins on wine protein stability, the thermal stability of two different hazing wine proteins, VVTL1 and chitinase, were tested by DSC after tannin addition. Before tannin addition some preliminary assays on the protein alone were performed. Generally, the thermodynamic model of unfolding for a protein considers only two transition stages, which includes the folded and the unfolded state of proteins (Cooper et al., 1999). Sometimes in particular conditions of the buffer the proteins can be better fitted with more than two transition stages (Barone et al., 1995;Michnik et al., 2005). This was the case of VVTL1, which did not show the classic two-state thermodynamic model, but rather it showed two small shoulders on the lower side of a main peak. This behavior suggested that the protein, at least in these experiment conditions, did not possess a classic two-state thermodynamic model (Cooper et al., 1999), rather showing the best fitting with a three-stage transitions (Fig. 4). The melting temperature of the main peak was around 60 °C, slightly lower compared to that described in a previous work (Falconer et al., 2010).

Probably this is due to the different composition of model wine, in fact, to mimic more closely the real wine conditions, our model solution contains also 40 mM of NaCl that provides 40 mM of ionic strength.

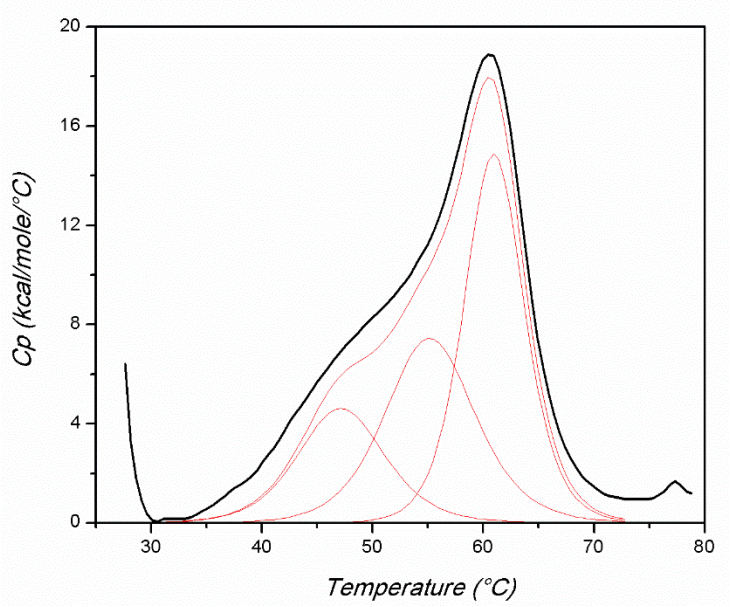

Fig. 4 *Thermogram scan for VVTL1 fitted with three transition stages. Protein concentration was 0.2 mg/ml. Scan rate 1 °C/min from 25° C to 80°C in model wine buffer composed by 5 g/L tartaric, 12 % ethanol acid and 40 mM NaCl, pH 3.2.*

The addition of tannins, irrespective of the time (T0, T8, T8 OX), decreased the melting temperature and induced thermodynamic changes. The thermograms with tannins seem to show irreversible aggregation of the protein as suggested by the baseline distortion (Fig. 5A-B-C), which can affect the shape of thermogram and causes distort DSC transition, leading to final wrong estimation of thermodynamic parameters (Cooper et al., 2009). For this reason, we avoid to report the thermodynamic parameters and concentrate our efforts to discuss the differences emerging in term of melting temperature, which is a robust parameter able to provide thermal information. The stability of VVTL1 after T0 and T8 addition showed the similar results with a T_m around 58,2 °C. Despite DLS results, even tannins T0 seem to interact with VVTL1, showing the ability to potentially affect the protein stability upon heating. The addition of tannins T8OX affected the thaumatin even strongly, further decreasing the melting temperature down to about 57,8 °C. In addition, the presence of T8OX induced stronger aggregation effect as suggested by the exothermic peaks during the scan (Fig. 5C).

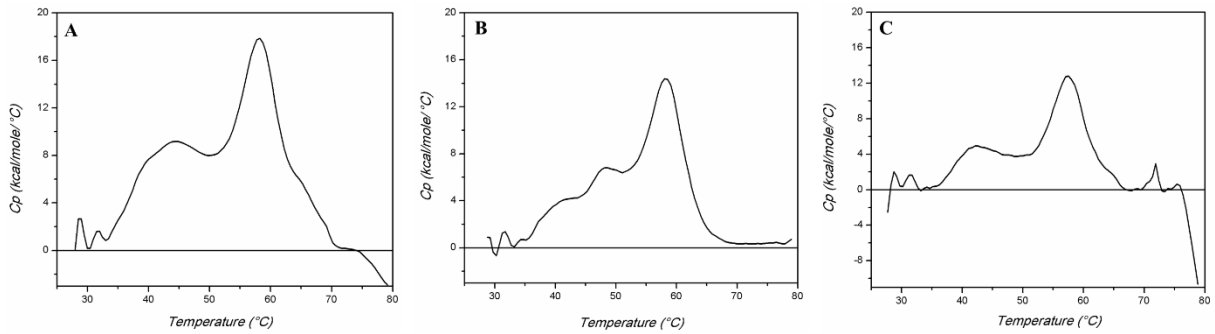

Fig. 5 *Differential Scanning Calorimetry thermograms for VVTL1 + Tannins T0 (**A**), VVTL1 + Tannins T8 (**B**) and VVTL1 + Tannins T8OX (**C**). Protein concentration was 0.2 mg/ml and tannin concentration 0.0625 mg/mL (T/P ratio = 0.3125). Scan rate 1 °C/min from 25° C to 80°C in model wine buffer composed by 5 g/L tartaric, 12 % ethanol acid and 40 mM NaCl, pH 3.2.*

On the contrary, chitinase revealed aggregation and precipitation already when tested alone (Fig. 6B). The chitinase thermograms showed slight shifts of the protein transitions after tannins addition. However, it was impossible to observe huge differences among the pool of tannins (Fig. 6A). The melting temperature assumes values around 50°C and did not change, irrespective of the added tannin. In addition, an exothermic trend is always revealed at the end of the thermograms, evidencing that precipitation occur. This might only confirm the major propensity of chitinase to denature upon heating in comparison to VVTL1 (Sauvage et al., 2010).

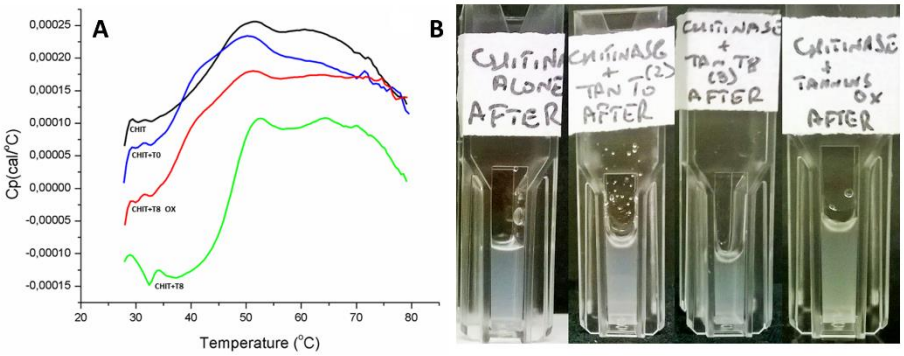

Fig. 6A) T*he raw thermograms of chitinase alone (black line) and after the addition of tannins T0 (blue line), tannins T8 (green line) and tannins T8OX (red line).* **6B)** *Visual assessment of samples after DSC analysis in the following order: chitinase alone, chitinase + tannins T0, chitinase + tannins T8, chitinase + tannins T8OX.*

In conclusion, the results with VVTL1 showed specific interactions between tannins and wine proteins. Only the tannins purified from half-filled bottles seem to have a major impact on proteins stability. On the contrary chitinase showed visible haze formation already when tested alone leading to a hard discrimination and impossible interpretation of the data.

Optic density at 540 nm before and after DSC experiments

The turbidity induced after DSC experiments were calculated measuring the absorbance at 540 nm before and after every DSC experiment (Table 1). This allows measuring the haze formed upon DSC experiment. Generally, values clearly above 0,02 a.u. indicate the presence of visible haziness (Pocock et al., 2003). While chitinase assumes always values much higher than 0,02 a.u. both with and without tannins (data not reported), VVTL1 showed net visible haze only after the addition of tannins T8 OX. VVTL1 alone did not show increase in haziness. After the addition of tannins T0 a little increase was observed, even if very close to 0,02. Tannins T8 assume values higher than tannins T0, slightly over 0,02. The most consistent results were obtained with tannins T8 OX, which showed net visible haziness assuming values significantly higher than 0,02. These data suggest a significant higher effect of these tannins compared to the others and confirm what observed by DLS and DSC.

Table 1. *The absorbance of VVTL1 in presence and absence of tannins after DSC measurement at 540 nm*.

SAMPLES	BEFORE	AFTER	SUBSTRACTION
VVTL1	0.019	0.022	0.03
VVTL1	0.026	0.019	N.A.
VVTL1 + T0	0.009	0.024	0.015
VVTL1 + T0	0.01	0.02	0.01
VVTL1 + T8	0.022	0.038	0.016
VVTL1 + T8	0.02	0.035	0.015
VVTL1 + T8 OX	0.038	0.115	0.077
VVTL1 + T8 OX	0.04	0.105	0.065

Conclusions

To correctly face the problem of wine protein stability it is necessary to know more in deep the mechanisms involved in protein interactions and precipitations in wine. One of the most important candidate to interact with wine proteins are the polyphenols, especially the tannins because the well known ability to bind the proteins. Protein-tannin interactions in wine are considered to be driven mainly by hydrophobic forces (Marangon et al., 2010). Moreover, the exposition of hydrophobic sites, which are normally buried in the internal part of protein, can be increased by high temperature, promoting hydrophobic-driven interactions (Marangon et al., 2010; Siebert et al., 1996). The work here presented seem to be in agreement with this affirmation. Indeed, the tannins purified from wine few days after bottling (T0) suggested to interact with VVTL1 upon heating, whereas they did not show effect on the total wine proteins at room temperature. Moreover, the data suggested that both the tannins purified from wine eight months after bottling (T8 and T8 OX) are capable to interact with wine proteins already at room temperature, especially those deriving from the bottle left half-filled. It can be then concluded that the tannins can undergo some oxygen driven evolutions in the bottled wine allowing them to enhance the reactivity with proteins over time. However, the oxygen concentration in white wine are generally very low because it is normally produced under non oxidative conditions (sulphur dioxide, contact with yeast…). Therefore, it is conceivable that these phenomena can occur in bottled wine, but with the involvement of a slow kinetics of interactions.

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CHAPTER III

EFFECTS OF POLYPHENOLS ON SECONDARY STRUCTURE OF VVTL1 WINE PROTEIN

Publication 3

Effects of polyphenols on secondary structure of VVTL1 wine protein

Mattia Di Gaspero, Paolo Ruzza, Rohanah Hussain, Simone Vincenzi, Diana Gazzola, Barbara Biondi, Giuliano Siligardi and Andrea Curioni

In preparation

Abstract

Polyphenols are important constituent of wines largely studied for their antioxidant propriety and human health benefits. The interest in white wines is much related to the capability of these compounds to bind proteins. The aim of the present work is gain additional information in understanding polyphenols and wine proteins interaction. We investigated the effects of single procyanidins B1, B2, tannic acid, quercetin, rutin as well as those of a total wine tannin extract on the conformational properties of VVTL1 (PBD file 4L5H), a major protein isolated from Manzoni Bianco wine. The secondary structure of VVTL1 was barely affected by the presence of the tested polyphenols. However, the presence of polyphenols negative affect the protein thermal transition behavior. Moreover, the investigation of protein in the near-UV region revealed an involvement of the aromatic side-chain residues of protein. Finally, the procyanidin B1 showed a higher propensity to interact with VVTL1 compared to the procyanidin B2. The results present in this work demonstrate the existence of interaction between VVTL1 and such polyphenols, highlighting a significant modification of the native state of protein.

Keywords:
Polyphenols, VVTL1, SRCD, Interactions, Secondary Structure, Wine

Introduction

Polyphenols are important secondary metabolites of higher plants largely studied for their intrinsic protection against oxidation and health benefits (Han, Shen, & Lou, 2007). These compounds, which are present in the grape skins and seeds, are released during winemaking contributing significantly to the organoleptic proprieties of wines (O'Connell & Fox, 2001). Moreover, the importance to study polyphenols is also due to their capacity to bind proteins. Food processing operations like clarification and fining treatments, which consist of adding proteins to beverages in order to precipitate tannins to improve haze stability and taste (Siebert, Carrasco & Lynn, 1996; Siebert, 1999), take advantage of the capability of polyphenols to interact with proteins (Ricardo-da-Silva, Cheynier, Souquet, Moutounet, Cabanis, & Bourzeix, 1991; Sarni-Manchado, Deleris, Avallone, Cheynier, & Moutounet, 1999; Sarni-Manchado, Cheynier, & Moutounet, 1999; Maury, Sarni-Manchado, Lefebvre, Cheynier, & Moutounet, 2001; Cosme, Ricardo-da-Silva, & Laureano, 2008). It is documented that flavan-3-ols, which are an important class of grape polyphenols mainly composed by (+)-catechin and (-)-epicatechin units, interact with salivary proteins conferring the characteristic mouth feeling of wine known as astringency (Pascal, Poncet-Legrand, Cabane, B., & Vernhet, 2008). In addition, flavan-3-ols seemed to participate to haze formation in white wines after interaction with proteins that limit beverage quality (Marangon, Vincenzi, Lucchetta & Curioni, 2010). Proteins involved in polyphenols/protein interaction have been shown to be prolin-rich and/or with a high basic residues content in protein relatively large and hydrophobic and having a conformational open and flexible structure (Simon et al., 2003; Richard et al., 2005). These interactions may be either reversibly or irreversibly and do not necessarily lead to precipitation. In reversible interactions, non-covalent forces such as hydrogen bonding, hydrophobic bonding and van der Waals forces are involved. NMR studies indicated the presence of stacking interactions of the phenolic rings with the proline residues and the stabilisation of the complexes through hydrogen bonding between the H acceptor site of the adjacent peptide bond and the hydrogen atom of the phenolic hydroxyl (Murray, Williamson, Lilley, & Haslam 1994). More recently, isothermal titration calorimetry studies showed that the interaction of flavanols with poly-L-proline involves both entropic (associated to hydrophobic effect and conformational changes) and enthalpic (attributed to

hydrogen bonding) phenomena (Poncet-Legrand, Gautier, Cheynier, & Imberty, 2007). The latter is prevalent in the case of flavanol monomers and the former in that of polymers. In irreversible interactions, covalent bonds are formed between proteins and by-product of polyphenols oxidation (Ozdal, Capanoglu, & Altay, 2013). On the other hand, Haze formation is attributed to some PR-proteins withstanding the vinification and persisting in the wine after bottling (Waters et al., 2005). Current model of haze formation suggests that the unfolding of these proteins, mainly due to inappropriate storage conditions, causes the exposition of the hydrophobic sites making the wine proteins more susceptible to aggregation. These aggregates gradually grow up through processes of cross-linking triggered by interactions with other wine components, especially polyphenols (Marangon, et al., 2010; 2011). However, to date there has not been a thorough study between polyphenols and single wine protein at molecular level. To this purpose our work wants to investigate the effects of some single polyphenols, including procyanidins B1 and B2, tannic acid quercetin, quercetin rutinoside (rutin) as well as those of a wine total tannin extract, on the secondary and tertiary structure of Vitis Vinifera thaumatin-like protein (PDB-file 4L5H), isolated from Manzoni Bianco wine, were studied by Synchrotron Radiation Circular Dichroism (SRCD). Tannic acid belongs to the family of hydrolysable tannins that are represented by molecules composed of simple phenolic acids such as gallic acid or hexahydroxydiphenic acid esterified to polyols, typically glucose (Figure 1). Proanthocyanidins B1 and B2 are dimers composed by (−)-epicatechin and (+)-catechin that belong to the family of condensed tannins and represents a large majority of tannins present in grape and wines. They may differ by the nature of the constitutive units, by their degree of polymerization, and by the type of linkage (C4-C8 or C4-C6) from subunits (Cheynier, 2005). Proanthocyanidins B1 and B2 are characterized by a C4-C8 linkage and differ by the presence of a catechin in the lower unit of B1 chain. These molecules may exist in two different conformations: compact and extended. In the compact form, the two catechol rings B and E are faced together thanks to π-π stacking, while this intramolecular interaction disappears in the extended and the two rings are free (Cala et al., 2010). These conformations are strictly related to the medium composition and in hydro-alcoholic solution procyanidin B1 adopt a dominant compact conformation (92%), whereas procyanidin B2 manifests both extended and compact form (45:55 %) (Tarascou et al., 2006). Quercetin and rutin are flavonol compounds characterized by the 3-hydroxyflavan backbone. Flavonols in grapes exist only as 3-glycosides, whereas

the corresponding free aglycones can be found in wines, along with the 3-glycosides, because of acid hydrolysis that occurs during winemaking and aging (Castillo-Muñoz et al., 2010). It can be assumed from the above that the selected phenolic compounds are candidates to interact with VVTL1.

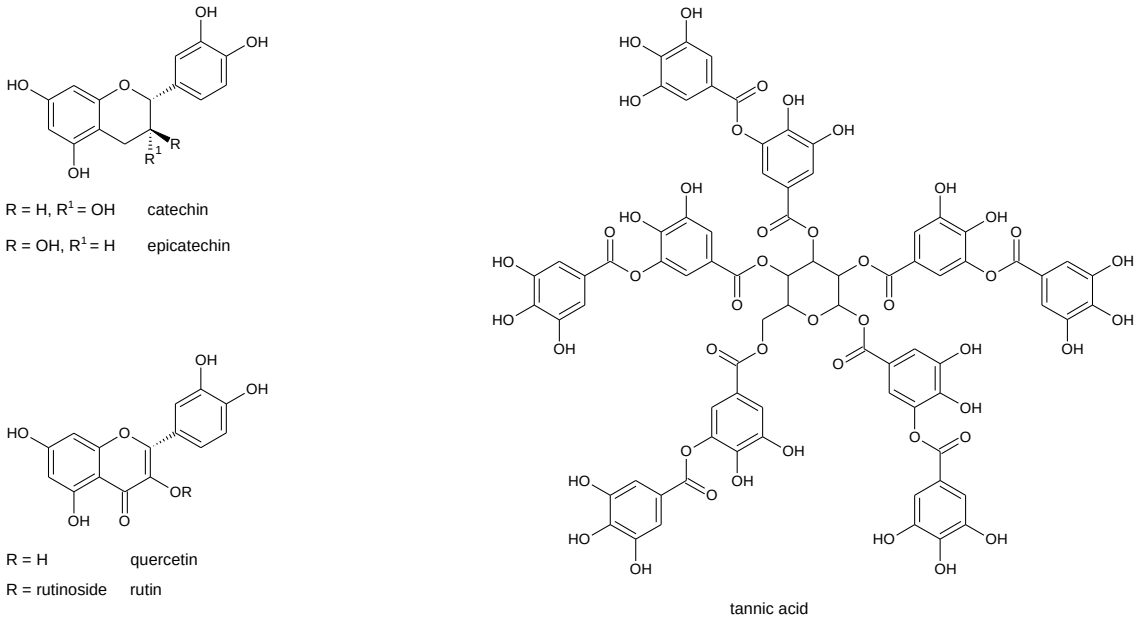

Figure 1. *Chemical structures of catechin, epicatechin, quercetin, rutin and tannic acid.*

Materials and methods

Samples preparation

Procyanidins B1 and B2 were purchased from Extrasynthese (Genay, France), tannic acid, quercetin and rutin were purchased from Sigma-Aldrich (Milan, Italy). The wine tannins extract was obtained from Sauvignon Blanc wine provided by commercial wineries in the Conegliano area (Italy). Briefly: 1.5 L of Sauvignon Blanc few days after bottling were eluted on a glass column (400 × 24 mm) packed with 20 g of Sephadex LH-20 resin (Sigma-Aldrich) equilibrated with 20 % ethanol in water. The resin was washed with 20 % ethanol in water, ethanol absolute and finally, 60 % acetone in water. The desired tannins were present in this final fraction that was evaporated under vacuum, diluted with water and lyophilized.

VVTL1 was purified from the Manzoni Bianco wine provided by commercial wineries in the Conegliano area and characterized as previously described (Di Gaspero, Ruzza, Hussain, Vincenzi, Biondi, Gazzola, Siligardi, & Curioni, 2017).

Synchrotron Radiation Circular Dichroism

Protein samples were prepared dissolving 0.4 mg/mL of VVTL1 in a model wine solution (MWS) composed by 12% ethanol in a meso-tartaric acid solution (5 g/L) adjusted to pH 3.2. The polyphenol stock solutions were prepared at the concentration of 0.038 mM in MWS.

SRCD spectra in the 180 to 260 nm range 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 monochromator slit widths (0.5 – 1.0 mm) were used according to the experiment. Spectra were measured using a 0.02 cm path length Suprasil cell (Hellma Ltd., UK). 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-five consecutive repeated scans for each sample at 20 °C. SRCD spectra were processed and analyzed using the CDApps software (Hussain et al., 2015).

Near-UV spectra were recorded using a nitrogen flushed Jasco J-715 spectropolarimeter (Tokyo, Japan) using a 1.0 cm quartz cells (Hellma Ltd.).

Results and discussion

To evaluate the effects of polyphenols on the secondary structure of VVTL1 protein, the far-UV region of VVTL1 alone was initially investigated using SRCD spectroscopy. At room temperature (25°C), the SRCD spectrum of VVTL1 is characterized by the presence of two positive peaks at about 195 nm and 231 nm and a negative peak at 213 nm. The peaks at 195 and 213 nm, attributable to the amide bond, is characteristic of a β-sheet conformation, while the band at 231 nm is due to the contribute of aromatic side-chains (Figure 2). Successively, the SRCD spectrum of VVTL1 in presence of different polyphenols has been recorded. To eliminate the contribute of tannins in the dichroic signals, the SRCD spectrum of VVTL1 in presence of polyphenols were subtracted by the corresponding spectrum of the single polyphenol in MWS at the same concentration. As show in Figure 2, the presence of polyphenols slight modified the SRCD spectra of VVTL1, indeed, we observed a decrease in the intensity of the positive band at 195 nm in presence of PB1, PB2, WTE and TA, while in the presence of Q and R this band slightly increased. Anyhow the negative band at 213 did not showed any appreciable change. This is confirmed by the analysis of the secondary structure content by the CONTINLL program (Provencher, & Gloeckner, 1981) present in the CDApps software (Hussain et al., 2015). As reported in Table 1, the β-sheet content of VVTL1 at 25°C in presence of tannins decreased less than the 10%. On the contrary, the 230-250 nm region of the SRCD spectrum, also containing the contribute of the aromatic transition of polyphenol, is strongly modified, suggesting that in the presence of VVTL1 also the ligand conformation is modified.

Table 1 *Secondary structure content of VVTL1 (0.400 mg/mL) with and without polyphenols calculated using CONTINLL (Provencher, and Gloeckner, 1981) present in the CDApps software (Hussain et al., 2015).*

Sample	% Secondary structure		
	β-sheet	turns	unordered
VVTL1 alone	46	21	31
VVTL1 + PB1	44	22	30
VVTL1 + PB2	41	21	34
VVTL1 + Q	45	23	28
VVTL1 + R	47	23	28
VVTL1 + WTE	42	22	32
VVTL1 + TA	43	22	30

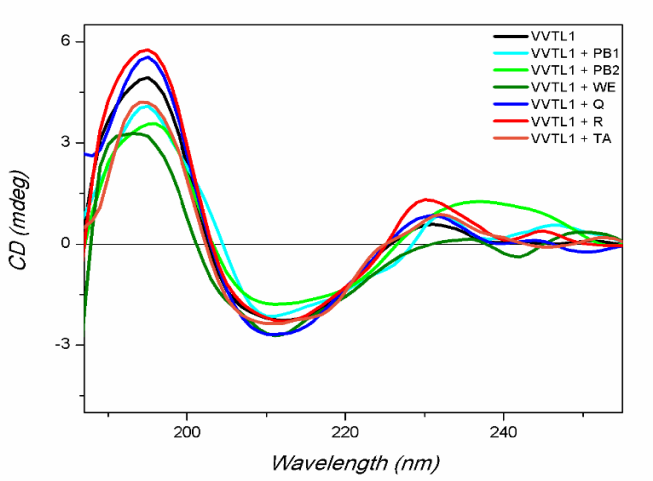

Figure 2 *Far-UV SRCD spectra of VVTL1 alone or in presence of 2 eq. of tannins (indicated) in model wine solution. SRCD spectra were recorded using a Suprasil 0.02 cm cell (Hellma) filled with 60 µL solution, integration time 1 s, 1 nm digital resolution, 39 nm/min scan speed and monochromator slit widths 0.500 mm. Protein concentration was 0.400 mg/mL.*

The effects of tannins on the stability of VVTL1 proteins were assessed by both thermal and UV-photo induced denaturation experiments.

Thermal stability studies were performed in the 5-70°C temperature range due to the presence of ethanol in the buffer composition. A drastic change in the SRCD spectrum of VVTL1 at temperature above the 55°C can be detected: the two positive bands at 195 and 231 nm disappeared and the negative band at 213 nm is shifted at about 202 nm with a shoulder at about 218 nm. The SRCD spectrum recorded at 20°C at the end of the thermal denaturation process (blue line) was not superimposable to that recorded at the same temperature during the experiment (orange line), suggesting the adoption of a non-native conformation by VVTL1, and consequently the non-reversibility of the process (Di Gaspero et al., 2017). The addition of tannins affected in different way the thermal denaturation of VVTL1 and in particular the SRCD spectrum recorded at 20°C at the end of the denaturation experiment. While in the presence of PB2 an increase the positive band at 195 nm can be detected (Figure 3A, blue line), in the presence of the other tannins a significant decrease of this band was observed (as example the SRCD spectra as function of temperature in presence of PB1 are reported in Figure 3B). Moreover, in presence of both PB1 and TA also the positive band in the 230-250 nm region disappeared.

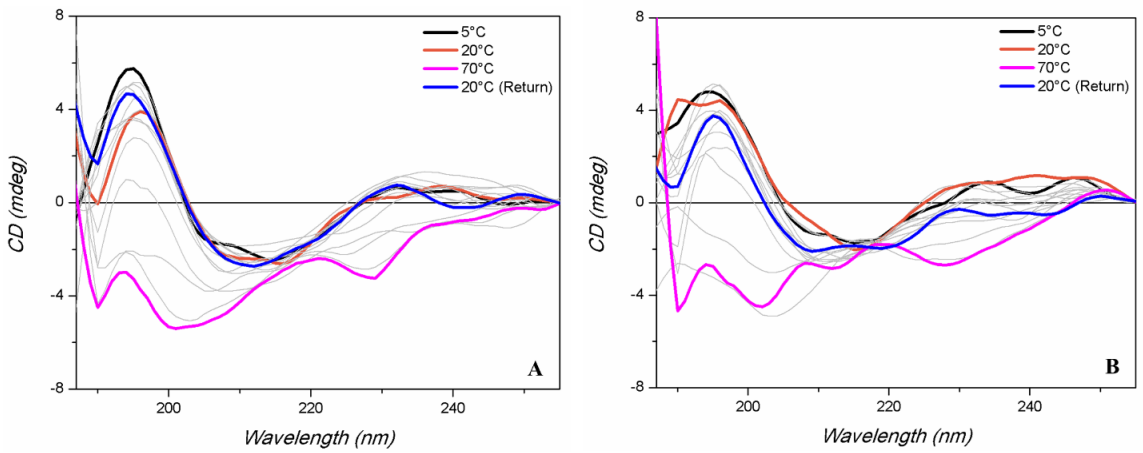

Figure 3. *Far-UV SRCD spectra of VVTL1 in presence of 2 eq. of* ***PB2*** **(A)** *and* ***PB1*** **(B)** *at different temperatures (indicated). SRCD spectra were recorded using a Suprasil 0.02 cm cell (Hellma) filled with 60 µL solution, integration time 1 s, 1 nm digital resolution, 39 nm/min scan speed and monochromator slit widths 0.500 mm to eliminate the effect of UV denaturation. Protein concentration 0.400 mg/mL in MWS.*

Analysing the secondary structure content of VVTL1 alone in function of temperature it was found that up to about 55°C the structure content of this protein did not change, while an increase in the unordered structure in the 55-60°C range was observed. In the presence of polyphenols, a similar behaviour was noted, but the transition temperature was lower than that observed for the VVTL1 alone. This was more evident in the presence of both TA and WTE (Figure 4A and 4B).

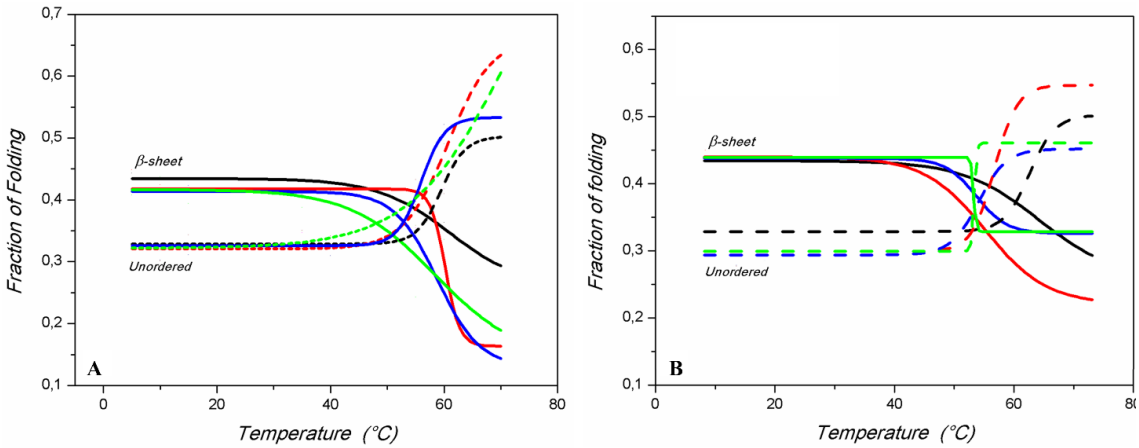

Figure 4. **(A)** *Plot of secondary structure content for VVTL1 (black), VVTL1 +* ***PB1*** *(red); VVTL1 +****PB2*** *(blue); VVTL1 +* ***WTE*** *(green).* **(B)** *Plot of secondary structure content for VVTL1 (black), VVTL1 +* ***TA*** *(red); VVTL1 +* ***Q*** *(blue); VVTL1 +* ***R*** *(green) determined with CONTINLL (Provencher, and Gloeckner, 1981) present in the CDApps software (Hussain et al., 2015) from SRCD data versus temperature. VVTL1 concentration was 0.400 mg/mL, polyphenols concentration was 0.038 mM in MWS. The SRCD spectra were measured using B23 module B, integration time 1 s, 0.500mm (1.2 nm bw) using a cylindrical Suprasil cell of 0.02 cm pathlength (Hellma) filled with 60 µl of solution.*

The near-UV CD spectrum of VVTL1 is characterized by a negative band at 290 nm with a shoulder at about 300 nm, while below 260 nm it exhibits a positive ellipticity. The addition of procyanidin B1 and B2 slight modified the near-UV CD spectrum of VVTL1 and more interesting the changes is not influenced by the different chirality of procyanidin moieties. Indeed, in presence of either PB1 or PB2 (Figure 5A) the pattern of the near-UV CD is characterized by a decrease in the intensity of both the negative band at 290 nm and the shoulder at 300 nm, while the positive ellipticity is shifted at higher wavelength (about 280 nm). A very similar behaviour is observed in the presence of addition of either polyphenol wine extract (Figure 5B) or quercetin (data not shown), while in presence of its glycoside rutin, the near-UV CD spectrum of VVTL1 has profoundly changed (Figure 5C). The intensity of the both the negative band and the shoulder at 290 and 300 nm, respectively, is greatly increased. Additionally, two non-resolved negative band appear at about 255 and 265 nm, respectively, and a negative signal is present in the 310-340 nm range. This may be attributable to the presence of the rutinose, which increase the solubility of quercetin favouring the interactions of this polyphenol with the VVTL1 protein. Also in presence of tannic acid the CD spectra of VVTL1 is modified (Figure 5D). It is characterized by two positive bands at about 275 nm and 309 nm, respectively, and negative bands at 261, 287 and 293 nm.

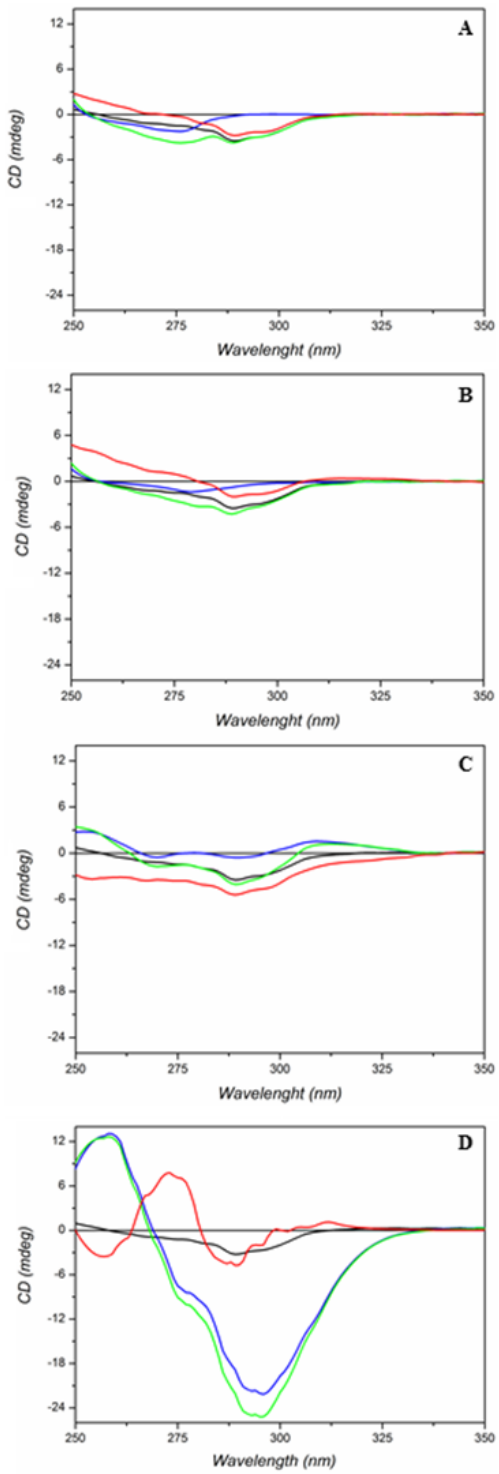

Figure 5 *near-UV CD spectra of VVTL1 alone (black line), in presence of 2 eq. of phenolic compound (red line), CD spectrum of the phenolic compound alone (blue line) and the sum of the single CD spectrum for VVTL1 and the selected phenolic compound (green line) in MWS. The polyphenols reported are procyanidin B2* (**A**)*, wine tannins extract* (**B**)*, rutin* (**C**) *and tannic acid* (**D**)*. VVTL1 concentration was 0.500 mg/mL, polyphenols concentration was 0.047 mM, protein/ligand ratio was 1:2. The CD spectra were measured using nitrogen flushed Jasco J-715 spectropolarimeter, scanning speed 50nm/min, data pitch 0.5 nm, response time 4 s, bandwidth 2 nm using a Suprasil 1.0 cm cell (Hellma) filled with 900 µl of solution.*

The high UV photon flux of synchrotron beamline can denature proteins (Clarke and Jones, 2004), and a significant decrease of secondary structure in structurally ordered proteins is well observable upon light irradiation using the B23 beamline (Hussain et al., 2012). This phenomenon provides g useful information on the protein stability and on the effects of both the presence of ligands and medium composition (Ruzza et al., 2015, 2016). The mechanism of photo-denaturation is likely to include free radical damage to the photosensitive residues of tryptophan and tyrosine, as well as the oxidation of amino acid side chains (Xu and Chance, 2007; Grosvenor et al., 2010). Aromatic residues are present in VVTL1, which may explain the irreversible loss of conformation during UV irradiation. A protein UV photo-denaturation assay was carried out by performing twenty-five consecutive repeated scans for each sample at 20°C (as an example the UV photo-denaturation assay of VVTL1 in presence of tannic acid is shown in Figure 6.

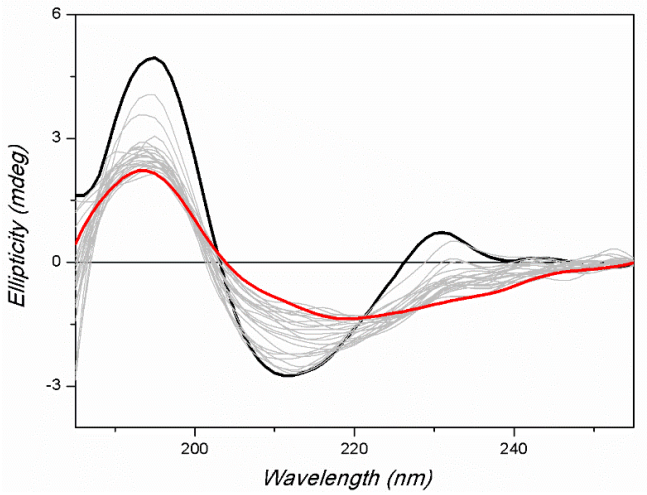

Figure 6. *VVTL1 UV photo-denaturation assay carried out with B23 module. Twenty-five repeated consecutive SRCD scans of VVTL1 (0.400 mg/mL) in MWS in presence of 2 eq. of* ***TA****. The solid black line indicates the first scan and the solid red line the 25th scan. Integration time 1 s, 0.02 cm cell filled with 60 µL solution, monochromator slit widths 1.000 mm (2 nm bandwidth), synchrotron ring current 300 mA.*

By analysing the secondary structure content of VVTL1 alone versus number of scans, a significant decrease in β-sheet content was noted already at the fifth scan, but from the seventh scan any further change in the secondary structure was observed (Figure 7). In the presence of procyanidins B2, quercetin or rutin, a small change in the secondary structure of VVTL1 was observed as reported in Table 2. On the contrary, when either procyanidins B1 or wine tannin extract were tested, a drastic decrease in the ordered conformation was detected. Surprisingly, the addition of tannic acid induced only a small change in the secondary structure of VVTL1 (Figure 7).

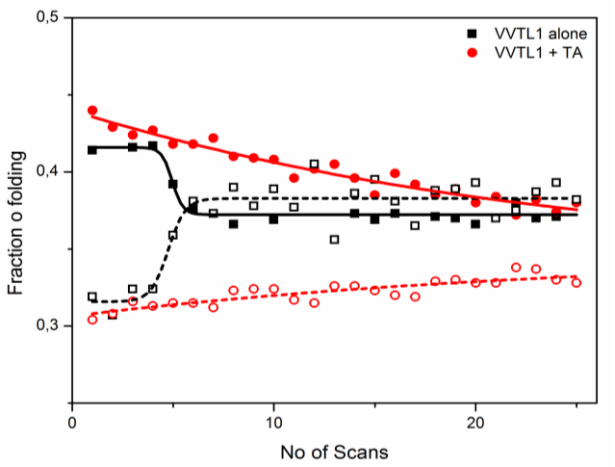

Figure 7. *Plot of the secondary structure content of VVTL1 alone (black) and in presence of tannic acid (red). Secondary structure was determined with CONTINLL (Provencher and Gloeckner, 1981) present in the CDApps software (Hussain et al., 2015) from SRCD data versus number of scans.*

The type and the mechanism of interactions between polyphenols and proteins, involving both hydrogen bonds and hydrophobic interactions, appears to be dependent by several factors such as experimental conditions as well as polyphenols and proteins properties, and there is still no clear evaluation of the relative role of these factors. In hydroalcoholic solution at pH 3.5, Simon et al. (2003) found that the principal driving forces toward polyphenols and proteins was governed by the hydrogen bond between the phenol OH groups of tannins and the carbonyl group of the peptide bond. However, NMR studies in 10% of DMSO showed that hydrophobic interactions were predominant in the interaction between gramicidin S and polyphenols (Zhang et al., 2002). Indeed, hydrophobic forces are predominant in interactions involving apolar polyphenol such as tannic acid (Hagerman et al., 1998). On the contrary, the more polar procyanidin B1 [(-)-epicatechin-C(−4)-C(−8)-(+)-catechin] forms preferentially hydrogen bond with protein and the simple epicatechin does not interact with protein by hydrophobic forces but by hydrogen bonding (Frazier et al., 2006). Also the stereochemistry of flavan-3-ol pyran rings, as well as the conformational flexibility of polyphenol appear to be important parameters of the capacity of polyphenols to interact with proteins. Experimental data showed that while (+)-catechin interact with polyproline producing haze, (-)-epicatechin did not interact (Poncet-Legrand et al., 2006). Moreover, (-)-epicatechin-C(−4)-C(−6)-(+)-catechin dimer showed a great affinity towards proteins than that of the dimer (-)-epicatechin-C(−4)-C(−6)-(-)-epicatechin (De Freitas & Mateus, 2001).

Table 2. *Secondary structure content of VVTL1 (0.400 mg/mL) alone or in presence of tannins at the start and after 25 scans in UV photo-denaturation assay. Secondary structure was calculated with CONTINLL (Provencher and Gloeckner, 1981) present in the CDApps software (Hussain et al., 2015).*

Sample	% Secondary structure					
	after 1 scan			*after 25 scans*		
	β-sheet	**turns**	**unordered**	**β-sheet**	**turns**	**unordered**
VVTL1 alone	41	22	32	38	19	38
VVTL1 + B1	43	24	28	24	24	44
VVTL1 + B2	43	22	31	38	22	30
VVTL1 + Q	43	22	30	39	20	35
VVTL1 + R	43	22	30	40	20	33
VVTL1 + WTE	43	23	30	21	18	44
VVTL1 + TA	44	22	30	38	23	33

Conclusion

Despite the relative abundance of polyphenols in white wines, these compounds, especially the polymers of catechin (+) and epicatechin (-) are associated to the precipitation of wine proteins (Batista, Monteiro, Loureiro, Teixeira, & Ferreira, 2010). Experimental data showed that protein/polyphenol interaction are favourite in presence of proteins having a high content of basic residues, a relatively large MW, hydrophobicity and showing a conformational open and flexible structure (Simon et al., 2003; Richard et al., 2006). In contrast, VVTL1 seem not to be favourable for the interaction with polyphenols. Indeed, it is characterized by a pI of 4.76 (Gasteiger et al., 2005) and a grand average of hydropathicity (GRAVY) of -0.438 (Kyte & Doolittle, 1982) in a rigid structure stabilized by the presence of eight disulphide bonds.

Nevertheless, the low propensity of VVTL1 to interact with polyphenols, collectively the data here presented demonstrate the existence of interaction. Quercetin did not strongly affect the stability of VVTL1, whereas the wine tannins extract negative affect the stability of protein, as showed by both thermal and UV-denaturation assay. Conversely, the presence of rutin seemed to show stabilizing effects on protein structure. Procyanidin B1 highlighted a higher propensity to interact with VVTL1 than procyanidin B2. While tannic acid revealed ambiguous effects, and need further investigation to be fully understood. Finally, the investigation in the near-UV region of VVTL1 evidenced a strong involvement of the aromatic side-chain residues in the interaction.

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CHAPTER IV

IN VITRO PRODUCTION AND CHARACTERIZATION OF GRAPE PROTEINS

Pubblication 4

In-vitro production and characterization of grape proteins

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In preparation

Abstract

An alternative source for grape proteins purification was proposed. This method allowed the isolation of grape proteins without the presence of interfering substances such as polyphenols and then in absence of agents responsible for decrease of the final proteins content. Grape callus tissues were initiated from the pulp of Manzoni Bianco grapes. The protein extracts revealed protein profiles very similar to those reported in literature for fresh grape tissue, with however a different proportion in PR-proteins. In addition, the quantification of proteins suggested a potential higher content of PR-proteins in the callus tissues. Finally, two main proteins were isolated from grape callus and analysed by LC-MS Q-TOF analysis. The two proteins were identified as class IV chitinase and VVTL1 (4L5H), confirming the sameness of proteins isolated from callus with those naturally present in grapes. Moreover, a quantification of polyphenols content was carried out revealing the absence of condensed tannins and hydrocinnamic acids.

KEYWORDS:

Grape callus tissue; wine; protein expression; *Vitis vinifera*; Thaumatin-Like Proteins; chitinases

Introduction

Winemakers constantly face the problem of protein instability of white wines. This defect, commonly named haze formation, is attributed to some proteins that persist after vinification in the bottle (Waters et al., 1992). Among the wide variety of wine proteins, some grape Pathogenesis Related-proteins, particularly chitinases and Thaumatin-Like proteins, have been shown to be the main components involved in the haze formation (Ferreira et al., 2001). Under determinate conditions, these grape proteins undergo structural modification that promotes their aggregation. These phenomena lead to the formation of visible floccules that scatter the light conferring to the wines that undesirable turbid appearance (Dufrechou et al., 2010). However, the proteins alone are not able to fully explain visible haze formation, thus, the involvement of non-proteinaceous compounds has been proposed (Pocock et al., 2007; Marangon et al., 2010; Dufrechou et al. 2015; Chagas et al. 2016). Commonly, to stabilize the wine the hazing proteins are removed before bottling through their adsorption onto bentonite. Its usage has several drawbacks, including the loss of wine quality and the huge management costs, which has been estimated to produce around $1 billion hidden cost per year (Majewski et al., 2011). The study of the mechanisms involved in haze formation requires the purification of the grape proteins, but this is a difficult task, due to the relatively low content of proteins in the grape juice and to the presence of different interfering substances, such as polyphenols (Water et al., 1992). To this purpose, the purification of proteins from grape cells cultures propagated *in vitro* could be a feasible solution. In vitro cell culture is a methodology that allow to retain organs or portion of plant tissue on a sterile growth medium composed by a determined chemical composition. Compared to the harsh conditions to which grape berries are normally exposed in plant, the cellular coltures provide several advantages because are independent of pedoclimatic conditions and seasonal variations. They offer a defined and constant production system, which ensures uniform quality and rapid yield. The first trials of plant cell cultures date to the beginning of twentieth century in Germany where the roots of plants were immersed in a thin growth medium, they were called hydroponics (Dörnenburg and Knorr, 1995). The revolution started with the standardization and the adjustment of the universal culture media (Murashige and Skoog, 1962). The major advantage of these techniques is related to the possibility to manipulate the production of bioactive secondary metabolites which are not originally

synthetized in plant (Ramaschandra Rao et al., 2002). The grape cell cultures have been used to investigate the metabolic pathway of glucose regulation transport (Conde et al., 2006) as well as the production of bioactive compounds such as stilbenes (Ferri et al., 2009) and assess the genes expression during the phenologic phases of grape ripening (Sharathchandra et al., 2011). To date grape berry cultures have not yet been specifically used as a suitable method to produce PR-proteins. In contrast to the conventional methods, the extraction from callus should avoid the polyphenols removal using polyvinylpolypyrrolidone (PVPP) or/and activated carbon charcoal (Van Sluyter et al., 2009; Marangon et al., 2010), which have been demonstrated to be able to interact with proteins (Israel et al., 2009; Mattew and Kozlov, 2014) or influence the protein recovery due to the removal of polyphenols likely associated with them (Waters et al., 1992). In addition, the isolation of proteins from grape juice or wines are strongly related to the grape cultivar, vineyard practices, climate and stress (Pocock et al., 1998; Meier et al., 2016) and limited to the harvest time (i.e. grape juice and wine production period). Conversely, the grape culture in vitro could standardize the production of protein and it is potential available all year. However, the callus tissues are the result of dedifferentiation of cells and during this phase the cells are so stressed that, frequently, their genome can result modified (Dörnenburg and Knorr, 1995). Thus, if the aim is to obtain proteins representing exactly those found in grape, and responsible for the wine hazing, it is crucial to assess whether the callus-collected proteins are the same as those found in fresh grape tissue.

Material and methods

Grape Explants

Several sections of ripe and unripe grapes of *Vitis Vinifera* cv. Manzoni Bianco (vintage 2014 and 2015), were used to obtain calli. The grapes were collected from the vineyards of the University campus of Conegliano at different times: before veraison (5 august), close to veraison (18 august) and at harvest time (27 august). The berries were washed with 20% commercial sodium hypochlorite for 15 minutes and then exhaustively washed with sterile deionized water. Then, the berries were cut with a blade and thin sections were placed on solid Murashige and Skoog medium (MS) (Moorashige and Skooge 1962), prepared with some modification. Briefly, the concentration of macroelements was halved, the 3-indoalacetic acid (IAA) and agar were replaced with 0.1 mg/L of 1-naphthaleneacetic acid (NAA) and 2.8 g/L of phytagel (Sigma, Milan), respectively. Finally, the plates were incubated at 25°C in the dark with the aim to minimize polyphenols synthetize. To freshly maintain the callus tissues, the cultures were divided and placed on fresh medium about every 45 gg, depending on the rate of development.

Protein extraction

Callus tissues were grinded by mortar with 1:5 ratio (w/v) of 100 mM sodium citrate pH 5.5, 67 mM phosphate buffer pH 7.8 and 33mM tartaric acid buffer adjusted to pH 3.5. The suspension was agitated for 1h and centrifuged at 3000g per 15 minutes. The pellets were further treated with a 1:2.5 (w/v) ratio of the same buffer, stirred overnight, and centrifuged as above. The surnatants were pooled, filtered at 0.45 µm (Sartorius, Roma) and stored at -20°C.

Protein recovery, qualitative and quantitative determination

Total protein content of callus extracts was determined in according to a previously method Vincenzi et al. 2005. The calibration curve was prepared by using a serial dilution in water of bovine serum albumin (Sigma, Milan, Italy) ranging from 62.5 µg/mL to 1000 µg/mL. Qualitative analysis of proteins was performed by SDS-PAGE in no reducing conditions (Laemmli et al. 1970).

The molecular weight standard proteins were Broad Range Molecular Weight Markers (Bio- Rad, Hercules, CA). After electrophoresis, the gels were stained for 18 h in colloidal Coomassie and then destained with water for 24 h.

Protein purification and Identification.

The proteins recovered from callus were purified following the procedure described by Van Sluyter et al. 2009 with some modification as reported by Gazzola et al., 2017 – *In press.* Each extract was loaded on a SP-Sepharose column (2.5 × 30 cm, GE Healthcare, Milan, Italy) using an Akta-purifier UPC-900 (GE Healthcare). The column was washed with 30 mM sodium citrate buffer, pH 3.0, and then eluted with a gradient from 0 to 40% of 30 mM MES buffer, pH 5.0, containing 1 M NaCl in 120 min. The fractions were analyzed by SDS-PAGE. To avoid the problem of high salt concentration in the fractions, causing problems in the electrophoretic migration, a 100µL aliquot of each fraction was diluted to 1 mL with water and precipitated with the KDS method (Vincenzi et al., 2005). The pellet obtained after KCl addition was directly resuspended in loading buffer. The fractions containing similar protein profile were pooled together, then, ammonium sulfate was added to achieve a final concentration of 1.25 M. Finally, after a filtration to remove eventually precipitated material, the solution was loaded on a Phenyl-Sepharose column (1.5 × 20 cm) equilibrated by two volumes of 1.25 M ammonium sulphate in 50 mM sodium citrate buffer, pH 5.0 and eluted with a linear gradient from 0 to 100% of 50 mM sodium citrate buffer, pH 5.0, in 120 min. The fractions were again analyzed by SDS-PAGE as above and those containing the proteins of interest were pooled, concentrated by Vivaspin (Sartorius) with 3 kDa cutoff and stored at -20°C.

Peptide mass fingerprinting analysis on trypsin digestion of the purified proteins was carried out using a LC MS/MS Xevo G2-S Q-TOF (Waters) mass spectrometer in order to identify the isolated proteins.

Polyphenols determination and tannins detection.

Total polyphenols were extracted treating the callus tissue with 1:2 (w/v) ratio of acetone 70% and centrifuged at 14000g per 15 minutes. The extraction was repeated twice, and the supernatants were pooled. The acetone was removed under reduced pressure by Büchi Rotavapor R-114, and the phenolic solution was resuspended with distilled water before being analyzed for both total polyphenols and condensed tannins using the colorimetric procedure of Folin-Ciocalteau (Singleton et al. 1965) and butanol-HCl assay (Porter et al. 1985), respectively.

Results and discussion

Induction and characterization of grape callus tissues

Although the growth of vegetable cell is very slow compared to those of animal or bacteria, when cultivated *in vitro* their metabolism is accelerated resulting in a rapid biomass proliferation (Dörnenburg and Knorr, 1995). The callus is considered as a disorganized mass composed essentially by undifferentiated cells with highly vacuolated parenchyma character. The genetic expression of these cells was not suppressed, but only temporarily inactivated and it can be reactivated after a certain combination of hormones. This phenomenon is a peculiar propriety of vegetable cells and it is named totipotency (Kieran et al. 1997). In theory, starting a callus from grape we can reproduce *in vitro* the protein expression of the initial grape berry, including the PR-proteins involved in the haze formation (Waters et al. 1993). It has been documented that VVTL1, which is the most representative PR-protein of grape, is found only in the berry and is encoded by a single gene that is expressed in conjunction with the onset of sugar accumulation and softening (Tattersall et al., 1997). Sarry and colleagues (2004) confirmed this statement finding that the PR-proteins, including β-glucanases, chitinases and a isoflavone reductase-like proteins, were largely expressed during ripening (19% of the total identified proteins). Thus that the production of these proteins is strongly correlated to the ripening of grape. To this purpose some explants of Manzoni Bianco grape berries, which has been found to be very rich in these proteins (Vincenzi et al., 2005), were collected from ripe grapes and grown on a solified medium (Murashige and Skoog, 1962).

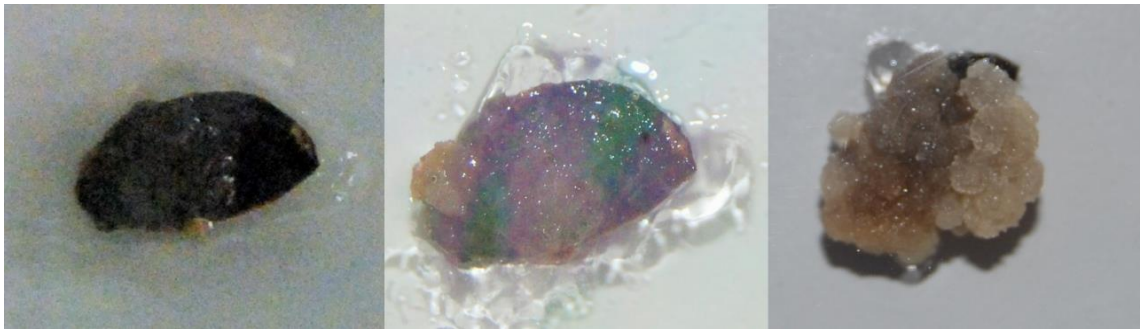

Fig.1 *An example of development of Manzoni Bianco explant after 5, 24 and 64 days, respectively. A slice of about 2 mm was putted on solified media (Murashige and Skoog, 1962)*

Observing the patterns of 1D SDS-PAGE gel, it's clear that tartaric acid buffer extracted the largest number of proteins. In particular, the tartaric acid extract revealed a protein profile very similar to that reported in literature (Sauvage et al., 2010). Actually, three major bands ranging between 18 and 31 kDa were detected, likely corresponding to TL-proteins and chitinases. In addition, also the presence of a faint band between 6.5 and 14.4 kDa corresponding to putative Lipid Transfer Proteins (LTP) was detected (Wigand et al., 2009). Finally, slight protein bands between 45 and 66 kDa were observed, probably indicative of the presence of β-glucanase and vacuolar invertase, which showed those molecular weights. The phosphate buffer suggested the extraction of a major number of proteins in the 20-25 kDa range, however their intensity was lower, and some band, such as the 31 kDa one, disappeared. Even lower amounts were detected with citrate buffer. This is unexpected, as the 5.5 and 7.8 pHs are not so extreme to induce protein denaturation and precipitation. This fact could be explained by the action of proteases, which can be co-extracted together with the other proteins after crushing of callus tissue. As a matter of fact, when the aim is to extract the totality of grape proteins the inhibition of potential proteolytic enzymes and/or interaction with polyphenols are required (Sarry et al., 2004; Deytieux et al., 2007). In particular, Sarry et al., (2004) developed a multi-steps method that implied the use of TCA and reducing agent to precipitate only the proteins coming from pulp avoiding the action of proteolytic enzymes.

Whereas Deytieux et al., (2007) used phenylmethanesulphonylfluoride (PMSF) and EDTA as protease inhibitors. In the present case, however, the aim of the work was the obtainment of PR-proteins, which are normally extracted in the real condition of winemaking, i.e. at low pH and without protease inhibitors. Even if the grape PR-proteins are well known for their high resistance to proteolysis (REF), it is clear that endogenous proteases are able to partially to totally degrade them when the pH was increased (pH 5.5 and 7.8). For this reason, the tartaric acid buffer was chosen as final buffer for the extraction of proteins from calli.

Characterization of protein expression of grape callus tissues

It has been reported that the isolation of cells in vitro could induce changes in the genome as a response mechanism to stress. This phenomenon has both positive and negative aspect because it leads to the formation of mutants and different cellular lines,

which can improve the genetic variability, but also it can negatively affect the genetic stability over time of the cellular lines (Dörnenburg e Knorr, 1995). In the present case, six callus tissues with different morphology and rapidity of growth were discriminated. In order to verify the presence of potential differences in protein expression among the callus tissues, the protein profiles of each cellular line was investigated by SDS-PAGE. 100 mg of each different initiated callus was separately minced by pestle in an Eppendorf tube and treated by 500 µL tartaric acid buffer pH 3.5. Subsequently, the proteins in the supernatant were precipitated by KDS assay (Vincenzi et al. 2005) and, then, the protein composition assessed by electrophoresis assay as reported in figure 3.

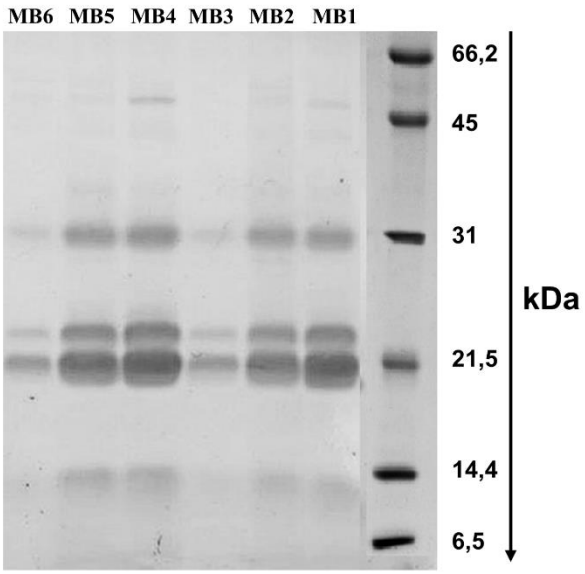

Fig.3 *SDS-Page in no reducing conditions of callus protein extract for the six cellular lines (MB 1→6). 100 mg of callus tissue was ground by mortar and treated by tartaric acid buffer pH 3.5. Then 200µl of protein extract were precipitated by KDS (Vincenzi et al., 2005).*

In all the samples a predominance of TLPs and chitinases was observed. In addition, the six grape explants showed almost identic protein profiles with differences only in term of abundance of proteins among the tissues. This result suggested the absence of different protein expression, but only quantitative difference among the grape tissues. However, the only use of SDS-PAGE analysis to discriminate and assess the protein expression of calli can be misleading. As a matter of fact, it is well known that grape proteins possess a wide range of different isoforms with similar molecular weight but different charge properties, not assessable by a simple SDS-PAGE (Monteiro et al., 2001).

With the aim to correctly assess the protein composition of each extract the collected solutions were fractionated by Cation Exchange Chromatography (CEC) and chromatogram compared (Fig. 4).

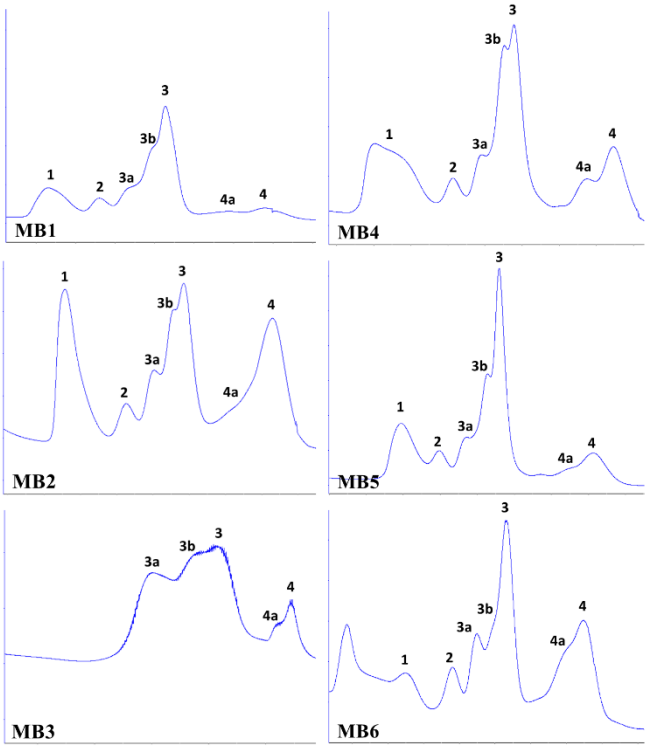

Fig.4 *Cation Exchange Chromatography (CEC) of the six protein extracts. The samples were eluted from 0 to 40% Buffer B composed by NaCl 1M pH 5 in 120 minutes.*

The data indicated approximately the same peaks for each sample, confirming what observed in electrophoresis gel with the only exception of MB3. In particular, the chromatograms reported in figure 4 showed four distinct peaks (1, 2, 3, 4) and three shoulders, two in correspondence of the third peak (3a and 3b) and one in correspondence of the last peak (4a). Conversely, MB3 cellular line did not show the first two peaks (1, 2). It is most likely that the very slow development of callus MB3 affect both the protein composition and the amount of proteins in the extract. This was consistent with the quantification of the total amount of proteins, which revealed that MB3 possess the lowest amount among the six extracts (Table 1). Observing the results deriving from BCA assay, it is also interesting to note that the amount of proteins extractable from calli is potentially higher than that obtained from fresh grapes during winemaking (table 1). It is reported that the proteins in white grapes achieve concentration between 191 to 251 mg/L (Pocock et al., 2000), whereas starting from

calli it is possible to reach a yield of extractable protein of almost 1g/kg of tissue (for MB2 line).

Table 1. *Quantification of total wine proteins for the six protein extracts by BCA assay. 100 mg of callus tissue was grounded by mortar and treated by tartaric acid buffer pH 3.5. Then 200µl of protein extract were precipitated by KDS (Vincenzi et al., 2005).*

Samples	Average Protein Concentration (µg/mL)	Dev. Standard	Total Protein / callus tissue (mg/g)
MB1	117.67	19.37	0.88
MB2	134.33	8.78	1.01
MB3	24.54	16.45	0.18
MB4	77.04	7.96	0.58
MB5	117.88	6.03	0.88
MB6	43.08	4.61	0.32

Purification and identification of proteins from callus tissues

On the basis of the preliminary characterization described above, the MB5 cellular line was selected for the purification of proteins. Although it was not the tissue with the largest amount of proteins, it has been chosen for its higher growth rate. After a propagation in large quantity, the proteins were extracted from MB5 tissue using tartaric acid buffer as reported above. The obtained protein solution was fractionated by cationic exchange chromatography and the peaks characterized from their profile in SDS-Page (Fig. 5). As expected the chromatogram of MB5 reflected those reported in figure 4 (data not shown). The data revealed the presence of two protein bands located between 6.5 and 14.4 kDa which, by comparison with literature data, could be associated to LTPs. These proteins were collected without other contaminant bands in the first eluting peak (Peak 1). Additional bands with the same MW but lower intensities appear also at the end of the chromatographic separation, in the peaks 3b and 3. Callus tissue seemed to express in relative good amount the LTPs, although they are generally found in trace in white wines (Wigand et al., 2009). LTPs are included in the big family of proteins devoted to plant defence, being classified as Pathogenesis Related

Protein-14 (Garcia-Olmedo et al., 1995). Being resistant to acidic conditions and proteolytic activity, these proteins possess all the characteristics to survive and persist within the wine after alcoholic fermentation. However only few cases reported the presence of Lipid Transfer Proteins in white wine (Okuda et al., 2006; Wigand et al., 2009; Lambri et al., 2012). This is probably due to the fact that LTPs are generally present in the skin and seeds of plants and, being the white wines fermented without the contact of the grape skin, the release of these proteins in the grape juice is negligible. This hypothesis seems to be confirmed by Wigand and co-authors (2009), which found a higher amount of LTPs in red wines compared to white wines. Lipid Transfer Proteins have been studied in grape mainly for their allergenic proprieties (Pastorello et al., 2003), whereas, given their almost absence in wine, their possible technological role in wine is still unknown. Therefore, the relative abundant proportion of LTP in the grape culture extract could make this method suitable to obtain and potential study this protein.

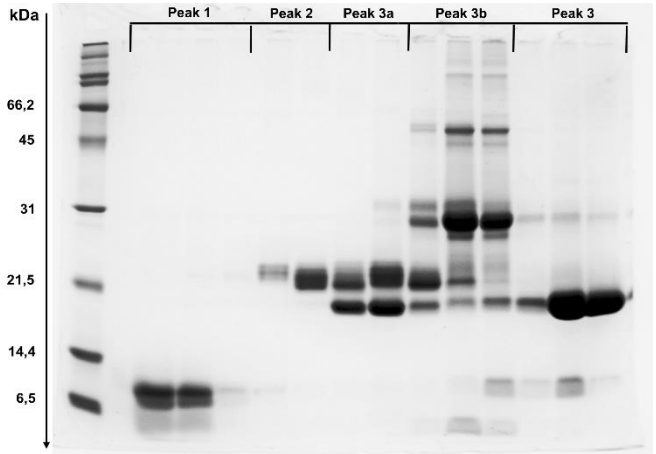

Fig.5 *Electrophoresis gel of the protein fractions obtained after MB5cation exchange. The proteins were precipitated by ethanol 80% and the protein profile assessed according to the procedure of Laemmli et al., 1970.*

The most abundant classes of grape proteins were found in the other chromatographic fractions, in particular the cation exchange was able to separate some TL-proteins from chitinases. Indeed, the peak 2 showed a single band at around 21 kDa, likely corresponding to a Thaumatin-Like Protein (TLP1). In the peak 3a a lower band (~19 kDa) was observed, highlighting the presence of another isomer of TLP (TLP2), whereas the peak 3 showed the presence of another abundant isomer of TLP with a MW similar to that of TLP2 (TLP3). The peak 3 was the main one detected in all the protein extracts (fig. 4), this mean that TLP3 is the most abundant protein found in callus

extracts. It is conceivable, thus, that this protein corresponds to the major Thaumatin-Like grape protein, the VVTL1 (Waters et al., 2005, Muhlack et al., 2007). The peak 3b contained a band at around 30 kDa that could correspond to the putative chitinase, contaminated by TLPs. As reported in literature the proportion in grape between chitinases and TLPs is variable depending on the seasonal variations and climate conditions, but generally is largely in favor to the latter ones (Monteiro et al., 2003; Van sluyter et al., 2009; Le Bourse et al., 2011). Monteiro et al., 2003 quantified the protein content in grapes harvested from the same vineyard in two consecutive years, revealing that the grape content drastically changes from year to year, especially that of chitinase. In the present work, from the obtained data the proportion between chitinases and TLPs is very similar, suggesting the presence of a high amount also of chitinase. Despite the use of grape juice, which protein content is strongly influence from the environmental conditions, grape cell culture allows to obtain almost the same proportion in proteins. In addition, it is interesting to note also the presence of a protein band at 50 kDa. This band is very similar to that reported by Vincenzi and Curioni, 2005, which observed an anomalous migration in Sodium Dodecyl Sulfate-Polyacrylamide gel for a chitinase isoform under no reducing conditions. They suggested the formation of a dimer of chitinase or an artefact due to no reducing condition of SDS-Page (Gazzola et al., 2017 – *In press*). The peaks 4 and 4a showed a faint mix of protein bands (data not reported). The subsequent stage of purification included the Hydrophobic separation of peaks 3 and 3b, which corresponded to those containing the main proteins of interest (Fig. 6A-B). This technique allows to separate the chitinases from the TLPs due to their different hydrophobicity with high protein purity (Vincenzi et al., 2014). Then, the collected protein fractions were checked using SDS-Page.

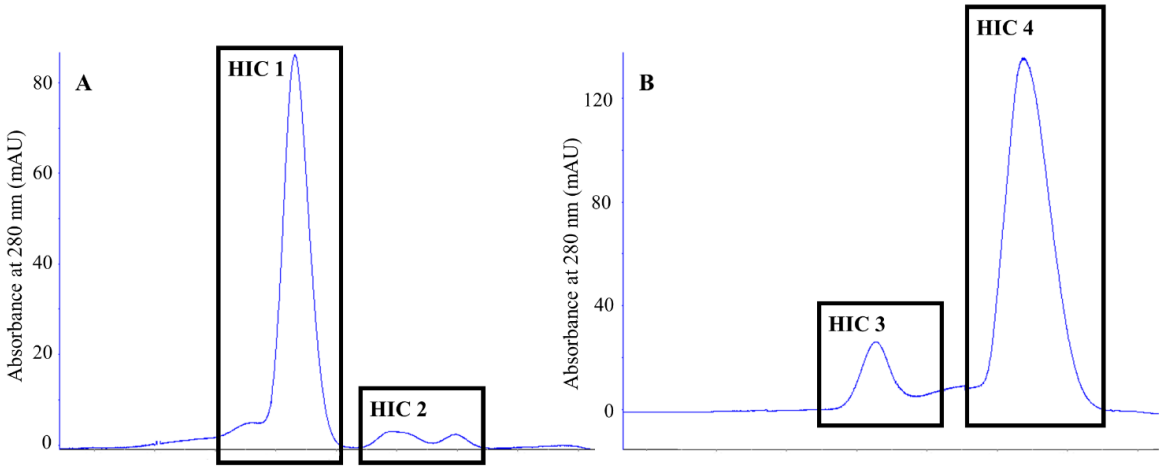

Fig.6 *Hydrophobic Interaction Chromatography of peak 3b (A) and peak 3 (B). 1.25 M ammonium sulphate was added to the protein fractions and the samples were eluted from 0 to 100% buffer B composed by sodium citrate 50 mM pH 5 in 120 minutes.*

Hydrophobic separation of peak 3 and 3b showed the presence of four different fractions (HIC 1, 2, 3 and 4). As expected the fractionation of peak 3b revealed the presence of a main peak composed by chitinase with a high rates of purity (HIC 1), whereas HIC 2 revealed mainly the presence of another isoform of chitinase and traces of TLP and LTP (data not shown). Also the separation of peak 3 revealed the appearance of two different fractions. The investigation of peak HIC 3 revealed a significant content of chitinase and LTP, whereas fraction HIC 4 showed the presence of a single band corresponding to TLP 3 (data not shown). The fractions HIC 1 and HIC 4 were dialyzed against water in order to remove ammonium sulphate and freeze-dried for mass-spectrometry analysis. In order to be sure that the isoforms expressed *in vitro* are identical to those expressed in the fresh grapes, the tryptic products and the molecular weight of the purified proteins from MB5 callus (HIC 1 and HIC 4) were compared with those of two known grape proteins previously purified from Manzoni Bianco grape juice (Vincenzi et al., 2014; Di Gaspero et al., 2017,) using a LC MS/MS Xevo G2-S Q-TOF mass spectrometer. The data revealed that the tryptic products and the weight of the proteins purified from calli are superimposable to those purified from Manzoni Bianco grape juice, indicating the same protein origin. It can be concluded that the grape callus can express the same protein isomers found in the grape and that this system can be a valid and alternative method to produce grape proteins.

Determination of phenolic content

The proteins purified from wine, but also from grape juice, being extracted in an environment rich in polyphenols (i.e. tannins), are likely to be complexed with these grape components since the moment of their extraction (Waters et al., 1992). This complexation leads to a modification of the protein structure and functionality. To this purpose the explants were grown in the dark in order to avoid the accumulation of these interference substances in the cells. The determination of phenolic component is fundamental in order to understand the conditions in which the proteins are extracted. The quantification of phenolic content and condensed tannins from a 70% acetone extract directly obtained from callus tissue (MB5) were carried out. In addition, an aliquot of the same extract was loaded on HPLC Reverse-Phase in order to assess the hydrocinnamic acids content. The results indicated that grape calli possess a very low amount of polyphenols (56.77±4.6 µg/g of fresh callus tissue). Some analyses performed on natural white grape berries by Folin Ciocalteau reported polyphenols content much higher than that observed in this work, achieving values between 1.5 -1.9 mg/g fresh weight (Ivanova et al., 2011). It is well known that grape must browning is correlated to the amount of hydrocinnamic acids, in particular of caftaric acid, and promoted by flavonols (Li et al., 2008). The cell cultures have been developed from the pulp of grape, thus it was expected to find such compound in the acetone extract, but the result of HPLC assay revealed the absence of this hydrocinnamates (Figure 7). Finally, the butanol-HCl assay suggested the absence condensed tannins (2.3 ± 1 mg/L).

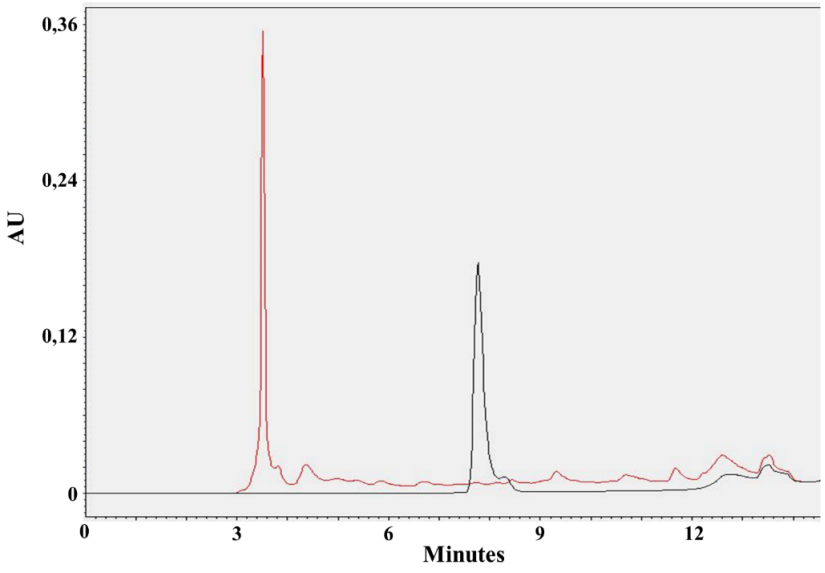

Fig. 7 *Superposition of HPLC chromatograms for the acetone extract from grape callus tissue (red line) and caftaric acid standard (black line).*

Conclusion and future prospective

In summary, this paper demonstrated that grape cells culture in vitro can be useful to obtain grape proteins in a relative good amount. Although the protein composition reflect that reported in literature for fresh grapes, its proportion is different, especially in the content of LTPs and chitinases. Contrarily to the use of grape juice and wine, whose protein content is strongly influenced by the environmental conditions, grape cell culture allows to standardize their production. It is well known that proteins undergo structural modifications after crushing grape berries due to the presence of interfering substances such as polyphenols, heavy metals, proteases (Ferreira et al., 2001; Volpe et al., 2009; Esteruelas et al., 2011). Thus, find a suitable system to isolate the proteins in a form as pure as possible is crucial. Because a very low amount of polyphenols and the absence of other interfering substances we can claim that grape-derived proteins can be collected really close to their native state. Therefore, this alternative system can be very interesting for the obtainment of grape proteins to be used for the characterization of their molecular and functional properties.

In addition, the advantage of this technique is the possibility to manipulate the expression of grape cells. Elicitors can be added in the culture medium in order to induce the production of other PR-proteins, which are well known to be synthesized in response to stress and pathogens (Martinez-Esteo et al., 2009). In addition, another promising application could be the cultivation of grape cellular tissues on a growth medium enriched in N^{15}. This idea should allow the production of labelled proteins for the study of their fine structure and mechanisms of interactions, giving additional knowledge on the mechanisms of wine protein haze formation in white wines.

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CONCLUSION AND PROSPECTIVE

Conclusions and prospective:

The work here presented can be located within the research aimed to study the problem of protein instability in white wines and its consequences on the organoleptic quality. This phenomenon is a major problem in wine sector because the development of haze must be prevented before bottling. To prevent the problem, commonly winemakers treat the wine with bentonite, which adsorb and eliminate the unstable proteins from the wine. However, although very effective in doing that, this adsorbent is unspecific, and has significant impact on the organoleptic profile of wines, mainly because a portion of the aroma compounds is also removed. Therefore, find valid alternatives for proteins stabilization is a fundamental issue for the wine industry and represents a challenge for wine scientists. However, a rational approach to reach this goal needs a clear knowledge of the molecular mechanisms and colloidal phenomena involved in the haze formation and also of the relationships existing between wine proteins and aroma compounds.

The first part of the research work was focused on the molecular mechanisms of interaction between proteins and aroma compounds. Actually, an earlier work observed a greater removal of fatty acid ethyl esters (FAEE), which are important aroma components formed during fermentation, by bentonite treatment in the presence of isolated wine proteins (Vincenzi et al., 2015). Thus it was hypothesised that bentonite adsorbs aroma not only with a mechanism of direct interaction (Lambri et al., 2013), but also indirectly, because removes also the FAEE associated with the proteins.

This hypothesis has been confirmed here, by showing that ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate are able to interact with the wine proteins. This interaction has been demonstrated by synchrotron radiation circular dichroism (SRCD), which is a very sensitive instrument enable to study protein-ligand interaction. The results suggested only a slight modification of the secondary structure of protein after the addition of the esters. However, the study suggested the existence of an interaction between such aroma compounds and VVTL1, confirming the hypothesis previously reported (Vincenzi et al., 2015). Moreover, the results led to the conclusion that the protein-FAEE interaction can be driven by hydrophobic forces acting between aliphatic chain of esters and the hydrophobic regions of VVTL1.

However, since the protein tested in this work was identified as the stable isoform 4L5H, which has a minor ability to interact with hydrophobic molecules (Marangon et al., 2014), it could certainly be interesting to further investigate the interaction with other wine proteins such as chitinase or more hydrophobic isoforms of TLP.

In conclusion the results here reported indicate that fermentative aroma compounds are associated with proteins in wine and that bentonite treatment can also impact the organoleptic profile of wine removing the proteins combined with them, demonstrating for the first time the existence of an interaction between wine proteins an aroma compounds of fermentative origin. Therefore, from a practical point of view, bentonite treatments to stabilise white wines should probably have a lesser impact when performed on the must where fermentative aroma are not present and this can be an important indication for winemakers who want to maximally preserve the aroma formed by the yeast during wine fermentation.

The second part was focused on the mechanisms of wine haze formation, which is known to be associated to the protein insolubilization in the bottled wine. It is established that non-proteinaceous compounds are involved in this phenomenon and that polyphenols are probable candidate for that. However, their role in haze formation in white wines is still not well clarified. In particular, it not known whether the onset of delayed hazing is exclusively due to changes in protein structure (unfolding) or also to changes occurring in polyphenols over time, the most important of which are polymerisation. Tannin polymerisation, which is enhanced by the presence of oxygen, can actually increase the reactivity of tannins with proteins. Therefore, in order to better understand the contribute of tannins over time in the precipitation of proteins, the effects of some tannins purified from wine at different times and condition were studied via reconstitution experiments by adding them to the wine proteins. The tannins were purified few days after bottling (T0) and eight months after bottling from full-filled bottles (T8, normal oxygen content) and half-filled bottles (T8 OX, excess of oxygen,). The onset of turbidity, monitored by Dinamic light scattering, confirmed that, compared to the tannins at T0, which did not give any light scattering the evolved ones were able to interact with proteins and form aggregates, especially those purified from the bottle left half filled. This led at the conclusion that the evolution of tannins in the bottle increase their reactivity, contributing to protein aggregation in the bottled wine and this

is strongly dependent on the oxygen content, as expected. In addition, the specific effects of these tannins on two pure proteins (a stable isoform of VVTL1 (I/4L5H) and a chitinase class IV) were investigated by differential scanning calorimetry. This experiment showed that also the tannins at T0 had an effect, decreasing the temperature at which the VVTL1 unfolds.

In conclusion all these findings showed that the very small quantity of tannins presents in white wines, which never has been considered previously, can affect the proteins behaviour, leading to major changes which can be responsible for haze formation. Moreover, the tannins left in the bottle for eight months in the presence of oxygen showed the highest effect evidencing the capability to precipitate the VVTL1 and forming haze. In contrast, the chitinase evidenced turbidity and precipitation already when tested alone, confirming the major propensity to aggregate than VVTL1. This confirms a different role of the two major proteins of the wine and suggest that their behaviour has to be studied separately.

These data suggest an evolution of tannins reactivity with proteins in bottle, suggesting a fundamental role of oxygen and allowed to gain new information on the mechanisms of protein precipitation in bottled white wines.

The third study was carried out in order to deepen the relation existing between polyphenols and wine proteins. To do it, the behaviour of wine VVTL1 was investigated by SRCD in the presence of some standards polyphenols (procyanidin B1, B2, quercetin, rutin, tannic acid and a tannins extract of wine). The wine tannins extract significant affect both thermal and photo stability of VVTL1. The addition of polyphenols significant affected the CD spectra of protein, but suggested also the modification of polyphenols, this being an important indication of their interaction with the protein. Moreover, Procyanidins B1 and B2 showed a different effect on VVTL1, and this result led to the conclusion that also the type of tannin is relevant, and, more generally that tannin like substances actually have the ability to interact with a protein in wine, confirming the conclusion reported above.

Thanks to this work new information on the interaction between tannins and wine proteins at molecular level has been gained.

The last part of the thesis focused on the possibility to produce in vitro the wine proteins to be studied for characterisation and functionality. Due to the complex protein mixture their isolation from grapes or wine is traditionally a difficult task, mainly because interfering substances lead to structural modification of these proteins. Successful obtainment of the grape protein of interest (TLPs, chitinases and LTPs) was achieved starting from the berry pulp tissue cultivated in vitro. Compared to those reported in literature has been noted that the proportion among the grape-callus protein classes was different and in particular the Lipid Transfer Protein were more expressed than those reported in literature. The interest of this latter protein, never studied in relation to wine, mainly regards their allergenic proprieties, as previously demonstrated (Pastorello et al., 2003), but also their possible involvement in foam formation has been showed in beer (Stanislava et al., 2007). Moreover, the quantification of polyphenolic compounds revealed a very low concentration in the callus tissues. This imply that the proteins can be produced in form certainly free from attached polyphenols. But the main advantage of the expression of the grape proteins in vitro is that they can be grown in the presence of labelled nutrients that in this way can be easily incorporated into the molecules of the different components. In such a way labelled proteins ca be produced to be used for analysis, such as NMR with the aim to define which are the protein regions involved in the interaction with ligands.

In summary the method of grape protein production developed here will represent a major tool to be exploited for the molecular and functional characterisation of the wine proteins and to study in depth their effects on wine quality with all the possible practical repercussions for the wine industry.

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APPENDIX

APPENDIX A: Circular Dichroism

An optically active molecule (chiral molecule) can preferably absorb right ended circularly polarized light (ER) or left ended circularly polarized light (ES), the differential of absorption between the two components generate a circular dichroism signal (CD) (Fig. 1).

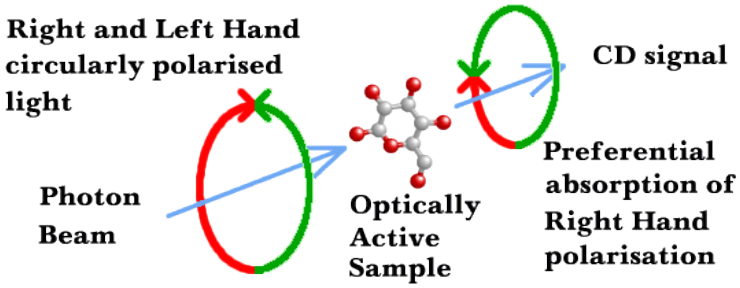

Fig. 1 *Scheme of Circular Dichroism*

All the amino acids with the only exception of glycine are chiral allowing the investigation by CD spectroscopy of protein structure. In proteins, the chromophores of interest include the peptide bond (absorption below 240 nm), aromatic amino acid side chains (absorption in the range 260 to 320 nm) and disulphide bonds (weak broad absorption bands centred around 260 nm. The most part of the studies focuses on the investigation of protein secondary structure in the Far-UV region. The absorption in this region is due principally to the peptide bond; there is a weak but broad n-π* transition centred around 220 nm and a more intense π-π* transition around 190 nm. Depending on the content of one of other secondary structure the protein assume characteristics CD spectra signals. Proteins with a predominance of α-helix are characterized by a large positive magnitude around 195 nm and two negative peaks at 208 and 220 nm with about half magnitude. For those composed by β-sheet the CD signal is approximately at half magnitude compared to those characteristics of helix conformation, and characterized by a positive peak around 192 nm and a negative peak at about 212 nm. Various other minor components such as turn, unordered or poly-proline helices (random coil) have characteristics CD spectra (Fig. 2).

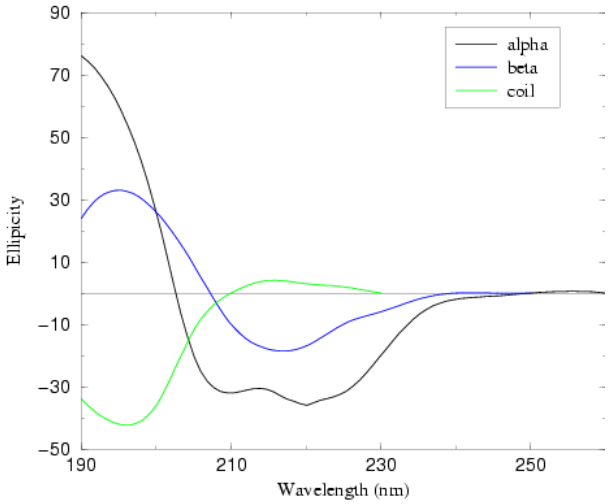

Fig. 2 *Characteristics CD spectra for the secondary structure components of proteins.*

The secondary structure of proteins can be estimated from the CD spectra using CONTIN, SELCON, and CDSSTR methods. However, the estimation of secondary structure is significantly more reliable when monitored at range below 170 nm and this represents a major problem for the conventional CD instruments: low photon flux, the occurrence of artefacts, single measurements, divergent beamline leads to low sensitive of protein structural studies in the far UV-region. This problem can be solved by the use of the Synchrotron Radiation Circular Dichroism (SRCD) (Fig. 3).

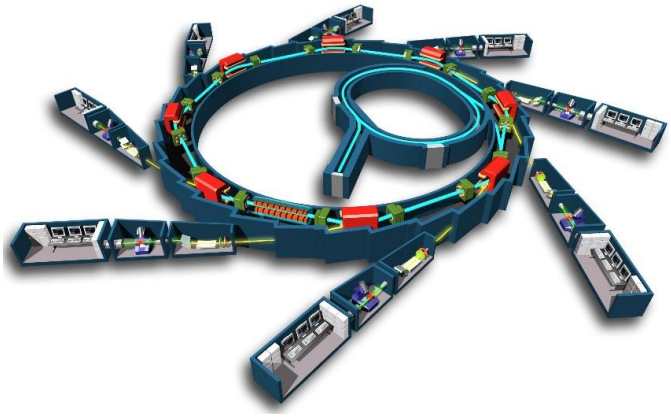

Fig. 3 *Synthetic scheme of a synchtron*

The synchtron radiation is the electromagnetic radiation generated when charged particles are radially accelerated. The light of synchtron promises high photon flux, broad spectrum (covers from microwave to hard X-rays), high brilliance (collimated photon beam) and high stability. Collectively these properties provide many advantages

in circular dichroism experiments such as the very little amount of sample required, short time-resolved measurements and minimization of noise.

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APPENDIX B: Dynamic Light Scattering

Dynamic light scattering, also called photon correlation spectroscopy (PCS), is a useful technique enable to investigate sphere particles in Brownian motion. A monochromatic light beam (i.e. laser) irradiate the particles in solutions that, being in motion, pass repeatedly through the light causing the phenomenon known as Doppler Shift (Fig. 1). Thanks to the assumption that the particles are spherical and in Brownian motion it is possible to calculate the diffusion constant (D), applying the formula based on Stokes-Einstein relation:

$$D=k_B T/6\pi\eta a$$

a Radius of the beads
K_B Boltzmann constant
T Temperature (K)
η Viscosity of the solution

The radius of beads (a) is none other than the hydrodinamic diameter (D_h) of particles. The light emitted by particles is converted in voltage variation by a photomultiplier (i.e. correlator). The result is expressed as a function of intensity fluctuation and time. Depending on the nature of particles the intensity of scattering (Is) assume distinct characteristics. Whether the particles in solution are small, the light is rapidly diffused in the solution assuming very rapid fluctuattion of intensity, whereas large particles move more slowly contributing to increase the scattered light. This fluctuation can be interpreted by the autocorellation function G(τ).

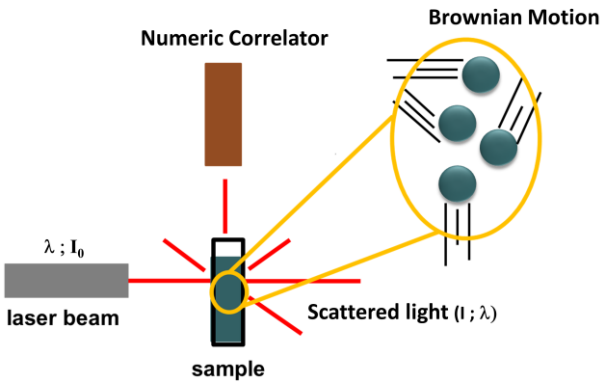

Fig. 1 *Synthetic scheme of operation of dynamic light scattering*

It is also possible measured the distribution of particle size in the background solvent through the calculation of polydispersity index (PI). It is defined as the variance of the distribution of particle size and ranging between 0 and 1. Values below 0.3 indicate relative monodisperse particles. This means that the size distribution is similar among the colloidal particles. On the other hand, if the value is above 0.3 the particles are polydisperse in the solvent. In this case the calculated size distribution is in advantage to the largest particles. The stability of colloidal system is dependent of size and concentration of the molecules in solution. A stable system no evidence increase in scattered light, whereas when a colloidal system such as a protein solution is unstable, the particles can aggregate and the diameter raise, scattering much more light. Sometimes they can generate metastable dispersion or phase separation leading to the formation of precipitates (Fig. 2).

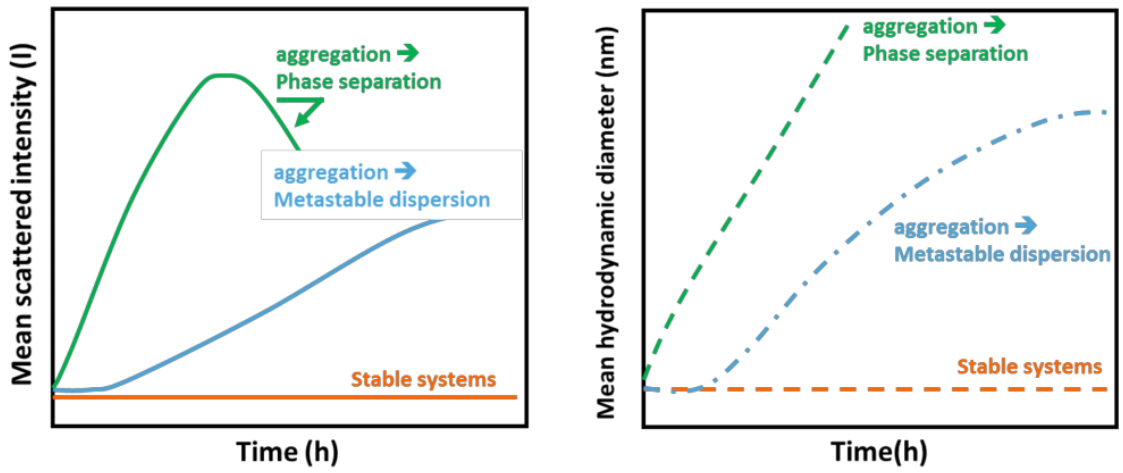

Fig. 2 *Stable and unstable systems observed from DLS assay*

DLS have several limitations that can be summarized as below:

- Size of particles: the upper limit of DLS is (8µm), however if the detected size of particles is routinely bigger than 1µm the laser diffraction must be considered.
- Effect of dust and impurity: misleading interpretation of data can be avoided filter the sample or use software able to eliminate noise.
- Sedimentation: when the experiment is performed in presence of dense particles.
- Low resolution: dynamic light scattering is not able to precisely assess a sample too polydisperse.

- Multiple light scattering: this occur when a particle overlaps another particle before light reaches the detector, resulting in very distortion results.

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APPENDIX C: Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a powerful tool to measure the heat energy generated during thermodynamic processes of proteins. Substantially energy is simultaneously introduced into the apparatus composed by the cell in which the protein of interest is dissolved and a reference cell (Fig.1). DSC measures the excess of heat energy generated during the thermodynamic process (DP = differential potential).

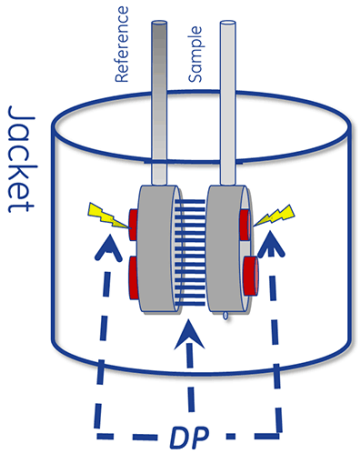

Fig. 1 *Scheme of DSC measuring cell*

Absorbed energy is indicative of endothermic process ($\Delta H > 0$), whereas release of energy is associated to exothermic process ($\Delta H < 0$). Changes in these parameters are associated to alteration of the forces stabilizing the native form of protein such as van der Waals, hydrophobic, and electrostatic interactions, hydrogen bonds, hydration of the exposed residues, conformational entropy, and the physical environment (i.e. pH, buffer, ionic strength, excipients). Exist an equilibrium in aqueous medium between native state and denatured state of protein, dependent to Gibbs free energy (ΔG) of the system and the thermodynamic parameters, which are enthalpy (ΔH) and entropy (ΔS).

$$\Delta G\,(T) = \Delta H(T) - T\Delta S(T)$$

The transition midpoint, also known as melting temperature (T_m) is considered the temperature equilibrium at which 50% of folded and unfolded protein are simultaneously present. Higher T_m values are indicative of more stable proteins.

Calorimetric enthalpy (ΔH_{cal}) is the total integrated zone below the thermogram peak, which indicates total heat energy uptake by the sample after suitable baseline correction affecting the transition. Van't Hoff enthalpy (ΔH_{VH}) is a thermodynamic parameter that

together the calorimetric enthalpy provides information on the transition state in particular when they are superimposable ($\Delta H_{VH} = \Delta H_{cal}$) the process evidence a two-state model transition (Fig. 2).

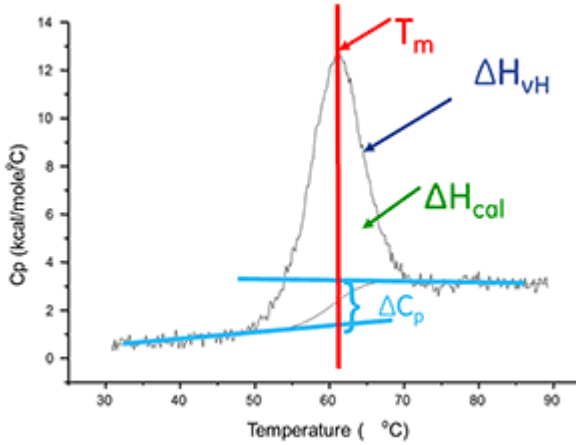

Fig. 2 *Thermodynamic parameters of a two-model transition*

References:

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