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Studies on grape, wine and grape seed proteins and development of methods for their biochemical and functional characterization

Studi sulle proteine dell'uva, del vino e dei vinaccioli e sviluppo di metodi per la loro caratterizzazione biochimica e funzionale.

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DATA CONSEGNA TESI

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Γένοιο οἷος εἶ

(Pindaro, Pitica II, v. 72)

THESIS SUMMARY

Grape and wine proteins represent a subject of study that in recent years has received increasing attention from the international research, mainly due to the fact that important issues on wine can be clarified through the study of the nature and the properties of these macromolecules. Among the reasons of increased concern for winemakers there is the problem of haze development in bottled white wines, known as “protein casse”, due to the presence of residual amounts of insoluble proteins that can become unstable and precipitate during wine storage, causing the appearance of sediments and turbidity. These precipitates are generally the result of denaturation and subsequent aggregation of heat-unstable wine proteins deriving from grapes and belonging to the functional category of plant pathogenesis-related (PR) proteins, namely thaumatin-like proteins (TLPs) and chitinases. It has been observed that these proteins are resistant to acidic pH, proteolysis and fermentation conditions, hence they survive the winemaking process.

In this thesis, firstly, the knowledge about chitinases was elaborated since recent scientific papers have described them as proteins potentially more susceptible to precipitation in white wines. In this context, several chitinase isoforms have been purified from Manzoni Bianco grape juice and their electrophoretic behaviour was characterized, deducing important functional and biochemical information on the properties of these enzymes.

Subsequently, the research has focused on white wine protein aggregation by means of an innovative instrument, the Izon qNano, for polydisperse nanoparticles detection and quantitation in heat-tested samples. In detail, the role towards aggregates formation upon heating played by TLPs, chitinase, phenolics and polysaccharides, all purified from the same unfined white wine, was investigated via reconstitution experiments to better understand the contribution of each compound on haze formation.

Taking into account both the high number and the big size of aggregates formed upon heating, the chitinase revealed to be easily unfolded by heat, thus making it more reactive with other wine macromolecules than TLPs. Among the latter, two isoforms showed to be more prone to form aggregates. It was then demonstrated that TLPs, being present in the starting wine at a much higher concentration than the chitinase, may

contribute to the problem of wine haze, even though recent studies revealed their secondary role in haze development.

Since the research in enology needs to find a precise method that allows an accurate quantification of the protein amount in wines and grape juices, in this study two colorimetric assays were compared: the Bradford method (based on the Coomassie Brilliant Blue, CBB) and the potassium dodecyl-sulphate (KDS) protein precipitation followed by the bicinchoninic acid assay (KDS/BCA).

Some main factors that can potentially affect protein quantification in wine and grape juices were analysed including ethanol, polyphenols and protein glycosylation. Moreover, the response of different proteins towards CBB and BCA reagents was studied. The Bradford assay did not prove to be accurate for wine protein quantification as it was affected by the presence of interfering substances in the matrices (ethanol and polyphenols) and by the aminoacid composition of the proteins tested. On the contrary, by applying the KDS/BCA method, the matrix didn't show any statistically significant effect on the slope of the protein calibration curve and there were less differences between the protein average responses. Furthermore, the BCA method, directly applied on the samples, was almost insensitive to the sugars present in glycoproteins and mannoproteins purified and resuspended in an aqueous medium.

Finally, the storage proteins expressed in the grape seed endosperms were studied systematically, through fractional extractions, electrophoretic analyses and mass spectrometry. These proteins are expressed independently from environmental conditions and their composition has been shown to be species-specific. In particular, the most represented proteins in grape seed endosperms were isolated and identified by mass spectrometry as 11S globulin-like proteins. For the first time, an apparent 7S globulin-like protein was discovered. Finally, it was verified that the doublet of 40 kDa, subunit of the 11S globulin-like protein of 65 kDa, according to its pronounced polymorphism, could be used as "molecular marker".

RIASSUNTO

Le proteine dell'uva e del vino rappresentano un argomento di studio che negli ultimi anni ha ricevuto crescente attenzione da parte della ricerca internazionale, soprattutto a causa del fatto che importanti aspetti enologici possono essere chiariti attraverso lo studio della natura e delle proprietà di queste macromolecole. Tra i motivi di maggiore apprensione per i produttori vi è il problema della formazione di torbidità nei vini bianchi imbottigliati, noto come "casse proteica" e dovuto alla presenza di quantità residue di proteine instabili che possono divenire insolubili e precipitare, durante lo stoccaggio dei vini, causando la comparsa di sedimenti e torbidità. Tali precipitati sono generalmente il risultato della denaturazione e successiva aggregazione delle proteine instabili del vino, identificate come derivanti dall'uva e appartenenti alla categoria funzionale delle proteine legate alla patogenesi (PR proteins) della pianta, in particolare proteine taumatina-simili (TLPs) e chitinasi. E' stato osservato che tali proteine sono resistenti a pH acidi, alla proteolisi e alle condizioni di fermentazione, risultando le più stabili al processo di vinificazione.

In questa tesi, in un primo momento, è stata approfondita la conoscenza delle chitinasi che recenti pubblicazioni hanno definito come le proteine potenzialmente più suscettibili alla precipitazione nei vini bianchi. In questo ambito, sono state purificate diverse isoforme di chitinasi dal mosto Manzoni Bianco ed è stato caratterizzato il loro comportamento elettroforetico, deducendo importanti informazioni sulle proprietà funzionali e biochimiche di questi enzimi.

Successivamente l'attività di ricerca si è focalizzata sullo studio dell'aggregazione proteica nei vini bianchi per mezzo di uno strumento innovativo, l'Izon qNano, in grado di individuare e quantificare nanoparticelle polidisperse in campioni testati al calore. In particolare, è stato analizzato il ruolo svolto dalle singole proteine (TLPs e chitinasi), dei polifenoli e dei polisaccaridi, tutti purificati dallo stesso vino, nella formazione di aggregati per mezzo di esperimenti di ricostituzione, con l'obiettivo di determinare il contributo di ogni componente alla formazione di torbidità, valutando le dimensioni e la concentrazione degli aggregati sviluppati nei campioni in seguito a riscaldamento.

Considerando sia l'elevato numero di aggregati formati che le notevoli dimensioni di questi ultimi, la chitinasi si è dimostrata facilmente denaturabile al calore e, come conseguenza di questo fatto, più reattiva con le altre macromolecole del vino rispetto alle taumatine. Tra queste ultime, due isoforme si sono rivelate particolarmente reattive.

Si è dimostrato pertanto che le TLPs, essendo tra l'altro presenti in quantità preponderante nel vino, possono contribuire al problema dell'intorbidamento anche se in misura minore rispetto alle chitinasi, nonostante studi recenti abbiamo rivelato un loro ruolo secondario nella formazione di torbidità.

Poiché è fondamentale nel campo della ricerca utilizzare un metodo preciso che stimi accuratamente la concentrazione delle proteine nei vini e nei mosti, è stato effettuato uno studio che ha messo a confronto due metodi colorimetrici per la quantificazione delle proteine nel vino: il metodo basato sulla colorazione di Bradford e il saggio dell'acido bicinconinico preceduto dalla metodica di precipitazione con potassio dodecyl solfato (KDS/BCA). Lo scopo di questo lavoro è stato quello di analizzare in dettaglio alcuni fattori che potenzialmente possono interferire nella quantificazione delle proteine nel vino, come l'etanolo, i polifenoli, la glicosilazione e la natura delle singole proteine. E' emerso che il metodo Bradford è inaffidabile nei confronti di una quantificazione proteica precisa in vino in quanto risente sia della presenza di sostanze interferenti nelle matrici (etanolo e polifenoli) che della composizione aminoacidica delle proteine utilizzate come standard. Di contro, il metodo BCA preceduto dalla precipitazione con KDS, si è dimostrato più affidabile in quanto le varie matrici non hanno influenzato la quantificazione e la differenza tra le risposte delle proteine è risultata più attenuata. Inoltre, la tecnica del BCA, applicata direttamente sui campioni, in quanto le mannoproteine non precipitano con KDS, è risultata pressoché insensibile nei confronti degli zuccheri presenti nelle mannoproteine purificate e risospese in mezzo acquoso.

Infine, sono state studiate sistematicamente, tramite estrazioni frazionate, analisi elettroforetiche e spettrometria di massa le proteine di riserva espresse nell'endosperma dei vinaccioli. Queste proteine vengono espresse indipendentemente dalle condizioni ambientali e sono tipiche delle diverse varietà. In particolare, sono state isolate ed identificate attraverso la spettrometria di massa le globuline 11S maggiormente rappresentate nell'endosperma dei vinaccioli e per la prima volta una probabile globulina

7S. Si è infine verificato che il doppietto di 40 kDa, subunità della proteina 11S di 65 kDa, visto il suo spiccato polimorfismo, può essere utilizzato come “marcatore molecolare” delle diverse varietà di *Vitis vinifera*.

LIST OF ABBREVIATIONS

AEC: Anion Exchange Chromatography;

BCA: *Bicinchoninic Acid;*

BSA: Bovin Serum Albumin;

CBB: Coomassie Brilliant Blue;

GC: Glycol Chitin;

HIC: Hydrophobic Interaction Chromatography;

KDS: *Potassium Dodecyl Sulfate;*

LTP: Lipid Transfer Protein;

LYS: Lysozyme;

Mr: Relative Molecular Mass;

MW: Molecular Weight;

MWCO: Molecular Weight Cut Off;

OVA: Ovalbumin;

PAS: Periodic Acid-Schiff staining;

PHE: Polyphenol;

pI: Isoelectric point;

PR proteins: Pathogenesis-Related proteins;

PS: Polysaccharide;

RMW: Real Model Wine;

RT: Retention Time;

SCX: Strong Cation Exchange Chromatography;

S-S: Disulphide (Bond);

THAU: *Thaumatin* from *Thaumatococcus daniellii*;

TLP: Thaumatin-Like Protein;

UF: Ultrafiltered;

VVTL: *Vitis vinifera* Thaumatin-Like protein.

CHAPTER 1

Literature review

INTRODUCTION

When talking about winemaking, many issues arise, linked to grape berry development, wine making and storage. The main concern for winemakers is the wine quality, which is determined by colour, clarity and organoleptic properties of wines (Ferreira *et al*., 2002) and in the case of sparkling ones, by the elegance of foam and effervescence (Blasco *et al*., 2011).

Proteins, with MW greater than 10 kDa (Hsu and Heatherbell, 1987a; Brissonet and Maujean, 1993; Waters *et al*., 2005) constitute one of the main grape juice and wine macromolecules together with polysaccharides and polyphenols. The most abundant proteins in grape juice are Pathogenesis-Related (PR) proteins, including chitinases and thaumatin-like proteins (Waters *et al*., 1996, 1998), along with invertase (Jégou *et al*., 2009). The same proteins are found in wines (Cilindre *et al*., 2008), in addition to mannoproteins coming from yeasts during the juice fermentation (Ferreira *et al*., 2002).

They play a leading part in the wine industry concern, despite their relatively low concentration, around ten to hundreds milligrams per litre (Bayly and Berg, 1967; Hsu and Heatherbell, 1987; Pocock and Waters, 1998). They are involved in a number of aspects that can impair the acceptance of the product by consumers, such as the haze formation in white wines attributed to the aggregation of some grape proteins, especially PR proteins (Pocock *et al*., 2007) during bottle storage. Therefore, treatments during the winemaking process, such as bentonite fining, have been used to lower the protein content in wine enhancing wine clarity and stability (Ferreira *et al*., 2002; Hoj *et al*., 2000). Proteins can however exhibit positive effects such as the stabilization of foam in sparkling wines (Senée *et al*., 1999; Girbau-Solà *et al*., 2002; Liger-Belair *et al*., 2008); their interaction with aroma compounds (Lubbers *et al*., 1994; Peng *et al*., 1997; Desportes *et al*., 2001; Jones *et al*., 2008) and the protection of wine against tartaric salt precipitation (Gerbaud *et al*., 1997; Moine-Ledoux *et al*., 1997). The phenomena in which proteins are involved are thus of

major interest but their understanding is not straightforward. They imply a full elucidation of grape juice and wine proteins and a deep characterization of each individual one.

This literature review contains a wide range of information concerning white wine protein occurrence, characterization and stabilisation as well as factors in wines affecting protein content and instability.

THE ORIGIN OF WINE PROTEINS

The origin of wine proteins is the subject that has occupied researchers since the fifties, with contradictory conclusions reported.

Wine proteins have long been considered a mixture of proteins from grapes and proteins from autolyzed yeasts (Ferreira *et al*., 2002). If the second option is worth, yeasts may affect the wine protein composition in two ways: through the transfer of proteins to the wine during the process of yeast autolysis and/or the presence of exocellular protease enzymes in the yeasts may contribute to the hydrolysis of the grape juice proteins (Feuillat *et al*., 1980). Bayly and Berg (1967) fermented a model juice solution and they concluded that the contribution by the yeasts to wine protein levels was negligible. Lee (1985) also suggested that the major source of wine protein is the grape and that the level of total protein is influenced by the grape variety, the stage of maturity and the pedoclimate conditions. Hsu and Heatherbell (1987a) concluded the same using polyacrylamide gel electrophoresis.

In more recent studies, Ferreira *et al*. (2000) showed that the vast majority of the polypeptides present in wines derive entirely from the grape pulp. Dambrouck *et al*. (2003) used modern immunological techniques to confirm that wine proteins originate predominantly from the grapes and many of them were glycoproteins. Some proteins came also from the yeasts and they were released during alcoholic fermentation and consisted of high molecular weight mannoproteins. In conflict with the authors mentioned above there was the opinion raised by Yokotsuka *et al*. (1991), who analysed the protein profile of *Vitis vinifera* var. orientalis cv. Koshu grapes as well as the resulting wine made from the same grapes. They found eight wine protein fractions not present in the juice and suggested they had come from yeasts.

Other sources contributing proteins to wine have also been identified. Kwon (2004) utilised nanohigh-performance liquid chromatography/tandem mass spectrometry to profile soluble proteins in a white wine. Twenty proteins were identified including five proteins derived from the grape, twelve from yeast, two from bacteria and one from fungi. However, the relative levels of proteins from microbiological sources were not established.

Tattersall *et al*. (1997) characterised a 24 kDa protein, *Vitis vinifera* thaumatin-like protein 1 (VvTL1), and found that it was highly expressed in conjunction with the onset of sugar accumulation and softening in the grape berry. Only the berry pulp and the berry skin extracts contained detectable amounts of VvTL proteins with the level of VvTL protein in the pulp extracts many times higher than the levels found in the berry skin. As above discussed, it is possible to affirm that proteins in wine are generally believed to come largely from grape berries (Marchal *et al*., 1996; Luguera *et al*., 1998).

Nevertheless, the proteins present in wines do not correspond to a representative fraction of the pulp proteins, since most of these are lost during vinification (Ferreira *et al*., 2000). Fermentation is primarily responsible for the difference between grape juice and wine protein content (Murphey *et al*., 1989). The lower protein levels typically found in wines are mainly due to proteolysis and denaturation of the grape proteins during fermentation, caused by proteases and changes in the pH, respectively (Bayly and Berg, 1967; Feuillat *et al*., 1980; Murphey *et al*., 1989). In fact, the proteins that end up in wines are those that are highly resistant to proteolysis and to the low pH values, characteristic of these beverages (Waters *et al*., 1992). In addition, it has been estimated that approximately half of total wine protein is bound to grape phenolics. During vinification, part of the soluble grape proteins are precipitated via interaction with tannins (Somers and Ziemelis, 1973).

CHARACTERIZATION OF WINE PROTEINS

The coming of modern analytical techniques improved considerably the knowledge about proteins in wine. At first, some researchers at UC Davis (Moretti and Berg, 1965; Bayly and Berg, 1967), by means of electrophoresis, were able to separate four different sized protein bands, with variable concentration within the same kind of wine and among wines from different cultivars of *V. vinifera*. They were also the first authors to hypothesize

that certain protein fractions, rather than total protein, could be responsible for protein instability in white wines. Somers and Ziemelis (1973) used size exclusion chromatography to separate wine proteins from other components and came to the conclusion that the wine protein size was between 10 and 50 kDa. Hsu *et al*. (1987), using polyvinylpolypyrrolidone to remove polyphenols from white wine before protein analysis, discovered many different protein fractions in the wide range of 11.2-65 kDa. A following study (Hsu and Heatherbell, 1987b) suggested that low molecular weight proteins (20-30 kDa) were the most important for haze formation in wines, compared to those with higher molecular weights. This assumption was later confirmed by Waters and colleagues (1991, 1992) who described two major wine protein fractions in *V. vinifera* cv. Muscat Gordo Blanco wine. By SDS-PAGE, these proteins had molecular masses of 24 and 32 kDa. By analysing the amino acid sequence of the proteins, Waters *et al*. (1996) showed that these 24 kDa and 32 kDa proteins shared high homology with thaumatins and chitinases respectively and were highly similar to other plant pathogenesis-related (PR) proteins. Besides, the 24 kDa fraction (thaumatin-like protein) gave rise to twice as much haze as the 32 kDa fraction (chitinase). In an electrospray mass spectrometry study of the proteins in the juice of 19 cultivars of *Vitis vinifera* (Hayasaka *et al*., 2001a) the range of masses 13-33 kDa was observed. The proteins were identified as mainly thaumatin-like proteins (range of 21,239-21,272 Da) and chitinases (range of 25,330-25,631 Da) and the small variations in the masses of the proteins were due to the robust method of varietal identification based on mass spectrometry. Moreover the molecular weight of identical proteins differed slightly depending on analytical methods used. For instance, the MW of thaumatin-like proteins and chitinases determined in SDS-PAGE was higher than that determined by mass spectrometry (Pocock *et al*., 2000).

Protein isoelectric point is another characteristic commonly studied. The isoelectric point of a protein is the pH at which the protein shows zero net charge and it is important because, at wine pH, wine proteins have a net positive charge. This allows their removal by bentonite (negatively charged) and might also have importance in interactions between the protein and other non-proteinaceous factors in haze production. Proteins with low isoelectric points were found to contribute significantly to total wine protein (Moretti and Berg, 1965) and to wine haze (Bayly and Berg, 1967). This work was confirmed by Hsu

and Heatherbell (1987a) who suggested, together with Lee (1985), that the majority of wine proteins have a low pI of 4-6. Dawes *et al*. (1994) fractionated wine proteins on the basis of their pI and found that proteins with high pI (7.0) developed a compact sediment; proteins with middle pI (from 5.94 to 4.65) flocculated a precipitate 4 to 5 times larger than that of the high pI group; proteins with low pI (< 4.65) formed a suspended haze. This observation led to the conclusion that other wine components, primarily phenolics, need to be considered to fully understand protein haze. An interaction effect between protein pI and wine pH on haze formation was also found with lower wine pH resulting in smaller particle size and when wine pH approached protein pI, more haze was formed (Batista *et al*., 2009).

Up to now, wines have been reported to contain polypeptides ranging in molecular mass from 9 to 62 kDa and isoelectric points from 3 to 9 (Brissonet and Maujean, 1993; Hsu and Heatherbell, 1987b; Lamikanra and Inyang, 1988). However, the vast majority of the wine proteins exhibit low molecular masses (20–30 kDa) and low isoelectric points (4.1<pI<5.8), possessing a net positive charge at the pH values encountered in wines (Brissonet and Maujean, 1993; Ferreira *et al*., 2000).

Proteins responsible for protein haze in the long term are, paradoxically, very stable themselves in the short term and survive the vinification process. It is, therefore, not surprising that wine proteins are highly resistant to low pH and proteolysis (Waters *et al*., 1992). This fact ensures that only proteins resistant to these conditions, such as PR proteins, survive the winemaking process, becoming the damaging proteins of wines (Ferreira *et al*., 2002). Limited proteolytic processing of the wine proteins can, however, occur during white table wine vinification (Waters *et al*., 1998) and during the Champagne winemaking process (Manteau *et al*., 2003).

Protein levels in white wine have been reported by several authors and have been shown to differ by variety. Lee (1985) reported a range of protein concentration from 18 to 81 mg L^{-1} in 14 wines from different Australian regions and made from different varieties. Some of these wines appeared to have been fined with bentonite prior to analysis. Pocock *et al*. (1998) reported concentrations in unfined Australian wines up to several hundred mg L^{-1}. Hsu and Heatherbell (1987b) found a range of 19-44 mg L^{-1} in four different unfined white wines, while a very large variation (20-260 mg L^{-1}) was noted by Bayly and Berg (1967). Typically, the protein content of unfined wines are in the range 15 to 230 mg L^{-1}

(Ferreira *et al*., 2002) and may be up to 300 mg L^{-1} (Waters *et al*., 2005). Juice and wine protein concentrations of up to 700 mg L^{-1} were also reported by Vincenzi *et al*. (2005a), depending on protein recovery and quantification methods.

PROTEIN HAZE FORMATION IN WHITE WINES

Winemakers constantly need to face the possible appearance of turbidity during the storage of white wines after bottling. This occurrence can be caused by the insolubilization of the grape proteins which remain in wine after the fermentation process (Ferreira *et al*., 2002; Waters *et al*., 2005). The proteins that are involved in this problem are pathogenesis-related (PR) proteins, namely thaumatin-like proteins and chitinases (Waters *et al*., 1996). Currently, protein instability in white wines is mainly seen as a two step phenomenon: protein unfolding, occurring under excessive temperatures caused by inappropriate storage conditions, and subsequent colloidal aggregation, related to intermolecular interactions (Dufrechou *et al*., 2010). The structural diversity of wine proteins that can lead to different conformational and colloidal stabilities is a crucial issue for the identification of the physicochemical mechanisms involved in haze formation (Dufrechou *et al*., 2010). Despite recent advances in this field of research, the protein stability/instability in wines remains a problem not fully explained. Issues regarding (i) the influence of wine composition and storage conditions (pH, ethanol content, ionic strength, presence of co-solutes) and (ii) the features of wine proteins (structure, molecular size, hydrophobicity) involved in their denaturation and interaction with polyphenols (Waters *et al*., 1995) and polysaccharides (Dupin *et al*., 2000; Carvalho *et al*., 2006), are not solved yet.

PROTEINS RESPONSIBLE FOR WINE HAZE

When plants are infected by pathogens, a number of genes encoding for proteins are transcriptionally activated and new proteins are synthesized. These proteins are called pathogenesis-related proteins. PR proteins have been defined as proteins encoded by the host plants but induced only in pathological or related situations, including fungal, bacterial, viral and viroid pathogens, nematodes and phytophagus insects (Antoniw *et al*., 1980). PR proteins were initially found to be typically acidic, of low molecular mass,

highly resistant to proteolytic degradation and to low pH values. The term PR-like protein was proposed to designate proteins that are present in healthy plants, being induced essentially in a developmentally controlled, tissue-specific manner. These proteins, which are not synthesized in response to pathogen infection, are predominantly basic (van Loon *et al*., 1990). The distinction between PR proteins and PR-like proteins became soon blurred.

The induction of some PR proteins under pathological conditions suggests, but does not prove, a role for these proteins in plant defence (van Loon, 1994). Therefore, these proteins have been generally considered as defence proteins, functioning in preventing or limiting pathogen invasion and spread. Nevertheless, if they are already present in a tissue, or if they have been induced in non-infected, distant tissues as a result of primary infection in the vicinity, then they confer an enhanced level of protection. PR proteins are also induced in response to various environmental stress factors, such as drought, salinity, wounding, heavy metals and plant growth regulators (Derckel *et al*., 1996; Xie *et al*., 1999; Yu *et al*., 2001).

In grapevine berries, PR proteins accumulate during the growing season (Tattersall *et al*., 2001). They are synthesized in healthy grape berries in a developmentally dependent way as a normal part of the ripening process, with véraison (the French term used by viticulturalists to denote the beginning of ripening) apparently being the trigger for PR gene expression (Ferreira *et al*., 2002; Robinson *et al*., 1997). The two most prominent soluble proteins accumulated in grapes during ripening have been identified as chitinase (PR3 family) and thaumatin-like proteins (PR5 family) (Robinson and Davies, 2000), with chitinase alone being reported to account for half of the soluble protein in ripe grapes (Waters *et al*., 1998). Sarry *et al*. (2004) found out that about the 19% of the total proteins from grape berry mesocarp belonged to the PR-protein group. Among these proteins, the most represented were TL proteins, chitinases, β-glucanases and an isoflavon reductase-like protein, probably involved in the synthesis of phytoalessins.

The total quantity of PR-proteins detectable in the ripe grape berries depends on the cultivar, pedoclimatic conditions where the vineyard is collocated and the agronomical practices (Ferreira *et al*., 2002, 2004). Also the post-harvest practices, as mechanical harvest, are known to lead to a general increase in the PR-protein content in the grape juice

because of the physical damages that mechanical operations can cause to plants (Pocock *et al*., 1998).

Chitinases (EC 3.2.1.14) represent the second largest group of antifungal proteins after the PR-1 family (Ferreira *et al*., 2007). They catalyse the hydrolytic cleavage of the β-1,4-glycoside bond present in chitin of N-acetyl-D-glucosamine (Kasprzewska, 2003). In general, these enzymes act most often as endochitinases and produce chito-oligosaccharides of 2-6 N-acetyl-D-glucosamine residues in length (Stintzi *et al*., 1993).

Chitinases have been found in a very wide range of organisms, containing or not chitin, such as viruses, bacteria, fungi, plants (gymnosperms and angiosperms) and animals (insects, snails, fish, amphibians and mammals) (Goormachtig *et al*., 1998). Chitinases, as many other PR proteins, may be synthesized in both a constitutive and an inducible manner. In fact, some chitinase forms are synthesized constitutively in healthy plants in a developmentally and tissue-specific way. Others are also up-regulated by biotic and abiotic stresses, such as fungal challenge, wounding, drought, cold, ozone, heavy metals, excessive salinity and UV-light, and treatment with phytohormones such as ethylene, jasmonic acid and salicylic acid (Kasprzewska, 2003). The antifungal activity displayed by many chitinases was initially assumed to derive from their ability to digest chitin, leading to a weakened fungal cell wall and subsequent cell lysis. However, recent evidence indicates that the mechanisms by which chitinases inhibit fungal growth seem to be more dependent on the presence of a chitin-binding domain than on chitinolytic activity (Ferreira *et al*., 2007).

Waters *et al*. (1998) found that chitinases account for 50% of the soluble proteins in the berries of the grape vine (*Vitis vinifera* L. Muscat of Alexandria). Four chitinases have been purified and characterized by both sequence and mass spectral analysis, showing extensive sequence similarity. Despite the presence of several chitinase isoforms, which can derive from the expression of different genes (Robinson *et al*., 1997) or from limited protein degradation (Waters *et al*., 1998), the most important chitinase isoform in grapes seems to be a class IV chitinase (containing a chitin-binding domain), which is highly expressed during ripening (Robinson *et al*., 1997).

The thaumatin-like proteins (TLPs) are basic, 24-kDa proteins belonging to the PR-5 family and sharing high sequence homology to thaumatin, a sweet-tasting (to humans)

protein from the South African Ketemfe berry bush (*Thaumatococcus danielli*) (van der Wel and Loeve, 1972). A 24-kDa TL protein is abundantly expressed in grapevine fruits in a berry- and ripening-specific manner (Tattersall *et al*., 1997) but TL proteins are also produced in plants under different stress conditions (Zhu *et al*., 1995).

They induce fungal cell leakiness presumably through a specific interaction with the plasma membrane that results in the formation of transmembrane pores (Kitajima and Sato, 1999; Roberts and Selitrennikoff, 1990). These proteins have also been reported to possess β-1,3-glucanase activity (Grenier *et al*., 1999) or bind to actin (Takemoto *et al*., 1997). The proteins exhibit antifungal activity in vitro (Liu *et al*., 1994; Melchers *et al*., 1993; Woloshuk *et al*., 1991) and show enhanced lytic activity when tested in combination with chitinases and/or β-1,3-glucanases (Lorito *et al*., 1996). The TL proteins are, after the chitinases, the second most prominent grape and wine proteins (Waters *et al*., 1998; Pocock *et al*., 2000).

In addition to these two main groups of grape PR-proteins, there are also invertases, Lipid Transfer Proteins (LTP) and β-glucanase. The grape invertase is a protein of 62-64 kDa. It is a N-glycoprotein originating from the plant, as demonstrated by using immunological methods. This enzyme keeps its activity in wine and presents a high hydrophobicity (1050 kcal/100 amino acid residues) and a pI of 3.9 (Marchal *et al*., 1996). The grape invertase is believed to be one of the most abundant proteins in wine (from 9 to 14% of the total protein content of a Chardonnay wine) (Puff *et al*., 2001), and to possess a pI close to the pH of wine and a high hydrophobicity, potentially confering good surface properties on this protein (Puff *et al*., 2001). Plant lipid transfer proteins (LTPs; PR-14) are small, basic proteins, stabilized by four disulphide bonds, which transfer phospholipids between membranes. LTPs contain typically an internal, tunnel-like hydrophobic cavity that runs through the molecule (Cheng *et al*., 2004; Selitrennikoff, 2001). The mechanism responsible for their antifungal activity remains unknown, although it was suggested that these proteins insert themselves into the fungal cell membrane with their central hydrophobic cavity forming a pore, allowing efflux of intracellular ions and leading to fungal cell death (Selitrennikoff, 2001). A LTP of 9 kDa with high homology to that of peach has been discovered and indicated as the main grape and wine allergen (Pastorello *et al*., 2003). Plant β-1,3-glucanases and their homologues are known as PR-2-type proteins

(van Loon *et al*., 1994). They are induced upon pathogen attack (Menu-Bouaouiche *et al*., 2003) and wounding treatment (Derckel *et al*., 1998), but their activity was low or undetectable in pre-veraison berries (Jacobs *et al*., 1999).

FACTORS IN WINE AFFECTING WINE PROTEIN INSTABILITY

Although pathogenesis-related proteins from the grape are considered certainly a prerequisite for haze formation, several papers in the literature suggest that other wine components are involved in wine protein instability.

The phenolic compounds are obvious candidates as it is well established that they are involved in protein hazes in beer and fruit juices (Siebert, 1999). The interaction of grape protein with tannin was suggested more than 40 years ago (Koch and Sajak, 1959) and, Somers and Ziemelis (1973) proposed that up to 50% of white wine protein was bound to flavonoid material. They used this information to explain the variations noted by Bayly and Berg (1967) in protein stability among wines with similar total protein concentrations, and they speculated that protein haze is due to the fractions of residual wine proteins which have been rendered prone to precipitation by interaction with phenolics. Yokotsuka and colleagues (1983) found that tannins isolated from wines interacted with isolated must proteins to form a haze and that proteins isolated from grape must did not produce a visible haze in the presence of non-tannin phenolics from wine. These studies were not, however, undertaken under conditions identical to those commonly encountered in commercial white wines. Waters *et al*. (1995) reported that both heat-induced and natural haze contained procyanidins with a content ranging from 0.02 to 4.9% (w/w). The presence of procyanidins was necessary for wine proteins to form turbidity as wine proteins alone (isolated and back added to a model wine) did not cause turbidity (Waters *et al*., 1995). Polyphenols carry no charge or negligible negative charges at the wine pH, so that the major interactions should involve hydrogen bonding and hydrophobic interactions, as it was shown by Vernhet *et al*. (1996) in the case of the complexation between proanthocyanidins and proteins.

In model system studies, the amount of haze formed depended both on the concentrations of protein and polyphenol and on their ratio, and a conceptual model for the interaction between haze-active polyphanol and haze-active protein was proposed (Siebert

et al., 1996a). Briefly, “haze-active” polyphenols are thought to have at least two sites that can bind to proteins, and “haze-active” proteins have a finite number of sites which polyphenols can bind to. Thus, the largest network, corresponding to the largest particle size and the greatest light scattering, would occur when the number of polyphenol binding sites matches the number of protein binding sites, whereas either protein-rich or polyphenol-rich solutions result in smaller particles and less light scattering (Siebert *et al*., 1996a). The analysis of a natural precipitate from a Sauvignon Blanc wine revealed that proteins (mainly VVTL1 proteins) and phenolics only contributed for 10% and 7% of the dry weight of the precipitate, respectively, with the remaining part represented by polysaccharides (4%) and other unknown components (Esteruelas *et al*., 2009).

The effect of wine polysaccharides on protein haze has also been documented. Some wine polysaccharides (such as yeast mannoproteins, and grape arabinogalactan proteins and rhamno-galacturonans) carry negative charges in the wine pH range. As a consequence, these wine polysaccharides may establish electrostatic and ionic interactions with other wine components (Vernhet *et al*., 1996), resulting in the formation of either soluble or insoluble complexes in a process that is strongly dependent on their net electrical charge and on the structure of their functional groups (Samant *et al*., 1993). Fifteen different polysaccharides from different sources were added to wines before protein hazes were induced, and they either did not affect or increase protein haze levels (Pellerin *et al*., 1994). Another study showed that polysaccharides increased protein instability, particularly at moderate to high temperatures (Mesquita *et al*., 2001). However, the level of polysaccharide in both studies was much greater than that reported (Doco *et al*., 2003) in wines. A multifactorial study (Fenchak, 2002) showed a particular polysaccharide (pectin) to be important in haze formation. However, because pectolytic enzymes are commonly used in white winemaking and ethanol precipitates pectins, the levels of pectins in commercial white wines are very low. Waters and colleagues (1993, 1994a, 1994b) describe the effects of the yeast-derived mannoprotein ‘haze-protective factor’ that protects wines from protein haze. This polysaccharide is seen as an exciting prospect for preventing protein haze formation in white wine (Waters *et al*., 2005).

pH and ethanol content are other two factors often implicated in protein haze formation. Maximum light scattering was detected at pH 4-4.5 for a model solution when

ethanol content was 12% (v/v) (Siebert *et al*. 1996b). The proteins in the Arinto wine were heat unstable between pH 2.8 and 6, but they became rapidly resistant to heat precipitation above pH 6. In contrast, the isolated Arinto wine proteins dissolved in water showed a broad peak of instability centered around pH 4.0 when subjected to the heat stability test, becoming gradually more stable towards lower and higher pH values and reaching heat stability at pH 2.8 and pH 6.0. Not surprisingly, this peak of instability coincided with the isoelectric point of most Arinto wine proteins, as determined by two-dimensional electrophoresis (Batista *et al*., 2009). Furthermore, in a model solution study, turbidity gradually increased (100 to 120 NTU) in response to increases in ethanol content (6 to 12% v/v) (Siebert *et al*. 1996b). In real wine, however, this ethanol effect may not influence wine protein instability. It was found that a wine (12.1% v/v ethanol) with slight alcohol additions (up to 2% v/v) did not interfere with the haze formation profile to heat tests (Mesquita *et al*. 2001). This was also supported by Sarmento *et al*. (2000) whose results indicated that the ethanol concentration showed no significant effect on turbidity development in wines.

Pocock *et al*. (2007) pointed out that sulphate was the unknown essential factor promoting haze formation in the absence of phenolic compounds in a model wine. The role assigned to sulphate was the acceleration of protein denaturation and/or competition between sulphate anions and proteins at wine alcohol concentrations for water of solvation that caused a loss of water from the protein surface, resulting in the protein aggregation (Pocock *et al*. 2007). Besides, the two main wine proteins, thaumatin-like proteins and chitinase, differed in their haze response in model wines containing sulphate.

PROTEIN STABILISATION TREATMENTS IN WINES

The addition of bentonite, a montmorillonite clay, is universally employed throughout the wine industry for the prevention of white wine protein haze, in a process known as bentonite fining. Bentonite, which carries a net negative charge at the pH of wine, interacts electrostatically with the positively charged wine proteins, inducing their flocculation (Lambri *et al*., 2010; Sauvage *et al*., 2010). Bentonite has been shown to be non specific for proteins, as it also removes other charged species or aggregates (Lambri *et al*., 2010). Some authors affirm that this treatment does not lead to sensible variations of

the aromatic profile of wines (Leske *et al*., 1995; Pocock *et al*., 2003), while others stated that bentonite addition on grape juices and wines leads to a decrease of aromatic compounds concentration (Rankine, 2007; Pollnitz *et al*., 2003). However, it is generally assumed that bentonite fining at typical addition rates has a detrimental effect on wine aroma and flavour. Moreover, it is not clear whether bentonite removes some protein fractions selectively (Sauvage *et al*., 2010) or whether this removal changes as a function of matrix parameters (Achaerandio *et al*., 2001; Batista *et al*., 2009). Sauvage *et al*. (2010) studied the sensitivities of specific protein fractions to heat treatment and correlated these sensitivities with their susceptibilities to bentonite adsorption.

Furthermore, because of bentonite swelling and poor settling characteristics, 3−10% of the wine volume is taken up by it and the quality of this "lees" wine is reduced (Tattersall *et al.*, 2001). In addition, handling and disposal of spent bentonite continues to be of concern, because of high labour input and associated costs, occupational health and safety issues, and the wine industry's environmental responsibilities (Høj *et al.*, 2000). Therefore, an increasing interest in alternative practices to bentonite fining for protein stability has been developed. Considerable attention was given to the use of proteolytic enzymes (Lagace and Bisson, 1990) during short term heat exposure, to induce PR protein degradation (hydrolysis into small peptides and their component amino acids). However, proteases seem not to be able to effectively degrade grape PR-proteins because of their intrinsic resistance to proteolysis and for the unfavourable conditions for the enzyme activity existing in winemaking conditions (low temperatures) (Waters *et al.*, 1992). Other methods for white wine stabilization alternative to bentonite fining are ultrafiltration (Hsu *et al*., 1987) and protein adsorption on different solid matrices (Vincenzi *et al.*, 2005b).

METHODS FOR THE QUANTIFICATION OF GRAPE JUICE AND WINE PROTEINS

Many different techniques are available for protein quantification in general, but three major disadvantages when dealing with wine and grape juice samples hinder the use of some of them (Moreno-Arribas *et al*., 2002). Proteins are typically present at very low concentrations in these media, which leads to the use of techniques with very low detection limits. The presence of interfering substances, such as phenolic compounds and ethanol can

distort the quantification (Marchal *et al*., 1997). Moreover, the absence of standard grape or wine proteins does not allow their direct quantification in a sample (Le Bourse *et al*., 2010).

In the literature, the most common technique for grape juice and wine proteins quantification is the Bradford method (Bradford, 1976) due to its simplicity, reproducibility and rapidity (Moreno-Arribas *et al*., 2002). Three forms (cationic, neutral and anionic species) of Coomassie dye exist in equilibrium with λ max at 470, 650 and 595 nm, respectively, and the development of the colour relies on the anionic species binding to proteins, resulting in absorbance increase at 595 nm (Compton and Jones, 1985). Disadvantages of this assay are the response variation of the reagent to different proteins (Ahmed, 2005), underestimation of protein concentration due to interfering substances in the medium (Waters *et al*., 1991) and longer incubation time (Murphey *et al*., 1989). In particular, Marchal *et al*. (1997) considered the interferences which may falsify the estimation of direct measurement of proteins with the Bradford method in Champagne Pinot Noir and Chardonnay wines and established that ethanol and exogenous and endogenous phenolic compounds seriously impaired the quantification of the wine protein content. Other colorimetric methods, such as Lowry (1951), Biuret (Gornall *et al*., 1949) or Smith (also called bicinchoninic acid method assay) (Smith *et al*., 1985) can provide interesting results despite their potential interferences with other compounds (Fusi *et al*., 2010; Moreno-Arribas *et al*., 2002).

Assuming that a rational approach to eliminate the interfering compounds might be to separate the proteins from the wine before proceeding with quantification, Vincenzi *et al*. (2005a) developed a new procedure for protein recovery and quantification in wine by consecutive addition of SDS and potassium chloride (KCl). The KDS-protein complexes so recovered were precisely quantified by the Smith assay, in accordance to the quantifications obtained by densitometric quantification of protein bands from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In addition to methods that allow total protein quantification in a sample, two other techniques can lead to the quantification of an individual protein in crude or pure sample. The most powerful method is the determination of protein concentration and purity by RP-HPLC, as described by Marangon *et al*. (2009). The quantification of individual proteins was achieved through comparison of their peak area to the peak area of two standard

proteins (cytochrome c and bovine serum albumin). Other techniques, such as densitometric measurement of bands from SDS-PAGE gels (after Coomassie Brilliant Blue or other stains, or antibody immunostaining) were described by Marchal *et al*. (2000) and Hsu and Heatherbell (1987a).

PREPARATIVE TECHNIQUES FOR FRACTIONATION AND PURIFICATION OF GRAPE JUICE AND WINE PROTEINS

After isolation and concentration procedures, proteins can undergo separation and characterization steps. This approach allows the study of individual purified proteins, in terms of structure and functional properties (Le Bourse *et al*., 2010).

Chromatography has become a key tool for the study of proteins and it is currently involved in the first steps of purification protocols. Fast protein liquid chromatography (FPLC) can be used with several techniques: ion exchange, hydrophobic interaction, affinity, gel filtration/size exclusion and chromatofocusing. The form of the sample loaded on the chromatography column varies according to each author, as well as the conditions of elution and detection techniques. Researchers can set up a one step chromatography technique or use a combination of two or more methods to take advantages of each one and thus achieve an efficient purification (Le Bourse *et al*., 2010). Different chromatographic techniques have been used for fractionation and purification of grape juice and wine proteins depending on the proteins characteristics. A wide number of authors used ion exchange chromatography for grape juice or wine proteins first fractionation (Luguera *et al*., 1998; Monteiro *et al*., 2007; Muhlack *et al*., 2007). It is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins are usually eluted by a continuous or stepwise salt gradient (NaCl). Both strong and weak cation and anion exchange techniques can be used. For instance, Jégou *et al*. (2009) used anion exchange FPLC to purify the grape vacuolar invertase, involving the use of a 7.5 pH elution buffer. On the other hand, Van Sluyter *et al*. (2009) used cation exchange FPLC for bulk grape juice fractionation. Cation exchange medium allows the use of an elution buffer pH close to original wine pH of around 3.0 (Ferreira *et al*., 2002). As a second step, they developed a method to purify thaumatin-like proteins and chitinases by

hydrophobic interaction chromatography (HIC), based on a previous work (Marangon *et al*., 2009). HIC separates proteins with differences in hydrophobicity.

HPLC, especially size exclusion (HPSEC), is also used by some authors to study proteins and peptides in berries, grape juice and wine (Moreno-Arribas *et al*., 1996; Yokotsuka and Singleton, 1997; Gonçalves *et al*., 2002; Marangon *et al*., 2009). Pocock *et al*. (2000) used for instance a semipreparative C18 HPLC column for the purification and characterization of different grape juice and wine proteins.

Affinity chromatography is also a common second step after ion exchange chromatography. Waters *et al*. (1993) separated a haze protective factor, a macromolecule fraction made up of a polysaccharide and a protein component, from other wine macromolecules by a combination of ConA and anion and cation exchange chromatography. Gel filtration chromatography is also used to separate proteins with different molecular size. It was set up a single step purification by Esteruelas *et al*. (2009) to isolate the natural haze protein in white wine. In addition to chromatographic techniques, electrophoresis is often used on preparative scale to study proteins. For instance, Dorrestein *et al*. (1995) used FPLC and PAGE to compare and analyze the soluble proteins of four white wines.

To date, there are only few references in the literature on the application of capillary electrophoresis (CE) techniques to grape juice or wine proteins (Luguera *et al*., 1998; Dizy and Bisson, 1999). CE can separate proteins according to their isoelectric point, molecular mass, or charge/mass ratio. A fast analysis of proteins in wines by capillary gel electrophoresis (CGE) has been performed by Rodríguez-Delgado *et al*. (2002) for the first time. The separation in CGE is based on a molecular sieving mechanism with large molecules being retardated. CGE is thus based on the same separation principle than SDS-PAGE, but displays certain advantages over it: rapid analysis time, low sample volume consumption, as well as automatic evaluation and quantification of the separated protein peaks (Guttman, 1996).

ANALYTICAL TECHNIQUES FOR GRAPE JUICE AND WINE PROTEINS

The fractions collected after the purification step can be characterized by different means. Waters and co-workers (Girbau *et al*., 2004; Marangon *et al*., 2009; Van Sluyter *et*

al., 2009) used RP-HPLC, based on hydrophobic interactions, to identify grape and wine proteins by the determination of retention time of individual proteins. Proteins are eluted in function of increasing hydrophobicity, using an elution gradient with decreasing polarity. This technique allows the determination of the fraction purity of the fraction, its identity, as well as its quantification, as described by Waters and co-workers (Marangon *et al*., 2009; Van Sluyter *et al*., 2009).

Electrophoresis can assess fraction purity and estimate proteins molecular masses and pIs (Le Bourse *et al*., 2010). Most of the studies on grape and wine proteins have been carried out using the conventional electrophoretic methods of native and SDS-PAGE and IEF. Actually, Cilindre *et al*. (2008) interestingly developed the use of two-dimensional electrophoresis (2D-E) and nano-LC-MS/MS and set up the first steps of proteomic approach to study wine proteins, in relation with *B. cinerea* infection. Vincenzi and Curioni (2005) revealed an anomalous electrophoretic behavior of a chitinase isoform in glycol chitin-containing SDS-PAGE gels. A progressive shift of the relative molecular mass of this enzyme (from 30,500 up to 57,700 Da) with increasing glycol chitin concentration in the gels up to 0.1% was revealed.

A separation of wine proteins by 2-DE and their identification by nano-LC-MS/MS or matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, as described by Cilindre *et al*. (2008) and Sauvage *et al*. (2010), or by N-terminal aminoacid sequences analysis, as described by Okuda *et al*. (2006), opens the way towards further proteomic developments.

Proteins separated by gel electrophoresis can be electrophoretically transferred onto a membrane to carry out immunodetection assays, such as western blot. Grape or wine proteins (bulk or purified after preparative technique) are utilized as antigens to immunize rabbits, by methods that ensure the production of highly specific polyclonal antibodies (Le Bourse *et al*., 2010).

PROTEOMIC ANALYSES OF GRAPE JUICE AND WINE PROTEINS

More recently proteomic approaches have been used to have a better understanding of grape juice and wine proteins characteristics.

First of all, MS can provide complementary information to SDS-PAGE by assessing molecular masses (Monteiro *et al*., 2003; Pocock *et al*., 2000) and providing structural information (identification of proteins). Different MS techniques have been successfully applied to study the grape and wine proteins. Liquid chromatography electrospray ionization LC-ESI/MS and nano-LC/MS have been used to identify grape and wine proteins and peptides (Jégou *et al*., 2009; Marangon *et al*., 2009; Van Sluyter *et al*., 2009; Wigand *et al*., 2009). Protein and peptide fingerprinting was achieved by different authors using MALDI-TOF and surface-enhanced laser desorption/ionization TOF (Weiss *et al*., 1998; Sauvage *et al*., 2010; Chambery *et al*., 2009).

MS is a powerful tool for amino acid sequence determination, in link with HPLC for amino acid analysis (Waters *et al*., 1992; Marchal *et al*., 1996; Waters *et al*., 1996; Muhlack *et al*., 2007). For the latter, purified proteins are subjected to SDS-PAGE and then electroblotted to a polyvinylidene fluoride (PVDF) membrane. Protein bands are visualized with stain and excised from the membrane. They then undergo Edman degradation (protein sequencer on line with HPLC analyzer). Waters and coworkers (1998) also offered a further contribution to the amino acid composition of that protein material presenting a sequence analysis of grape berry chitinases.

A main tool for the study of the three dimensional structure of a protein is the determination of its crystal structure. Van Sluyter *et al*. (2009) recently managed to produce four crystals of thaumatin-like protein isoforms. These crystals need to be subjected to X-rays for structural determinations. Protein structure can also be determined by other techniques. *Gonçalves et al*. (2002) examined the structure of a white wine mannoprotein by 1H and 13C nuclear magnetic resonance (NMR) spectroscopic techniques such as 1-D or 2-D total correlation spectroscopy (TOCSY) and 2-D heteronuclear multiple quantum coherence (HMQC). Although NMR allows the determination of three-dimensional structure in a liquid form, assessed to be more accurate than the one determined by crystallography, no reports of the use of this technique were found in the literature for grape proteins.

Recently, Falconer *et al*. (2010) published an innovating work, which provides a great contribution to the study of the secondary structure study of wine proteins. They used

differential scanning calorimetry and circular dichroism spectrometry to study the role of chitinase in wine protein haze and the kinetics of its unfolding.

BIOCHEMICAL PROPERTIES OF PLANT STORAGE PROTEINS

The fractionation of seed storage proteins based on solubility criteria (Osborne, 1924) is not absolute but is still used for convenience (Tandang-Silvas *et al.*, 2011). Seed storage proteins are classified into (*a*) albumins or water soluble fraction, (*b*) globulins or salt soluble fraction, (*c*) prolamins or alcohol-soluble fraction and (*d*) glutelins or dilute acid/alkali fraction (Tandang-Silvas *et al.*, 2011). Globulins are subdivided based on their sedimentation coefficients into 7S and 11S, the most extensively studied seed storage proteins because of their predominance in nature. The 7S globulins are trimer molecules with molecular weights of 150–200 kDa and have 40–70 kDa monomers. The 11S globulins, on the other hand, are hexamer molecules involving two trimers, have molecular weights of 300-400 kDa, and have 50-60 kDa monomers (Utsumi, 1992). They are synthesized, processed and accumulated during seed development. Their monomers are translated into a single prepropeptide in the rough endoplasmic reticulum. After cotranslational cleavage of the signal peptide in the endoplasmic reticulum, the propeptides accordingly assemble into trimers (Tandang-Silvas *et al.*, 2011). Unlike 11S globulins, 7S globulins are generally cotranslationally glycosylated at Asn residues of the consensus sequence Asn-X-Ser/Thr (Katsube *et al*., 1998). Glycosylated (Derbyshire *et al*., 1976) and unglycosylated (Kimura *et al*., 2008) pea 7S globulins and unglycosylated coconut 7S globulins (Garcia *et al*., 2005) have been reported as well. The 7S globulins are usually lacking in cysteine residues, hence, they are devoid of disulfide bridges. On the contrary, 11S globulins have two conserved disulfide bridges. Storage proteins are kept indefinitely in mature seeds in various organelles depending on the crop. Rice accumulates prolamins in protein bodies (Shewry and Halford, 2002) whereas soybeans store 7S and 11S globulins in protein storage vacuole (Mori *et al*., 2009). At the onset of germination, they are rapidly mobilized, used, and depleted.

CHARACTERIZATION OF *VITIS VINIFERA* GRAPE SEED STORAGE PROTEINS

To date, the majority of studies on grape seed proteins has focused on the optimisation of protein extraction, the protein composition of grape seeds (Gianazza *et al*., 1989; Zhou *et al*., 2010) and grape varieties differentiation by seed protein composition (Pesavento *et al*., 2008; Bertazzo *et al*., 2010). In the chemical approach to systematics, seeds have long been recognized as the most suitable material. In fact they correspond to a well defined step in the vegetable cycle and their composition has been shown to be species-specific and invariant under different growing conditions (Ladizinsky, 1983). Moreover, studies on physicochemical and functional properties of grape storage proteins were carried out by Zhou and colleagues (2011) to discover whether grape storage proteins can be exploited as potential food additives.

One of the problems in the analysis of seed proteins is the presence of interfering material (Gianazza *et al*., 1989) including tannins, which can bind and remove some specific seed proteins. Gianazza and coworkers (1989) showed that a protein extraction from entire seeds did not permit to obtain a sample suitable for separation in SDS-PAGE. Applying the protein extraction directly to a fraction represented by the endosperm deprived of the seed coat, they were able to determine the chemical parameters (charge, mass, subunit structure) and some biological activities of the endosperm proteins from *V. vinifera* seeds. In particular, they discovered that the major protein of the grape endosperm is a globulin with *Mr* 65 kDa, which in turn is composed of disulfide-bridged peptides, *Mr* 19-21 kDa and 38-44 kDa. Besides, the quali-quantitative variability among proteins extracted from individual seeds accounted for approximately 10%; they deduced that only large samples, including 20-30 seeds, were thus likely to be representative of the genetic set-up of a given Vitis clone.

In the study carried out by Zhou *et al*. (2010), an 11S globulin-like protein was isolated and purified from grape (*Vitis vinifera* L.) seeds by two consecutive cation exchange and size exclusion chromatography. The protein consisted of two subunits with molecular masses of 25.5 and 40.0 kDa, respectively.

Pesavento and co-workers (2008) proposed a method potentially suitable for the grape varieties differentiation based on the analysis MALDI/MS of the grape seed proteins. The hydrosoluble protein profiles of seed extract from three different *Vitis vinifera* grape

(red and white) varieties were analyzed and compared. In order to evaluate the environmental conditions and harvest effects, the seed protein profiles of one grape variety from different locations and harvests were studied. The results obtained seemed to prove that MALDI/MS can well characterize different grape varieties on the basis of the protein profile contained in the grape seeds.

Bertazzo and coworkers (2010) evaluated the power of seed protein profiles obtained by matrix-assisted laser desorption/ionization MALDI/MS for parentage investigation. The three cultivars considered lead to very similar spectra with differences in the relative intensity of the most abundant species. The results provided evidence for the ability of MALDI/MS to individuate minor differences in protein profiles of complex protein mixtures.

Grimplet and colleagues (2009) investigated the tissue-specific differences in protein using pericarp (skin and pulp) and seeds of berries from vines grown under well-watered and water-deficit stress conditions. Of 1047 proteins surveyed from pericarp by 2-D PAGE, only 90 (8.6%) identified proteins showed differential expression between the skin and pulp. Of 695 proteins surveyed from seed tissue, 163 were identified and revealed that the seed and pericarp proteomes were nearly completely distinct from one another. Only 19/163 proteins had the proteome in common, i.e. over 88% of the grape seeds proteins were tissue-specific. Moreover, water-deficit stress altered the abundance of approximately 7% of pericarp proteins, but had little effect on seed protein expression.

RESEARCH OBJECTIVES

This thesis aims to improve the knowledge on grape, wine and grape seed proteins by applying and developing methods for their biochemical and functional characterization.

Some studies indicated that the chitinases are the major haze-forming protein and they are more prone to form visible haze in model wine than thaumatin-like proteins. They also revealed the high number of chitinase isoforms present in grape juice and wine. For this reason, these enzymes were purified from grape juice and characterized for their better knowledge.

The nature of wine protein instability is sometimes difficult to explain due to the many factors involved. Proteins differ as a result of grape variety, maturity, climate, molecular size, and electrical charge. To make the problem even more complex, wine proteins can interact and precipitate with other components, usually forming complexes of protein, polyphenol and polysaccharide. The goal of the second work was to examine, *via* reconstitution experiments, both the size and concentration of individual aggregates formed by five purified wine proteins when heated in presence or absence of wine phenolics and polysaccharides. This lead us to understand which protein classes and isoforms were more involved in forming turbidity and to elucidate their mechanisms of interaction with other wine macromolecules.

Besides, since proteins are involved in a number of aspects linked to wine quality, their accurate quantification is thus crucial, but major drawbacks when dealing with grape juice and wine samples can impair the use of available techniques, such as the presence of contaminants which can distort the measurement and the absence of standard grape or wine proteins. The aim of the third work was to investigate the accuracy of two colorimetric methods used in enology research and the possible interferences in these assays that can potentially affect the quantification of proteins in grape juice and wine.

In conclusion, the storage proteins expressed in the grape seed endosperms were systematically studied for the first time since there is little information on extraction and isolation methods, subunit composition and structure of these proteins. In this last work we reported the SDS-PAGE banding patterns of seed storage proteins of different *Vitis vinifera* cultivars to check if they can be used to distinguish the cultivars of this species.

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CHAPTER 2

Purification and characterization of chitinase isoforms from Manzoni Bianco grape juice

ABSTRACT

The fractionation of *Vitis vinifera* L. cv. Manzoni Bianco grape juice using anion exchange chromatographic technique (AEC) allowed to obtain a fraction enriched in chitinases but still contaminated by other proteins. The following purification was achieved by hydrophobic interaction chromatography (HIC) obtaining six different protein fractions that were analyzed by SDS-PAGE and zymography. In particular two fractions (3 and 4) were composed exclusively of chitinases (range 31-34 kDa and ≈ 50 kDa bands with chitinolitic activity).

Coomassie staining of SDS-PAGE gels containing increasing amounts of glycol chitin (0, 0.01 and 0.05%) under non-reducing conditions showed a progressive shift of almost all the bands. Instead reduced samples showed the same migration pattern independently from the quantity of glycol chitin incorporated into the gel. It seems interesting to underline also the retarding effect of glycol chitin on 50 kDa bands suggesting that they could be chitinases.

Then five bands with chitinolytic activity and different electrophoretic mobility were selected to be analysed by mass spectrometry (MALDI-TOF/TOF MS). All the bands analyzed were found to belong to *Vitis vinifera* class IV chitinase (according to database Mascot), but the sequence coverage obtained with trypsin cleavage was not sufficient to discriminate the differences between bands.

Keywords: chitinase, electrophoresis, glycol chitin, grape juice.

INTRODUCTION

The problem of protein haze formation in white wines is still unsolved, despite wine hazing could be a serious quality defect because consumers perceive hazy wines as faulty products. Protein haze is caused by the presence of relatively low concentrations (from 15 to 300 mg L^{-1}) of pathogenesis-related (PR) proteins, namely thaumatin-like proteins and chitinases (Ferreira *et al.*, 2002; Waters *et al.*, 2005).

Chitinases are the most represented protein components in grape juice (over 50% of the total protein content) (Waters *et al.*, 1998) and also the most active in causing wine turbidity (Falconer *et al.*, 2010; Marangon *et al.*, 2011). Chitinases survive the winemaking process and remain in the finished wine, being stable at acidic pH and resistant to proteolytic enzymes, as most of the PR proteins (Waters *et al.*, 1996). These grape enzymes seem to maintain their activity in wine at least for some months after alcoholic fermentation (Manteau *et al*., 2003) and the consequences of this activity on wine quality are unknown. Chitinases have antifungal properties resulting from their activity toward chitin, a major structural component of many fungal cell walls (Graham and Sticklen, 1994). Moreover, their isolation, separation and characterization is a difficult task due to their low concentration and strong interaction with endogenous polyphenols and other non-protein compounds (Ferreira *et al.*, 2002).

The study of wine and grape chitinases by SDS-PAGE and detection of chitinolytic activity on gels has been reported only once (Vincenzi and Curioni, 2005), although this approach can give useful information for the characterization of these enzymes. This time the study was deepened coupling the analyses already mentioned with protein identification using the MS (MALDI-TOF/TOF).

According to the aim to study single purified proteins involved in white wine haze-formation, this study presents the purification and characterization of chitinase isoforms from Manzoni Bianco grape juice. This preliminary basic research is needed for further investigations about the role played in wine by ionic strength, sulfate, temperature fluctuations, pH and redox potential in the aggregation of wine chitinases.

MATERIALS AND METHODS

Materials

The grapes utilized in this work (*Vitis vinifera* cv. Manzoni Bianco, vintage 2008) were kindly supplied by the winery of "Scuola Enologica G.B. Cerletti" of Conegliano (TV, Italy).

Protein extraction from grape juice

15 kg of Manzoni Bianco grapes were manually crushed and treated with 7.5 g kg^{-1} polyvinylpolypyrrolidone (PVPP) (Fluka), 15 g 100 kg^{-1} ascorbic acid (Baker) and 37.5 g 100 kg^{-1} potassium metabisulfite (Carlo Erba). The grape juice (10 L) was treated with 3 g hL^{-1} of pectolytic enzymes (Pectazina DC, Dal Cin), decanted for a night at 4°C, and centrifuged (5000 g, 20 min, 4°C). The free run juice was dialysed (3.5 kDa cutoff dialysis bags) against distilled water, concentrated by ultrafiltration (3 kDa cut off) and freeze dried, giving 2.6 g of protein powder.

Protein separation by chromatography

Chromatographic separations were performed in two steps using an ÄKTA purifier FPLC (GE-Healthcare) equipped with an UV detector (λ Absorbance Detector). Data were processed by the Unicorn 5.11 software (GE-Healthcare). Each solution utilised and samples to load were previously filtered with cellulose acetate filters (Millipore) with 0.20 µm pore size and degassed.

Anion Exchange Chromatography (AEC): ≈50 mg of freeze dried wine macromolecules were dissolved in 20 mM Tris-HCl pH 9.0 (Buffer A) and loaded onto a Tricorn MonoQ 5/50 column (GE-Healthcare) equilibrated with the same buffer at a flow rate of 1 mL min-1. Bound proteins were eluted at 1 mL min-1 with 20 mM Tris-HCl, 1 M NaCl, pH 9.0 (Buffer B) using the following gradient: 0 to 14% B in 70 min, 14 to 100% B in 3 min. AEC fractions were pooled on the basis of elution profiles at 280 nm absorbance and SDS-PAGE protein patterns, concentrated and dyalized against water with Vivaspin 50 (Sartorius) with cutoff 3000 Da.

Hydrophobic Interaction Chromatography (HIC): AEC fractions were further fractionated through a HIC BioSuite Phenyl 10 µm HIC 7.5 x 7.5 mm column (Waters).

Buffer A was 20 mM tartaric acid containig 1.25 M ammonium sulfate, pH 3.5 and buffer B was 20 mM tartaric acid, pH 3.5. The flow rate was set up to 0.5 mL min^{-1} and the gradient was as it follows: 0 to 100% of eluent B in 60 min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970) in a Mini-Protean III apparatus (Bio-Rad). Samples were prepared by precipitating proteins from 5-50 µL (depending on the case) of pooled fractions by the KDS method (Vincenzi *et al.*, 2005a). Precipitated proteins were resolubilized in 20 µL of 0.5 M Tris-HCl buffer, pH 6.8, containing 15% (w/v) glycerol (Sigma) and 1.5 % (w/v) SDS (Bio-Rad) and heated at 100°C for 5 minutes before loading. For analysis under reducing conditions 4% (v/v) β-mercaptoethanol was added to the loading buffer.

Electrophoretic analyses were carried out at 25 mA constant current until the tracking dye Bromophenol Blue ran off the gel. The molecular weight standard proteins were purchased from Bio-Rad (Broad Range Molecular Weight Markers). 1.5 mm thick gels were prepared with T = 12%, 14% (acrylamide-N, N' metilenbisacrylamide 29:1; Fluka) according to the needs and alternatively stained with Coomassie Brilliant Blue G-250 (Sigma) or Coomassie brilliant blue R-250 (Sigma) or with the PAS (Periodic Acid-Schiff) stain procedure to stain glycoproteins as suggested by Segrest and Jackson (1972).

Chitinolytic activity detection on SDS-PAGE gels

Chitinolytic activity detection was assayed according to Trudel and Asselin (1989). Samples were prepared with the same reagents used for SDS-PAGE and loaded into a gel (T = 14%) containing glycol-chitin (0.01% or 0.05% w/v). After protein separation, the gel was incubated overnight at room temperature in a 50 mM sodium acetate buffer pH 5.5 containing 1% (w/v) Triton X-100 (Sigma). Afterwards, gels were incubated for 20 minutes with 0.5 M Tris-HCl buffer, pH 8.9, containing 0.01 % (w/v) Calcofluor White MR2, followed by a wash in distilled water (for at least 1 h). Protein bands with chitinolytic activity were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

Protein identification and Database searching

The selected bands were excided from the gel, subjected to trypsin cleavage, analyzed using a MALDI-TOF/TOF 4800 Analyzer. After protein quantification, the sample was diluted to a concentration of 1 mg mL^{-1} in 50 mM NH_4HCO_3, reduced with 10 mM Dithiothreitol (DTT) (1 h, 37°C, dark) and alkylated with 30 mM iodoacetamide (30 min, room temperature, dark). Sequencing grade modified trypsin (Promega, Madison, Wisconsin, USA) was added at an enzyme:protein ratio of 1:50 (w/w) for digestion overnight at 37°C. The digested protein was mixed with an equal volume of matrix solution (a-cyano-4-hydroxycinnamic acid, 5 mg mL^{-1} in 70% acetonitrile, 0.1% TFA) and 1 mL was spotted on a 384-well AB OptiTOF MALDI stainless steel target plate. Sample was analysed using a MALDI-TOF/TOF 4800 Analyzer (Applied Biosystems, Toronto, Canada) with 4000 Series Explorer v3.5.3 software. Mass spectrometry (MS) data were acquired automatically over a mass range of 900–3500 Da in the positive-ion reflector mode. In the MS spectrum, the 10 most abundant MS peaks were selected for MS/MS.

MS/MS data were searched using the Mascot search engine (Matrix Science, London, UK) against the MSDB database (3239079 sequences; 1079594700 residues; Taxonomy: Viridiplantae, 247880 sequences). Enzyme specificity was set to trypsin with one missed cleavage using a mass tolerance window of 50 ppm for the precursor ion and 0.3 Da for the fragment ions and carbamidomethylcysteine.

RESULTS AND DISCUSSION

Protein extraction from grape juice

For the grape protein extraction, *Vitis vinifera* cv. Manzoni Bianco (Riesling Renano x Pinot Bianco) was chosen because it is a variety with a high protein content (Vincenzi *et al*., 2011) and whose wine generally requires fining treatments with significant amounts of bentonite for its stabilization.

The grape crushing was performed manually with particular attention to avoiding oxidative processes potentially responsible for the formation of stable complexes between proteins and polyphenols. For this last reason Manzoni Bianco grapes were treated with polyvinylpolypyrrolidone (PVPP), ascorbic acid and potassium metabisulfite. The

polyvinylpolypyrrolidone was used in order to lower the concentration of phenolic compounds (Pereira and Moretti, 1997) that can bind proteins.

Ascorbic acid has been widely used in winemaking, especially white wine production, for more than 25 years. The basis for the application of ascorbic acid in winemaking lies in its ability to scavenge molecular oxygen (Peng *et al*., 1998) with the consequent advantage of a reduction in the amount of sulfur dioxide, the latter being not particularly efficient as an oxygen scavenger (Singleton, 1987). On the other hand, it has always been argued that some sulfur dioxide is necessary in combination with ascorbic acid as the oxidation of the latter produces dehydroascorbic acid and hydrogen peroxide (Zoecklein, 1995). The oxidative capacity of hydrogen peroxide is well established, but it is readily removed by reaction with the added sulfur dioxide (Rankine, 2007).

The free run juice was dialyzed (3.5 kDa cut off) against distilled water, concentrated by ultrafiltration (3 kDa cut off) and freeze dried, giving 2.6 g of protein powder. The characterization of the crude extract was obtained using an aliquot of freeze-dried protein powder, which was resuspended in ten volumes of distilled water and precipitated with 60% ammonium sulfate. This concentration was chosen because preliminary experiments showed that it was sufficient to precipitate all grape juice proteins without precipitating other macromolecules, i.e. polysaccharides. Both the pellet and the supernatant were dialyzed against distilled water and lyophilized, obtaining a yield respectively of 26% and 38%.

The two fractions thus obtained were compared to the total protein prior to precipitation by SDS-PAGE analysis. Protein samples were not reduced before the electrophoretic separation, because in this condition a higher number of bands could be detected compared to SDS-PAGE in reducing conditions (Vincenzi *et al*., 2005b). Gels were stained with Coomassie Blue R-250 which binds to proteins and with PAS (Periodic Acid-Schiff) which detects polysaccharides. Lanes CE show the protein pattern of the crude extract (quantities: a) 10 µg and b) 25 µg) (Fig. 1, gel A). The region between 20 and 30 kDa is particularly rich in protein bands which have been identified as being pathogenesis-related (PR) proteins including thaumatin-like proteins and chitinases (Waters *et al*., 1996; Monterio *et al*., 2001; Monteiro *et al*., 2007). High molecular weight protein bands are also evident (45-66 kDa region). The protein with apparent MW of 66 kDa is most likely a

grape vacuolar invertase which is known to be one of the most represented protein in grape juice and wine, reaching 14% of Chardonnay wine proteins (Dambrouck *et al*., 2005). Protein bands with MW ranging from 45 to 60 kDa have also been identified by proteomic analysis in a Semillon grape juice as "unnamed protein product (*Vitis vinifera*)" and class IV chitinase (*Vitis vinifera*) (Marangon *et al*., 2009). Finally, the low MW protein band of 12 kDa probably corresponds to a Lipid Transfer Protein (LTP), whose presence has already been reported in grapes and it is considered one of the major grape allergens (Pastorello *et al*., 2003).

The protein composition of the pellet obtained by precipitation with 60% ammonium sulfate was similar to that of the starting crude extract, indicating that the amount of salt used was enough to recover all the proteins (Fig. 1, Gel A, lanes P). The SDS-PAGE analysis of the supernatant, as expected, showed to contain a small amount of proteins, only visible in the lane in which the protein content was higher (25 µg) (Fig. 1, Gel A, lanes S). PAS staining confirmed that the supernatant (Fig. 1, Gel B, lanes S) was the richest in polysaccharides, with a residual amount of high MW sugars detectable also in the crude extract (Fig. 1 Gel B, lane CEb). On the contrary, the pellet obtained by ammonium sulfate precipitation proved to be almost completely without polysaccharides.

Considering the yields mentioned above, it is important to note that about 1/3 of the crude extract weight was lost during protein precipitation (likely due to dialysis). For this reason the chromatographic fractionation was performed using the crude extract, without applying the precipitation process with 60% ammonium sulfate. Moreover, the high ionic strength that would have been created during the precipitation could have caused structural changes in proteins, such as the break of any aggregates, altering the usual protein pattern of grape juice proteins.

Protein fractionation and characterization

The grape juice macromolecules (> 3.5 kDa) were initially fractionated using an Anion Exchange Chromatography (AEC) column. Since the grape proteins have very similar MWs and different pI (Monteiro *et al*., 2001), this chromatographic technique that fractionates molecules based on surface charge, proved to be very effective at this stage, compared to a separation by gel filtration chromatography. Moreover, since most grape proteins have an acidic pI (Pueyo *et al*., 1993), it was chosen to make a separation in

MonoQ Anion Exchange column. At pH 9.0, proteins are positively charged and are selectively eluted in decreasing order of pI by an increasing salt gradient. This chromatographic fractionation has already been applied in many works (Waters *et al*., 1992; Dorrestein *et al*., 1995; Pastorello *et al*., 2003), allowing to obtain a good resolution of protein peaks, although some researchers have obtained good protein separations in cation exchange chromatography (Van Sluyter *et al*., 2009). In fact, cation exchange medium allows the use of an elution buffer pH close to original wine pH around 3.0 (Ferreira *et al*., 2002).

A representative AEC chromatogram for ≈ 50 mg of grape juice macromolecules loaded is shown in Figure 2. The material not retained by the column (FT) was very little or at least had a low absorption in the UV. Almost all proteins, however, were eluted from the column at relatively low concentrations of NaCl, while another peak (probably contaminated by polyphenols) was obtained with higher salt concentrations (1 M NaCl).

Six separated fractions were considered (Fig. 2) and a non-reducing SDS-PAGE analysis was performed for each of them (Fig. 3). As expected, the unretained peak (FT) did not contain any proteins. The first peak consisted of two combined peaks (1a and 1b). Fractions 1a and 1b displayed bands between 20 and 26 kDa, most probably TLPs according to literature data (Waters *et al*., 1996). Fraction 1b contained a 40 kDa MW band which could correspond to a β-glucanase (Esteruelas *et al*., 2009; Sauvage *et al*., 2010). The second peak contained only one band which showed a MW (21 kDa) similar to that of thaumatin-like proteins. The third peak was divided into two different pooled fractions: 3a and 3b. Both fractions showed to contain several TLP isoforms and a protein with a MW of ≈ 66 kDa, probably an invertase (Marchal *et al*., 1996). It must be underlined the presence of bands with MWs of 31 and 32 kDa which, according to literature data, could correspond to grape chitinases (Waters *et al*., 1996; Marangon *et al*., 2009). In this case, the enzyme seems hardly present, while it has been reported that it can reach up to 50% of total grape proteins (Waters *et al*., 1998). From preliminary analyses, the same amount of chitinases loaded in SDS-PAGE in reducing and non-reducing conditions, showed a much more intense staining in the presence of the reducing agent in the sample. One possible explanation could rise from the fact that performing the SDS-PAGE analysis under non-reducing conditions limits the dye binding to the protein, resulting in an underestimation of

the enzyme concentration. Moreover, fractions 3b contained a ≈ 50 kDa, whose identity was clarified in this work. Finally, the last peak (Lane 4) contained two proteins: another TLP isoform and a band that for its low MW was thought to correspond to the putative LTP.

By combining the chitinase-containing peaks, still contaminated by other proteins (Fig. 3, Fractions 3a and 3b), from 15 chromatographic runs, each starting from 50 mg of protein, a sufficient amount of freeze dried sample was recovered.

With the aim of obtaining a better separation of the different proteins present in the pooled peak 3a-3b (from now on named “peak 3”), a different chromatographic method based on another principle of interaction was used: hydrophobic interaction chromatography (HIC) that separates proteins according to surface hydrophobicity (Le Bourse *et al*., 2010). HIC of the protein peak 3 from AEC gave six peaks differing in surface hydrophobicity (Fig. 4). The single fractions were collected into 6 groups corresponding to HIC peaks. The pooled fractions were then dialyzed to remove the salt and concentrated by ultrafiltration.

HIC fractions were analysed by SDS-PAGE under reducing (Fig. 5, Gel A) and non-reducing (Fig. 5, Gel B) conditions, showing to contain several protein bands, differing in both relative mobility (*Mr*) and staining intensity. In every fraction except for fraction 4, bands at 21.5-27 kDa were detectable both in reducing and non-reducing conditions. Several authors indicated grape and wine proteins with these MWs as belonging to the thaumatin-like proteins class (TLP) which can present different isoforms (Peng *et al*., 1997; Tattersal *et al*., 1997; Davies and Robinson, 2000). Bands with MW of ≈ 31 kDa and ≈ 30 kDa (reducing conditions gel) and bands with MW of ≈ 32 kDa and ≈ 31 kDa (non-reducing conditions gel) were detectable respectively in fractions 3 and 4. It is interesting to underline the different migration rate observable for these bands comparing the gel in reducing conditions to that in non-reducing conditions. In fact the bands with apparent MW of ≈ 31 kDa moved to ≈ 32 kDa in non-reducing conditions, that one of ≈ 30 kDa to ≈ 31 kDa. This behaviour for proteins with MW of about ≈ 30 kDa agrees with that previously observed by Vincenzi and Curioni (2005). In fractions 3 and 4 (gel in non-reducing conditions) were also present bands with ≈ 50 kDa MW. The presence of a protein band of 52 kDa MW was also found in NuPAGE (under non-reducing conditions) by Marangon *et*

al. (2011) who identified it by NanoLC-MS/MS as class IV chitinase (*Vitis vinifera*). The band with 40 kDa MW present in fraction 2 could correspond to a Putative Thaumatin-like Protein as reported by Marangon *et al*. (2009) or to a β-glucanase (Esteruelas *et al*., 2009; Sauvage *et al*., 2010).

An additional analysis was performed to better understand the nature of the proteins fractionated on the basis of hydrophobicity. Chitinolitic activity on gel was assayed in the 6 HIC fractions (Data not shown) according to Vincenzi and Curioni (2005a). The chitinase activity was present in all the HIC fractions, although with low staining for fractions 1 and 6. In particular, the main chitinolytic activity belonged to the bands in the range 30-32 kDa and at 50 kDa detected in fractions 3 and 4.

Anomalous electrophoretic behaviour of chitinase isoforms in glycol chitin-containing SDS-PAGE gels

Afterwards only two fractions (3 and 4) were taken into account for a further characterization since they showed to contain more bands with chitinolytic activity than the other fractions (Data not shown).

Fractions 3 and 4 were analyzed by SDS-PAGE under reducing and non-reducing conditions in the presence of increasing amounts of glycol chitin (0, 0.01 and 0.05%) (Fig. 6A, 6B and 6C respectively). Then the chitinolytic activity detection after SDS-PAGE in reducing and non-reducing conditions was tested only in gels containing 0.01 and 0.05% glycol chitin (Fig. 6D and 6E respectively). Comparing the protein pattern profile of fractions 3 and 4 in reducing conditions to that in non-reducing conditions, a higher number of protein bands was detected when the samples were not reduced, confirming previous results (Vincenzi and Curioni, 2005).

The samples analyzed in reducing conditions showed the same migration pattern independently from the quantity of glycol chitin incorporated into the gel. In particular, fraction 3 always showed two major proteins with MW of 31 and 29 kDa, whereas in fraction 4 a single band at 30 kDa was evident, regardless of the different concentrations of glycol chitin to which samples were subjected.

In contrast, Coomassie staining of HIC fractions 3 and 4 in SDS-PAGE gels containing increasing amounts of glycol chitin (0.01% and 0.05%) under non reducing conditions showed a progressive shift in the *Mr* of almost all the bands except for a faint

band (≈ 30 kDa) in fraction 4. The retarding effect of glycol chitin suggests that the proteins could be chitinases (Vincenzi and Curioni, 2005) that interact with their substrates during the electrophoretic migration under non-reducing conditions. This interaction did not seem to involve the catalytic site of the enzyme, because no smears were detected in the gel region above the final position of the chitinolytic band, indicating that the enzyme does not degrade its substrate while migrating into the gel. Surprisingly the *Mr* shift regarded also a ≈ 50 kDa band present in both fractions. The disappearance of the ≈ 50 kDa band in reducing conditions suggested that the polypeptide could be a dimer of chitinases linked by S-S bonds. All the bands showed chitinolytic activity after staining the gels (Fig. 6D and 6E) (Trudel and Asselin, 1989), confirming fractions 3 and 4 as being composed exclusively of chitinases. At the end the interaction between these isoforms of chitinase and the substrate should involve a chitin-binding domain different from the catalytic site. In fact, some chitinase enzymes of plant origin (class I and class IV chitinases) are characterized by having a chitin-binding domain of the hevein type in the N-terminal region (Collinge *et al*., 1993).

Bands identification by MALDI-TOF/TOF MS

Five chitinase bands showing different *Mr* (from gel 0% GC) and one band (from gel 0.01% GC) whose *Mr* was unaffected by glycol chitin, were excised and analysed by MS after trypsin cleavage (Fig. 7).

All bands were found to belong to *Vitis vinifera* class IV chitinases (Mascot database), corresponding mainly to two possible isoforms (accessions O24530 and Q7XAU6). Only for the band named ‘CHI DIMER II’ there was only one sequence matched (Q7XAU6), and even three in the case of ‘CHI 3’ (Table 1). These results are in agreement with those of Marangon *et al*. (2011) who used peptide nanoLC-MS/MS to establish the identity of the proteins in the natural wine haze. Results in this work indicated that almost every excised band with heterogeneous mobility contained the same chitinase.

Three reasonable hypothesis can be given to explain why there are bands with different electrophoretic mobility which, however, are recognised as chitinases corresponding to two possible isoforms: i) the MALDI-TOF/TOF MS data do not provide complete coverage of any sequence. Therefore, the low percentage of sequence coverage did not allow to assign an identification with certainty. In addition, because of the great

lack of available grape protein sequences, there is a chance that the selected proteins do not exactly match corresponding database entries; ii) the bands with different electrophoretic migration could be fragments with different MW deriving from the same original protein. In fact it has been shown that some partial degradation of the chitinases could happen during juice preparation, producing fragments of different size (Waters *et al.*, 1998); iii) performing the electrophoresis under non-reducing conditions can favour differences in the hydrodynamic volumes of the protein derived from structures stabilized by S-S bonds (Vincenzi and Curioni, 2005; Marangon *et al*., 2011).

Moreover, chitinases seem to be present in the grape juice also in the form of S-S linked dimers (CHI DIMER I and CHI DIMER II), as documented elsewhere (Vincenzi and Curioni, 2005; Marangon *et al*., 2011) and whose significance and effects remain to be established. It is not yet clear whether the dimers are naturally present in grape juices or they are artefacts that occur during the protein extraction.

CONCLUSIONS

By means of chromatographic and electrophoretic techniques, at least 5 different grape chitinase isoforms were identified in the grape juice. MS analysis by means of MALDI-TOF/TOF MS allowed to confirm that all isoforms belonged to class IV chitinases, although it is not yet possible to establish if these proteins are indeed different or if they come from the protein degradation of the same original protein. Moreover, chitinases seem to be present in the grape juice also in the form of S-S-linked dimers, whose significance and effects on wine remain to be established. Thus the consequences of the presence of these isoforms of active enzymes in relation to wine quality warrants further investigation.

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TABLES AND FIGURES

Table 1. Selected proteins identified by MALDI-TOF/TOF MS.

Sample	Protein identification name	NCBI accession number	Sequence coverage (%)	Number of peptides matched
CHI 1	class IV endochitinase [*Vitis vinifera*]	>gi|2306811|gb|AAB65776.1|	17%	5
	class IV chitinase [*Vitis vinifera*]	>gi|33329392|gb|AAQ10093.1|	17%	5
CHI 2	class IV endochitinase [*Vitis vinifera*]	>gi|2306811|gb|AAB65776.1|	20%	6
	class IV chitinase [*Vitis vinifera*]	>gi|33329392|gb|AAQ10093.1|	20%	6
CHI 3	class IV endochitinase [*Vitis vinifera*]	>gi|2306811|gb|AAB65776.1|	15%	5
	class IV endochitinase [*Vitis vinifera*]	>gi|2306813|gb|AAB65777|	15%	5
	class IV chitinase [*Vitis vinifera*]	>gi|33329392|gb|AAQ10093.1|	15%	5
CHI not DELAYED	class IV chitinase [*Vitis vinifera*]	>gi|33329392|gb|AAQ10093.1|	20%	6
	class IV endochitinase [*Vitis vinifera*]	>gi|2306811|gb|AAB65776.1|	20%	6
CHI dimer I	class IV chitinase [*Vitis vinifera*]	>gi|33329392|gb|AAQ10093.1|	17%	5
	class IV endochitinase [*Vitis vinifera*]	>gi|2306811|gb|AAB65776.1|	17%	5
CHI dimer II	class IV endochitinase [*Vitis vinifera*]	>gi|2306811|gb|AAB65776.1|	18%	5

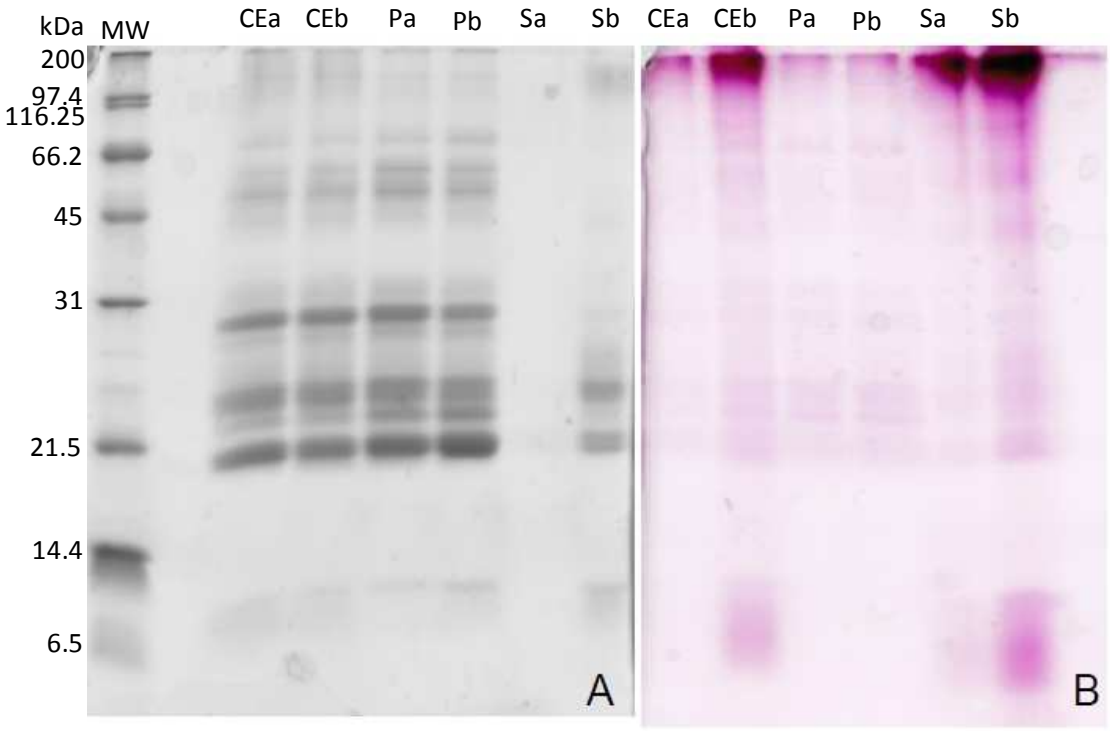

Figure 1. SDS-PAGE (T = 14%) in non-reducing conditions of the protein crude extract (CE), the pellet recovered after ammonium sulfate 60% precipitation (P) and the supernatant (S). Amounts loaded: 10 µg (a) or 25 µg (b). Left panel (A): samples stained for proteins with Coomassie Brilliant Blue R-250. Right panel (B): samples stained for glycoproteins with PAS.

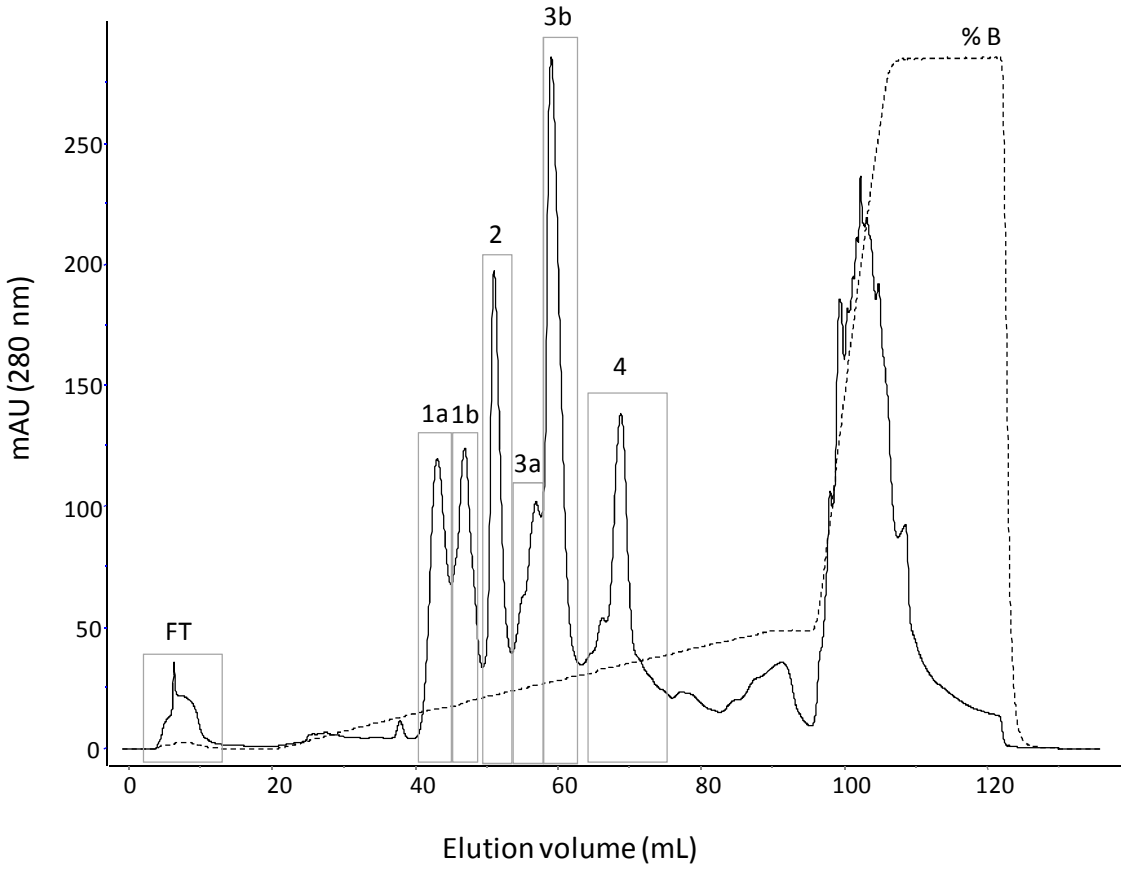

Figure 2. Anion exchange chromatogram (AEC) for Manzoni Bianco crude extract (50 mg). Collected fractions are indicated by numbered boxes. The dotted line indicates the salt gradient.

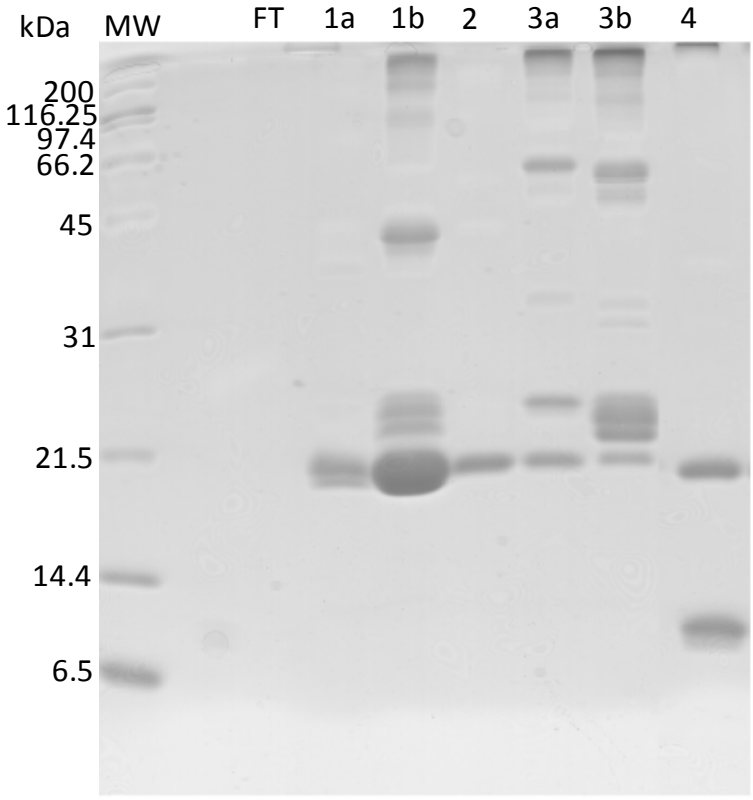

Figure 3. SDS-PAGE analysis (T=14%) in non-reducing conditions (Coomassie Brilliant Blue R-250 staining) of pooled fractions from Anion Exchange Chromatography.

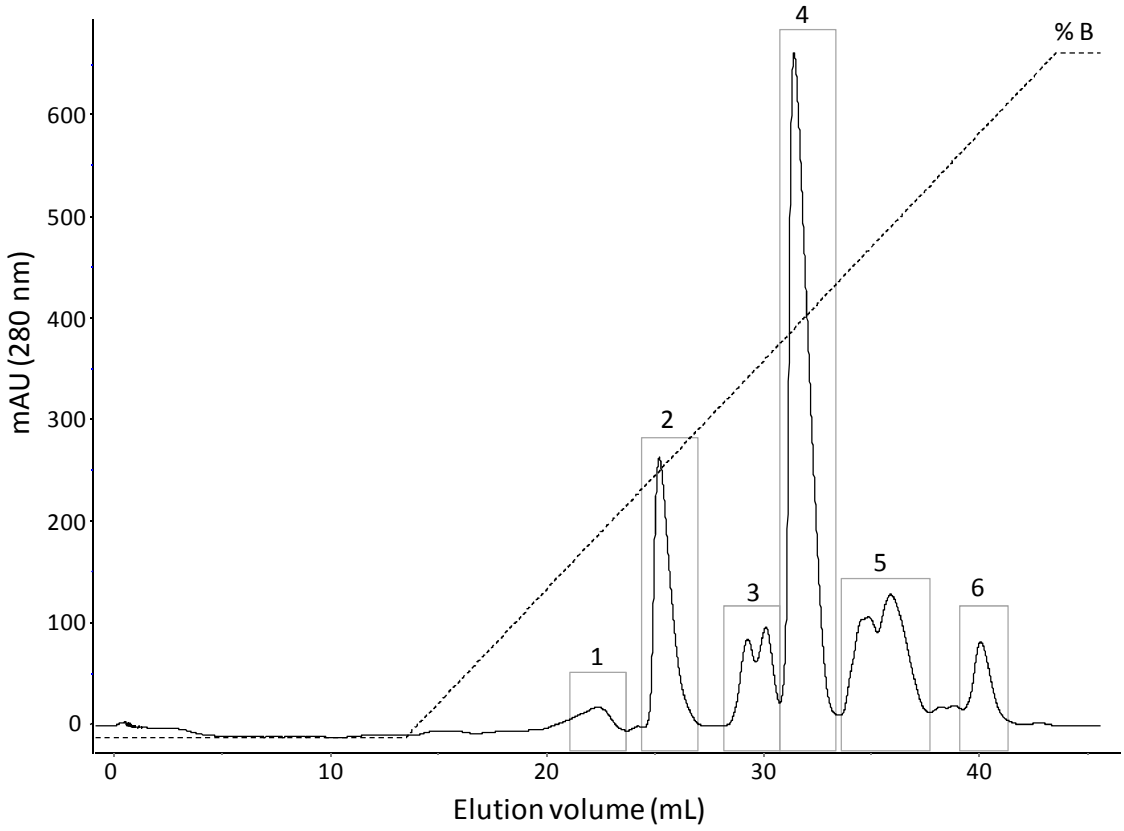

Figure 4. Hydrophobic interaction chromatography (HIC) of AEC fraction 3. Collected fractions are indicated by numbered boxes. The dotted line indicates the salt gradient.

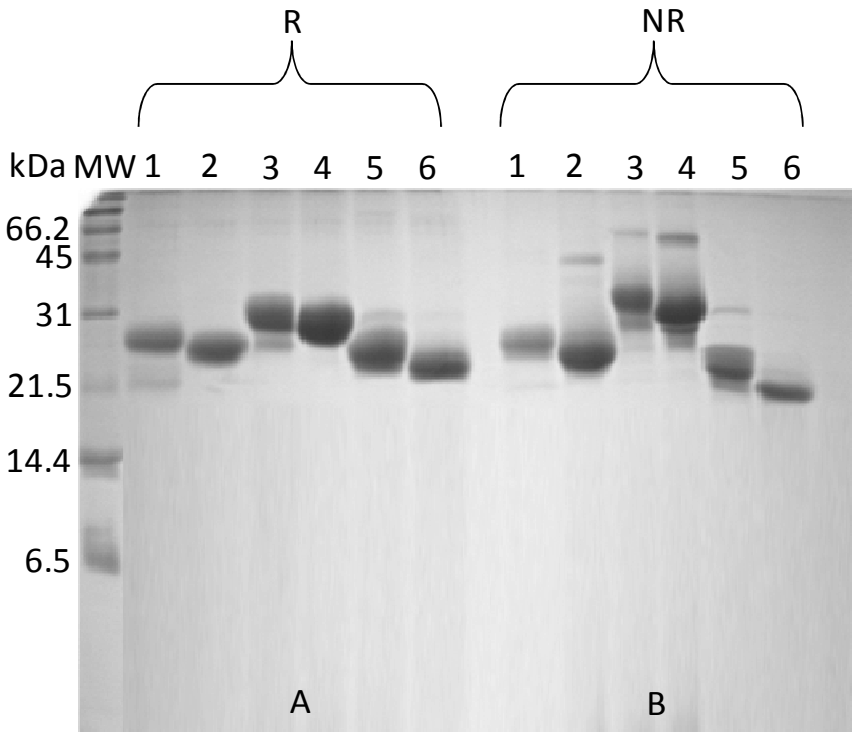

Figure 5. SDS-PAGE analysis (T=12%) in reducing conditions (R; left) and in non-reducing conditions (NR; right) of the fractions from Hidrophobic Interaction Chromatography. Gels were stained with Coomassie Brilliant Blue R-250.

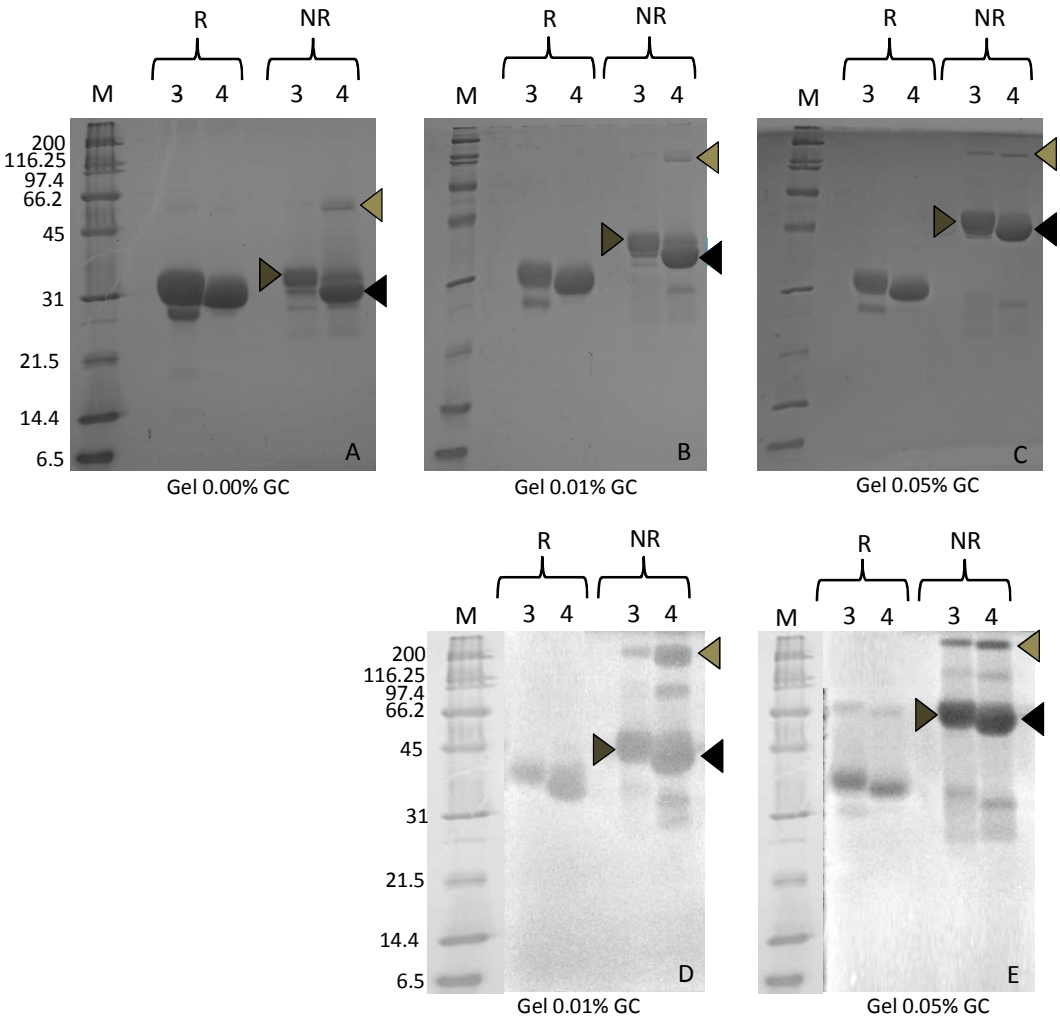

Figure 6. SDS-PAGE analysis of fractions 3 and 4 under reducing (R) and non-reducing (NR) conditions. Gels contained 0.00 (A), 0.01 (B), 0.05% (C) glycol chitin (GC). Chitinolytic activity detection of fractions 3 and 4 under reducing (R) and non-reducing (NR) conditions. Gels contained 0.01 (D) and 0.05% (E) glycol chitin (GC). Molecular weight standard proteins are in lanes M. The arrowheads indicate bands retarded in the presence of glycol chitin.

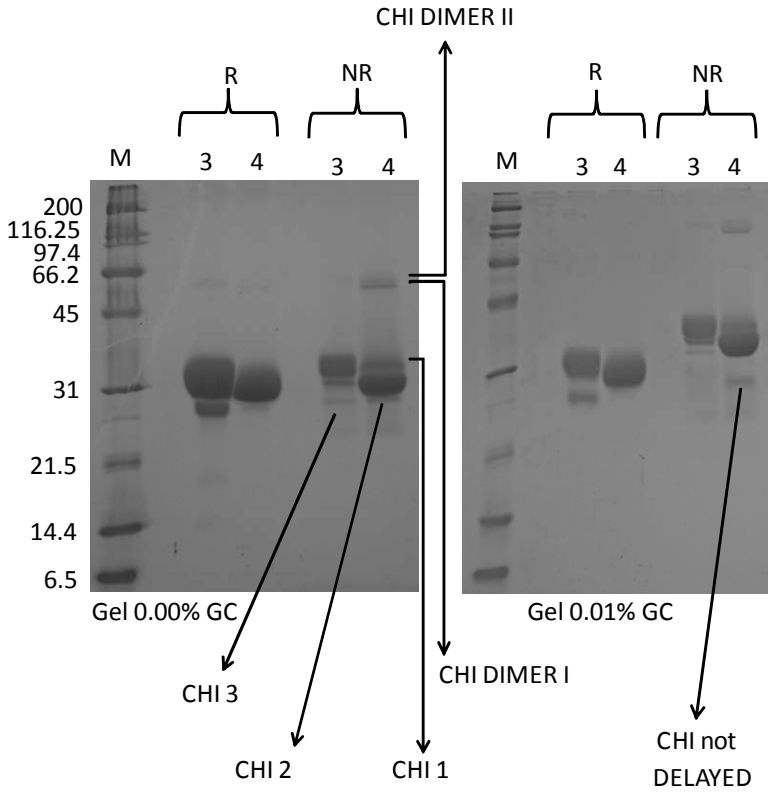

Figure 7. Bands selected to be analysed by MALDI-TOF/TOF MS. Gels were stained with Coomassie Brilliant Blue G-250.

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CHAPTER 3

Role of purified wine proteins, polysaccharides and phenolics on haze formation in white wine via reconstitution experiments

ABSTRACT

White wine protein aggregation was studied by means of an innovative instrument (Izon qNano) to detect and quantify nanoparticles formed upon heating. The role played by thaumatin-like proteins (TLPs), chitinases, phenolics and polysaccharides, all purified from the same unfined white wine, towards aggregate formation was investigated *via* reconstitution experiments. Five purified proteins, one chitinase (CHIT C) and four *Vitis vinifera* Thaumatin-Like isoforms (VVTL1 C, D, H and I), were dissolved in the starting wine from which proteins, polysaccharides and phenolics had been previously extracted (named RMW, real model wine), and heat tested alone or in combination with the other macromolecules. The number of aggregates formed upon heating indicated that CHIT C was more reactive with other wine macromolecules than TLPs. Among the four TLPs tested, two (VVTL1 I and C) proved to be more reactive than the others (D and H). In terms of aggregates size, CHIT C formed the largest particles, while differences were found among the 4 TLP isoforms.

In general chitinase was the protein that in absolute value mostly accounted for haze formation, while some TLPs isoforms showed the potential of playing a role on haze formation too.

Key words: Aggregate; chitinase; phenolics; polysaccharides; thaumatin-like protein; white wine; wine haze.

INTRODUCTION

Protein haze formation in white wines is a serious quality defect because consumers perceive hazy wines as faulty. Protein haze is caused by the presence of pathogenesis-

related (PR) proteins, namely thaumatin-like proteins (TLPs) and chitinases (Ferreira *et al*., 2002; Waters *et al*., 2005), which can aggregate into light-dispersing particles during the storage of wines. Therefore PR-proteins need to be removed and this is performed through bentonite fining. Research into alternatives to bentonite fining has been stimulated by the fact that this method presents several drawbacks an (Marangon *et al*., 2011b). In order to find a valid substitute to bentonite, a better understanding of the mechanism of protein haze formation is required.

Currently, protein instability in wines is mainly seen as a two steps phenomenon: protein unfolding, occurring under high temperatures during storage, and subsequent colloidal aggregation, due to intermolecular interactions (Dufrechou *et al*., 2010). Understanding how differences in protein structure impact on stability is key to identifying the physicochemical mechanisms involved in haze formation. In addition, non proteinaceous wine components have been shown to modulate protein hazing (Pocock *et al*., 2007; Dufrechou *et al*., 2010; Batista *et al*., 2010; Marangon *et al*., 2011a; Marangon *et al*., 2011b). Despite recent advances in this field of research, protein stability/instability in wines remains a problem not fully understood. Issues regarding (i) the influence of storage conditions and wine composition (temperature, pH, ethanol content, ionic strength, presence of co-solutes) (Mesquita *et al*., 2001; Dufrechou *et al*., 2010; Marangon *et al*., 2011b) and (ii) the features of wine proteins (structure, molecular size, hydrophobicity) that are involved in their denaturation and interaction with other wine components, including polyphenols (Waters *et al.*, 1995) and polysaccharides (Dupin *et al.*, 2000; Carvalho *et al.*, 2006), are not solved yet.

The interaction of wine proteins with phenolic compounds has been the focus of extensive research for more than 40 years (Koch and Sajak, 1959). In 1973, Somers and Ziemelis tried to explain the variations in protein stability among wines with similar total protein concentrations by speculating that protein haze is modulated by the presence of flavonoid material bound to the proteins. Yokotsuka and colleagues (1983) found that wine tannins interacted with must proteins to form a haze, while the same proteins did not produce turbidity in the presence of non tannin phenolics. However this study was not undertaken under conditions identical to those commonly encountered in commercial white wines (Waters *et al*., 2005).

The effect of wine polysaccharides on protein haze has also been documented. Fifteen different polysaccharides from different sources were added to wines before inducing protein haze, and they either did not affect or increased protein haze levels (Pellerin *et al*., 1994). Another study showed that polysaccharides increased protein instability, particularly at moderate to high temperatures (Mesquita *et al*., 2001). However, the level of polysaccharides in both studies was much greater than that reported in wines (Doco *et al*., 2003). Only a very specific group of polysaccharides, naturally present in wines at trace levels, have been shown to reduce protein haze (Waters *et al*., 1994; Moine-Ledoux and Dubourdieu, 1999; Brown *et al*., 2007). An essential step To studying the interactions between wine proteins and other wine macromolecules responsible for haze formation in white wines is to have an accurate characterization of the size and concentration of aggregates formed in samples upon heating because both parameters determine the degree of wine turbidity.

In previous studies, two techniques have mainly been applied to study the appearance of insoluble aggregates: nephelometry (Carvalho *et al*., 2004) and dynamic light scattering (Dufrechou *et al*., 2010; Marangon *et al*., 2011b). Data on particle size can be also obtained using methods such as disc centrifugation (Bondoc and Fitzpatrick, 1998), gel electrophoresis (Alberts *et al*., 1994) or electron microscopy. Besides conventional techniques such as flow cytometry (Shapiro, 2003) showed a number of limitations, including the need for large sample volumes and an inability to accurately detect particles smaller than ~400 nm (Bayley and Martin, 2000). The concentration of nanoparticles is more difficult to determine (Roberts *et al*., 2011). Biological nanoparticle concentrations has been quantified by qPCR (Ma *et al*., 2001), ELISA assays (Johansson *et al*., 1980) and UV/vis spectroscopy (Maizel *et al*., 1968), but while these methods are able to deal with small molecules, they do not directly detect particles.

Quantitative resistive pulse sensing using Coulter-type counters has been shown to hold promise as a fast and accurate alternative to established sizing methods for nanoparticles (Henriquez *et al*., 2004; Ito *et al*., 2004). A new technology, the IZON qNano, which utilises Scanning Ion Occlusion Spectroscopy (SIOS) to allow the detection of both size and concentration of individual particles/aggregates is among the instruments that use this system. The Coulter technique is a method that has been predominantly used

by researches to measure the size and concentration of biological molecules (viruses and bacteria) and other particles that are suspended in an electrolyte solution (Deamer and Branton, 2002; Ito *et al*., 2003; Li *et al*., 2003).

The qNano instrument (Fig. 1) incorporates: (i) a tunable nanopore around which there is the membrane, a septum at the centre of a cross-shaped stretching platform known as 'cruciform'; (ii) a fluid cell where the cruciform is placed for ionic current measurements through the pore by using Ag/AgCl electrodes; (iii) a U-tube manometer which applies a pressure across the membrane, enabling pressure-driven particle transport (Willmott *et al*., 2010). An obstruction in the nanopore creates a resistance in the electrical current and this resistance is the key information in analysing the particles or molecules under investigation, giving data on their size, concentration, and mobility.

The goal of the research presented in this chapter is to examine, *via* reconstitution experiments, both the size and concentration of individual particles/aggregates formed by five purified wine proteins when heated in presence or absence of wine phenolics and polysaccharides.

MATERIALS AND METHODS

Materials

The wine used was produced in 2010 from a Chardonnay juice sourced from the Barossa Valley region (South Australia) and kindly donated by Orlando Wines. No bentonite fining was performed. Conventional enological parameters were analyzed according to the Vine and Wine International Organisation methods (Table 1).

Purification of wine proteins

Chitinases and TLPs were purified as described by Van Sluyter *et al*. (2009). Briefly: 36 L of Chardonnay wine were loaded on an XK50 column (Amersham Biosciences) packed with 150 mL Macro-Prep High S resin (Bio-Rad) previously equilibrated with 30 mM sodium citrate, pH 3.0. Bound proteins were eluted with 30 mM MES/1M NaCl, pH 6.0. Strong cation exchange (SCX) fractions were adjusted to pH 5.0 (NaOH) and 1.25 M ammonium sulfate and further fractionated by hydrophobic interaction chromatography (HIC) on *2.6 cm diameter, 110 mL, Phenyl Sepharose HP column* (GE

Healthcare). After equilibration with 50 mM sodium citrate containing 1.25 M ammonium sulfate, pH 5.0, proteins were eluted with a linear gradient from 1.25 to 0 M ammonium sulfate in 50 mM sodium citrate (pH 5.0). SCX and HIC fractions (10 mL each) were pooled on the basis of elution profiles at A_{280} and Reverse-Phase (RP) HPLC analysis. Purity and identity of collected fractions were assessed by SDS-PAGE, RP-HPLC, nanoLC-MS/MS. Proteins were stored as ammonium sulfate suspensions at 4 °C.

Protein preparation

Ammonium sulfate suspensions of purified proteins were centrifuged (13000g, 15min, 4°C) and the protein pellet dissolved in deionised water. Salt removal and protein concentration were achieved by centrifugation with Nanosep ultrafiltration devices (3 kDa MWCO) (Pall Corp. Glen Cove, NY). Concentrated proteins were dissolved in "real model wine" (RMW, the starting wine after removal of proteins, polysaccharides and phenolics), and stored at 4°C.

Protein content determination

Protein content was determined by EZQ protein quantification kit (Invitrogen) following the manufacturer's instructions. The calibration curve was prepared using serial dilution from 0 to 250 mg L^{-1} of thaumatin from *Thaumatococcus danielii* (Sigma-Aldrich, Castle Hill, NSW, Australia). Fluorescence measurements were conducted using excitation/emission settings of 450/618 nm with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). In other cases proteins were quantified by UV absorption at 260/280 nm (Scopes, 1987).

Heat test conditions

The unfined Chardonnay wine was heated at 80°C for 2 h and cooled in ice for 2 h. After equilibration at room temperature, the haze was measured by calculating the difference in nephelometric turbidity units (NTU) between heated and unheated samples by means of a nephelometer (Pocock and Rankine, 1973). Samples were considered to be protein unstable when the difference in absorbance between heated and unheated samples was greater than 2 NTU.

Reconstituted samples to be analyzed by qNano were heated at 70°C for 1 h and

than cooled at 25°C. After 15 h, the diameter and concentration of the aggregates were measured.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

PAGE analyses were performed with NuPage 4-12% Bis-tris gels (Invitrogen) (1.5 mm thick, 15 wells) using an XCell SureLock Mini Cell (Invitrogen). Approximately 50 mg of $Na_2S_2O_5$ were added to the top reservoir to prevent cysteine oxidation. Samples were prepared dissolving approximately 3 µg of protein in 20 µL of loading buffer containing 5% 2-mercaptoethanol (Sigma). Precision Plus Protein unstained MW standards were from Bio-Rad laboratories (Regents Park, NSW, Australia). Proteins were stained with Pierce Imperial Protein Stain (Quantum Scientific, Sydney, NSW, Australia), according to the manufacturer's instructions.

Reverse Phase (RP)-HPLC

The purity of proteins was determined by RP-HPLC with a Vydac 2.1 × 250 mm C8 column (208TP52, Grace Davison Discovery Sciences, Baulkham Hills, NSW, Australia) on an Agilent 1200 system (Agilent Technologies, Santa Clara, CA) according to the method of Marangon *et al*. (2009) with modifications as suggested by Van Sluyter *et al*. (2009). Injection volumes were 25 µL of sample. Protein identity was assigned by comparison of the retention times (RT) with those of previously reported for purified grape PR proteins as follows: peaks with a RT between 12 and 16 min were assigned to the TLP class, whereas peaks eluted from 24 to 28 min were classified as chitinases.

Peptide NanoLC-MS/MS and Database Searching

Bands from SDS-PAGE were excised and used for peptide nanoLC-MS/MS according to the method of Van Sluyter *et al*. (2009). A ThermoFinnigan LTQXL linear ion trapmass spectrometer was used. To create a wine protein database, all 76552 Vitis protein entries and 5693 EC1118 yeast proteins in NCBI were downloaded on July 21, 2011, and used with X!Tandem according to the procedure of Van Sluyter *et al*. (2009).

Purification of polyphenols

Total polyphenols were captured passing the unfined Chardonnay wine through a FPX66 amberlite column (Rohm and Haas Company, Philadelphia, USA) and eluted with

80% ethanol. The ethanol was evaporated under reduced pressure by means of a rotavapor Laborota 4010 digital (Heidolph), and the phenolic solution was freeze dried.

Purification of polysaccharides

The deproteinized wine (flow through from SCX step) was passed through the FPX66 amberlite column to remove polyphenols. The resulting wine was then concentrated 30 times under reduced pressure and total polysaccharides were then precipitated with 3 volumes of ethanol. The pellet collected by centrifugation was dissolved in water, dialyzed against water (7 kDa MWCO) and freeze dried.

Preparation of the "Real Model Wine" (RMW)

The flow through from the unfined Chardonnay wine passing through the FPX66 amberlite column was recovered and macromolecules were removed by ultrafiltration (Stirred Cell System, Amicon) through 3 kDa MWCO membranes.

Analysis of protein aggregates with the IZON qNano™

The instrument was used following the manufacturer's instructions.

q-Nano and Membranes. Once the lower fluid cell was in place, 75 µL of RMW (used as the electrolyte) were placed into the centre channel. The upper fluid cell was then set into place and 40 µL of RMW were added to it. The tunable nanopore 1000 (diameter range 500-2000 nm, target particle diameter 1000 nm) was stretched to 50 mm. Voltage was adjusted until the current in the signal trace graph was approximately 140-150 nA and samples were loaded.

Electrolyte and standard nanoparticles. Carboxylated polystyrene calibration standard particles with diameter range of 500-2000 nm were diluted at the concentration 5 x 10^7 particles/mL in RMW. The solution was sonicated for at least 5 min prior to use. Calibration measurements were taken with the same settings of the samples to convert relative data to absolute values.

Data conversion. Data were digitalised and interpreted using Izon's customized v.2.2 instrument control software.

Experimental Procedure

Five proteins (named CHIT C, VVTL1 C, VVTL1 D, VVTL1 H, VVTL1 I), total

polysaccharides (PS) and total phenolics (PHE), all purified from the same Chardonnay wine were, characterized and used in reconstitution experiments using RMW as medium. Each protein was heat tested singularly or in combination with PS and/or PHE for a total of 4 treatments for protein (protein alone without PS and PHE, -PS -PHE; protein with addition of only PS, +PS -PHE; protein with addition of only PHE, -PS +PHE; protein with addition of both PS and PHE, +PS +PHE). Each compound was added to RMW at the approximate concentration it was found in the wine: proteins at 100 mg L^{-1}, polysaccharides at 170 mg L^{-1}, and phenolics at 225 mg L^{-1}. After the heating/cooling cycle (70°C for 1 h, 25°C for 15 h), the diameter (nm) and the concentration (particles/mL) of the aggregates formed in the different samples were determined by the qNano instrument.

Statistical analysis

Each experiment was performed in duplicate while the analyses were done in triplicate. Data collected from the qNano experiments were organized to be submitted to two-way analysis of variance (ANOVA) to examine the main effects “Protein” (variable A) and “Treatment” (variable B) and the effect of the interaction “Protein x Treatment” on the parameters considered (concentration and size of aggregates formed). Means were compared by the Tukey test at 5% probability. The statistical design according to which data were analyzed with CoHort Software (CoStat, version 6.4) is presented in Table 2.

RESULTS AND DISCUSSION

PR-proteins purification

The wine used was heat unstable (16.8 NTU upon heat test), contained 104 mg L^{-1} of proteins and needed 1.2 g/L of bentonite to be stabilized (data not shown). The SDS-PAGE protein pattern of the wine (Fig. 2) was typical of those reported in the literature (Dufrechou *et al.*, 2010; Sauvage *et al.*, 2010; Le Bourse *et al.*, 2011), with a major protein band, tentatively identified as TLP, within the MW range of 21-24 kDa. The band with apparent MW between 25 and 30 kDa was supposed to be a chitinase. Other proteins at MWs of 12, 13, 16, 35 and 65 kDa were also detected. From the literature, bands with MWs of 12 and 65 kDa were consistent with those of a lipid transfer protein (Pastorello *et al.*, 2003) and of a grape vacuolar invertase (Jégou *et al.*, 2009), respectively. Following the

method of Van Sluyter and co-workers, the wine was adjusted to pH 3.0, fined with 10 g/L polyvinylpolypyrrolidone and then filtered by vacuum through 0.8/0.2 µm filters. This process caused a 15% protein loss from the starting wine (Table 3).

Proteins were captured from 36 L of Chardonnay wine by SCX (Fig. 2). Cation exchange is a convenient method to fractionate captured proteins because it binds, at a pH value close to that of wine, the majority of PR-proteins without binding grape-derived polysaccharides (Van Sluyter *et al*., 2009). The separation started in non denaturing conditions at pH 3.0 and 50 mM NaCl. Proteins were eluted with a gradient of salt (0 min, 50 mM NaCl; 90 min, 300 mM NaCl, then 1 M NaCl) and pH (pH 3.0 to pH 6.0). SCX step yielded several peaks (Fig. 2), with the two major ones (named C and D) eluted at the beginning of the gradient followed by other four main fractions washed out at 1M NaCl and pH 6. A total of nine fractions were collected. Results from the EZQ assay indicated that the two major fractions SCX-C and SCX-D contained respectively a total of 102 mg and 492 mg of protein while fractions E, H and I contained 75, 72 and 73 mg of total protein, respectively; fractions A, B, F and G contained a low amount of protein and therefore were not used in following experiments (Table 3).

The protein composition of SCX fractions was than assessed by SDS-PAGE (Fig. 3) and RP-HPLC (Fig. 4) analysis.

SDS-PAGE showed that SCX fraction C contained three main bands with apparent MWs of 65, 26 and 21 kDa. The 65 kDa band was assumed to be an invertase (Jégou *et al*., 2009) and had a RT of 19.73 min by RP-HPLC. The 26 kDa band was supposed to be a chitinase and had a HPLC RT of 24.5 min. The thickest band at 21 kDa probably was composed of more than one TLP isoforms. As a matter of fact it showed three peaks by HPLC (with RT of 12.06, 12.74 and 15.07) all in the range of elution of TLPs.

By SDS-PAGE SCX fraction D showed two main bands with apparent MW of 65 and 21 kDa. As mentioned before, the faint 65 kDa band, whose peak did not appear in RP-HPLC SCX-D chromatogram, was probably a vacuolar invertases, while the 21 kDa band was likely TLP as confirmed by its retention time (12.72 min) by RP-HPLC analysis.

In SCX fraction E, the 65 kDa band was still detectable. Other two bands were revealed by SDS-PAGE: one, with apparent MW of 32 kDa, had a RT of 27.58 min by HPLC and was likely to be a different chitinases from that one present in SCX fraction C;

the second one, with an apparent MW of 21 kDa and a RT of 12.76, was supposed to be a TLP.

SCX fraction H and I both contained a band of apparent MW of 21 kDa that by HPLC showed up as a single peak (respectively with RT 12.76 and 12.74 min), recognised as TLP.

SCX fractions were further fractionated by hydrophobic interaction chromatography (HIC). It was confirmed that HIC following SCX is a convenient step because the different mechanisms of separation are orthogonal; it means that SCX separates on the basis of surface charge which is unrelated to surface hydrophobicity (Van Sluyter *et al*., 2009). Five of the nine SCX fractions were submitted to HIC, from which six pure proteins were obtained.

From the HIC fractionation of SCX-C (Fig. 5a) nine fractions were collected. Each one was analysed by RP-HPLC to determine how they could be pooled. The first 2 were pooled since they had the same RT, 24.6 minutes (Fig. 5b), a RT consistent with that of chitinases. The supposed identity for protein C 1-2, as well as for those that will follow on this discussion, was confirmed by nanoLC-MS/MS (Table 4), approach that has recently become frequent in grape and wine protein studies (Cilindre *et al*., 2008; Van Sluyter *et al*., 2009; Wigand *et al*., 2009; Marangon *et al*., 2009; Falconer *et al*., 2010). This chitinase, from now on named 'CHIT C', showed by HPLC a purity ~ 90% (Fig. 5d) and a MW of 22.5 kDa (Fig. 10). HIC fraction C4 was kept separated from the others (Fig. 5e); it showed to have 90% purity by HPLC, and to have a RT in agreement with that of TLPs (12.8 minutes). This protein was named 'VVTL1 C'; it had an apparent MW of 22.5 kDa (Fig. 10), and was recognised as a VVTL1 (*V. vinifera* TLP 1) by nanoLC-MS/MS (Table 4). Other two HIC fractions (6 and 7) were at first considered and grouped together since they had the same RT (15.2 minutes) by RP-HPLC (Fig. 5c). HPLC (Fig. 5f) and SDS-PAGE (Fig. 10) indicated that the pooled peak was not pure as fraction C 6-7 contained 3 bands (Fig. 10). Both the 65 kDa (C 6-7α) and the 26 kDa (C 6-7β) bands were identified by nanoLC-MS/MS as vacuolar invertases (Table 4). Despite these 2 bands showed up with the third 21 kDa band as a single HPLC peak (RT 15.24 minutes) (Fig. 5f), they behaved differently in reducing SDS-PAGE (65 and 26 kDa respectively). It is likely that the 26 kDa band is a fragment of invertases, a phenomenon previously reported by others (Okuda *et al*,

2006; Marangon *et al*, 2009).

When fractionated by HIC, the other main peak considered, SCX-D, separated in two major peaks, which were collected into 8 sub-fractions (Fig. 6a). As discussed previously, fractions were pooled on the basis of their RP-HPLC profile. SCX-D fractions 5 to 8 did not contain purified proteins while HIC fractions D1, D2, D3, and D4 all included the same protein with HPLC RT of 12.7 minutes (Fig. 6b). Therefore those fractions were pooled. SDS-PAGE analysis revealed only one band with apparent MW of 21 kDa (Fig. 10). This band was identified as a VVTL1 (*Vitis vinifera*) by nanoLC-MS/MS analysis (Table 4) and was named "VVTL1 D".

HIC fractionation of SCX fraction E yielded 12 sub-fractions (Fig. 7a), with only E1, E2 and E3 showing to contain pure proteins by RP-HPLC (Fig. 7b). This protein eluted early (HPLC RT of 8.47 minutes, Fig. 7b) and had a SDS-PAGE apparent MW of 11 kDa. This band (E 1-2-3, Fig. 10) was recognised as lipid transfer protein (LTP) by nanoLC-MS/MS analysis. Unfortunately the abundant HIC fraction E6 (potentially a TLP) could not be used because HPLC analysis showed that it was contaminated with a chitinase (RT 27.7 minutes) (data not shown).

HIC fractionation of SCX-H resulted in 4 peaks (Fig. 8a), with the main one (HIC-H 4) showing >99% purity by HPLC with a RT of 12.7 minutes (Fig. 8b) and an apparent MW of 21 kDa (Fig. 10). Through nanoLC-MS/MS analysis it was identified as VVTL1 (*Vitis vinifera*) (Table 4) and named "VVTL1 H".

HIC chromatogram for SCX fraction I showed two main peaks (Fig. 9a). RP-HPLC analysis of HIC fraction I 1 revealed a peak with RT of 12.9 minutes and >99% purity (Fig. 9b). By SDS-PAGE I 1 showed an apparent MW of 21 kDa (Fig. 10); by nanoLC-MS/MS analysis it was identified as VVTL1 (*Vitis vinifera*) (Table 4) and named "VVTL1 I".

There are some other important considerations to be done. Firstly it is essential to underline that the nanoLC-MS/MS data do not provide complete coverage of any sequence. Therefore, resulting sequences from this analysis are homologous and not the actual sequences of the purified proteins because of the great lack of available grape protein sequences and the high heterozygosity of grapes (Velasco *et al*., 2007). For this reason there is a chance that purified proteins do not exactly match corresponding database entries. Only the availability of crystal structures will solve the problem of comparisons between

purified proteins and database entries (Van Sluyter *et al*., 2009).

The fact that purified "VVTL1 C", "VVTL1 D", "VVTL1 H" and "VVTL1 I" were recognised as VVTL1 (*Vitis vinifera*) by nanoLC-MS/MS does not mean that we are necessary dealing with the same protein. Most probably they are different isoforms of the same protein. As a matter of fact Van Sluyter and colleagues (2009) purified two VVTL1 proteins (named H2 and I) that behaved differently in terms of both purification and crystallisation. It is likely that small variations in disulfide bonding and in aminoacid composition could result in conformational differences, even among proteins with identical primary structure, that lead to different SCX and HIC retention times. As a matter of fact, the 4 TLPs purified in this work (C, D, H and I) had different surface charge and hydrophobicity.

Characterization of haze aggregates using the nanopore instrument IZON qNano

The qNano technology was used to measure the size and concentration of aggregates formed upon heating the samples containing individual purified wine proteins, alone and in the presence of wine polysaccharides (PS) and/or phenolics (PHE).

Measure of aggregates concentration

Blank runs, i.e. samples where proteins were not included, always resulted in a very low aggregates formation. In particular in samples containing PHE, PS or both aggregates had an average concentration of 210,000, 200,000 and 416,667 particles/mL, respectively (data not shown). This indicates that the presence of protein in the medium is crucial for the onset of aggregation.

By testing the protein containing samples, several statistically significant differences in particles concentrations were found: i) among the five proteins, regardless of whether polysaccharides and/or phenolics were also present or absent (main effect "protein", Fig. 11); ii) among the four treatments (inclusion or not of polysaccharides and/or phenolics in the protein samples), regardless of the single protein behaviour (main effect "treatment", Fig. 12); iii) among the treatments to which each protein reacted differently (interaction effect "protein x treatment", Fig. 13).

Regarding the main effect "protein" (Fig. 11), CHIT C was more prone to form a high number of aggregates upon heating compared to TLPs independently from the treatment applied (absence or presence of polysaccharides and/or phenolics). Among the

TLPs, VVTL1 C and particularly VVTL1 I formed a larger number of aggregates compared to VVTL1 D and H, indicating different behaviors for the different TLP isoforms present in the wine.

The effect of the four treatments (main effect "treatment") on the number of aggregates formed is shown in Figure 12. Proteins alone (-PS-PHE) formed an intermediate number of aggregates (blue bar), while the addition of phenolics (-PS+PHE, green bar) resulted in an increase of the number of aggregates. in contrast, the addition of only polysaccharides to the samples halved the number of aggregates both in presence or absence of phenolics, indicating a direct effect of polysaccharides on protein aggregation.

The "protein x treatment" interaction effect is shown in Fig. 13, which reports the full data set obtained by the analysis of the different samples. CHIT C, VVTL1 C and VVTL1 I aggregated when heated alone in RMW, while VVTL1 D and VVTL1 H did not (Fig. 13, blue bars). These results are in agreement with previous data indicating that the RMW contained one or more factors contributing to the aggregation of heat unfolded proteins. One of these factors is sulfate, a compound shown to be required for haze formation in wine (Pocock *et al*., 2007). Indeed, the RWM here used contained 310 mg L^{-1} of sulfate and this can explain the aggregation of the single proteins here shown. In particular, Pocock and colleagues (2007) demonstrated the involvement of sulfate in protein hazing for both purified chitinases and TLPs in model wine. Adding sulfate to model wine triggered heat-induced protein aggregation, and the heat-induced haze increased with increasing sulfate concentrations.

More recently sulfate was proved to strongly affect chitinases aggregation in model wine upon heating, where it modulated both the rate of aggregation and the size (1-5 µm) of aggregates formed (Marangon *et al.*, 2011b). This is in agreement with results obtained in this work for CHIT C alone which formed big aggregates (721.8 - 4050.3 nm diameter range, see next section and Table 6). Previous DLS experiments carried out by Marangon and co-workers (2011b) showed only limited aggregation for the two TLPs isoforms tested indicating a very different behaviour between TLPs and chitinases self aggregation modulated by sulfate. On the contrary, in this work two of the TLPs isoforms (VVTL1 C and VVTL1 I) behaved similarly to CHIT C, giving more than 5,000,000 particles/mL, while the other two TL-proteins tested (VVTL1 D and VVTL1 H) formed a significantly

lower ($P \leq 0.05$) number of particles (93,600 and 176,667 particles/mL, respectively) (Table 5 and Fig. 13).

The lowest number of aggregates was observed by adding only polysaccharides to the RMW containing TLPs (Fig. 13, red bars), showing a significant effect of polysaccharides in reducing the aggregation of these proteins. This result is in agreement with the literature in which polysaccharides were shown not to increase haze during heat testing (Pellerin *et al.*, 1994). In contrast, adding polysaccharides to CHIT C, resulted in the formation of a number of aggregates (7,566,667 particles/mL) significantly higher than that of the four VVTL1 (Fig. 13, red bars; Table 5).

The addition of phenolics to the proteins (-PS + PHE) was the treatment that caused the formation of the statistically highest number of aggregates (4,731,538 particles/mL), regardless the nature of the single proteins (Fig. 12, green bar). In particular, among TLPs, VVTL1 I and C, in the presence of phenolics, showed a significantly higher number of aggregates compared to H. CHIT C did not differ statistically from the most reactive TLP isoforms VVTL1 I and C, neither from VVTL1 D. Among the five proteins tested, CHIT C was the only one to form a number of aggregates not significantly different for all treatments examined (Fig. 3).

This behaviour of proteins towards polyphenols is not unknown to the literature. Interactions between tannins and proteins have been extensively studied, owing to their role in haze formation. In fact substantial evidence exists to suggest that proteins and polyphenols interact together. Kock and Sajak (1959) were among the first investigators to determine that aggregates responsible for wine haze contained tannins; Somers and Ziemelis (1973) found that up to 50% of wine protein was bound to flavonoid material; Waters and co-workers (1995) detected the presence of procyanidins (0.02-4.9% w/w) in recovered wine haze.

The addition of PS to samples containing PHE and the proteins resulted in a reduction of the number of particles for VVTL1 D, VVTL1 H and VVTL1 I but not for VVTL1 C nor CHIT C (Fig. 13, compare green and yellow bars). Other studies have shown that the presence of polysaccharides in solution affects the interaction between tannins and proteins (Soares *et al.*, 2009) and the ability of some polysaccharides to reduce the formation of protein/tannin aggregates has been demonstrated (De Freitas *et al.*, 2003;

Mateus *et al*., 2004; Carvalho *et al*., 2006). Two mechanisms have been proposed to explain the inhibitory effect of carbohydrates towards protein-tannin aggregation: (i) a direct competition between polyphenols and carbohydrates for binding sites on the protein; (ii) the formation of a highly soluble ternary protein/polyphenol/carbohydrate complex resulting in a lower number of aggregates (Soares *et al*., 2009). Our results indicate that those mechanisms can be both valid for TLPs but not for CHIT C whose protein/tannin aggregates number was not decreased by the presence of polysaccharides. Therefore it seems that the effect of carbohydrates is governed by the structure and the chemico-physical parameters of the individual wine proteins, with chitinases unaffected and TLP isoforms differently affected.

It is likely that once CHIT C is in its irreversible heat-unfolded state many hydrophobic binding sites are exposed, so that it can be bound indiscriminately by phenolics or polysaccharides. VVTL1 D and VVTL1 H, during the cooling phase, are probably characterized by a partial refolding (Falconer *et al*., 2010). According to the two mechanisms described above the small portion of unfolded TLPs is mostly interacting with phenolics and only in a negligible way with polysaccharides. On the contrary, the two most unstable TLPs (VVTL1 C and I) likely have exposed more hydrophobic binding sites when in heat-unfolded status, resulting in a higher capability to bind phenolics. On the other hand, as discussed for the other VVTL isoforms, the presence of polysaccharides is able to reduce the formation of aggregates. As a matter of fact TLP unfolding temperatures have been reported to range between 56 to 62°C (Falconer *et al*., 2010), with one isoform having almost the same melt temperature as chitinases. This suggests that there are TLP isoforms with behavior similar to that of chitinases, fact that could indicate that some TLPs might play a role in haze formation.

Measure of aggregates size

In absence of proteins only few small aggregates were formed, with samples containing PHE, PS or both forming aggregates with average size of 431 nm, 482 nm and 474 nm, respectively (data not shown). The presence of proteins in the medium triggered the formation of aggregates of more than 500 nm.

Several statistically significant differences in particle size were found: i) Among the five proteins, regardless of the treatment to which they were subjected (main effect

“protein”, Fig. 14); ii) Among the four treatments, regardless of single protein behaviour (main effect “treatment”, Fig. 15); iii) Among the treatments, to which each protein reacted differently (interaction effect “protein x treatment”, Fig. 16).

When comparing the mean size of the aggregates formed in the presence of the different proteins (Fig. 14), it was observed that CHIT C produced bigger aggregates than TLPs. VVTL1 I, i.e. the protein that gave the highest number of aggregates among TLPs (Fig. 11), was also the TLP forming the largest aggregates (1086 nm mean diameter, Fig. 14), while the other three TLPs (VVTL1 C, D and H) formed aggregates with a mean size smaller than 1000 nm. It is noteworthy the comparison between VVTL1 I and C: both formed large numbers of aggregates (see Fig. 11) while in terms of size they greatly differed, with I forming the largest particles and C the smallest among TLPs. This might be due to the different surface charge of these two proteins. As a matter of fact, VVTL1 C was eluted at the beginning of the SCX fractionation (with 0.05 M NaCl), while VVTL1 I was eluted at the end (with 1 M NaCl), indicating different charge properties between the two proteins, which could affect their aggregative behaviour.

The evaluation of the main effect “treatment” (Fig. 15) showed that addition of phenolics did not affect the size of the particles formed by the proteins (Fig. 15, compare green and blue bars). In contrast, proteins formed the largest aggregates in the presence of polysaccharides (1149 nm) (Fig. 15, red bar) while the addition of both phenolics and polysaccharides yielded smaller particles than polysaccharides alone (Fig. 15, compare yellow bar with red bar). However, these particles were larger than those formed by in the presence of phenolics. These findings are in disagreement with the idea that PS reduce the size of the aggregates formed by proteins and tannins (Gonçalves *et al.*, 2011). In their experiment the ternary complex between protein/polyphenol/carbohydrate appeared to have a spherical diameter smaller (lower size) than that of a protein/polyphenols complex, which was thought to be more extended and less soluble. However, the discrepancy may be due to the differences in the materials used: effect of two carbohydrates (pectin and arabic gum) on the aggregation between α-amylase and procyanidin for Gonçalves group, wine proteins, polyphenols and polysaccharides in our study. Our data indicate that complexes formed by PRT and PHE had a medium size (934 nm) smaller than those produced when also PS were present (1007 nm).

Comparing the general trends of individual proteins towards single treatments results in large heterogeneity of the data (Fig. 16).

VVTL1 C and I formed the largest aggregates when only PHE were present in the matrix (821 nm and 1148 nm, respectively), while VVTL1 H gave the largest particles in the presence of PS (977 nm). In contrast, for CHIT C and VVTL1 D both PHE and PS were needed to reach the maximum particle size (1484 nm and 962 nm, respectively) (Fig. 16; Table 6), indicating a similar behaviour for these proteins. As seen for CHIT C, even for VVTL1 D, the addition of PHE in the mixture caused the formation of smaller size aggregates (807 nm) compared to those where both PHE and PS were inside the sample (962 nm).

Chitinase aggregates formed in presence of PS had sizes almost three times higher than those formed by VVTL1 C under in the same conditions. In addition CHIT C aggregates in the presence of both polysaccharides and phenolic more than doubled in size compared to VVTL1 C with the same conditions (Fig. 16; Table 6). Since aggregates are visible to the naked eye only when they exceed 1000 nm size (Ferreira *et al.*, 2002), it seems that VVTL1 D and VVTL1 H are likely to be less involved in wine haze than the other proteins here studied. VVTL1 C, which formed a large number of aggregates but of intermediate size (852 nm) and VVTL1 I, which gave a very high number of particles detected with medium size higher than 1300 nm, must be considered as proteins potentially able to play an active role on wine hazing. CHIT C, for all the treatments considered, was the only protein giving both the highest number of aggregates (Fig. 11) and the largest average sizes (Fig. 14), thus revealing its crucial role in haze formation in white wines.

Generally speaking these data highlighted the fact that the five proteins examined had a very different aggregation behaviour, confirming results reported in the literature where different protein classes have shown different physicochemical behaviours after their heat-induced unfolding (Dufrechou *et al.*, 2010; Marangon *et al.*, 2011b). Besides, it was demonstrated that within a given protein class (VVTL1), it is possible to have great differences in behaviour likely due to the differences existing among protein isoforms.

CONCLUSIONS

The use of qNano, a new technology to our knowledge never used in the study of

protein hazing in wines before, showed the potential to give useful information for elucidating the mechanism of protein hazing. Moreover, the coupling of this technique with reconstitution approach aimed to study protein aggregation in model systems proved to be suitable to obtain information potentially relevant for what really happens in wines. In general the observed data highlighted how differently the proteins tested behaved, confirming results reported in the literature where different protein classes have shown different physicochemical behaviours after their heat-induced unfolding.

CHIT C was more easily unfolded by heat and, probably as a consequence of this fact, more reactive with other wine macromolecules than VVTL1 C, D, H and I. However, VVTL1 proved to be more reactive than the other TLPs. Therefore some isoforms of TLPs, which in recent studies were considered as having a less relevant role than chitinases on haze formation in wines, showed the potential of contributing to this phenomenon. A possibility is that the more reactive forms of TLPs are present in larger quantities in certain wines, and this may explain why there are conflicting reports in the literature about the role of TLPs in haze formation. This theory is supported by both qNano data and by the notion that, despite being heat unstable, the wine used contained a low level of chitinases.

Future studies will be focused on elucidating the nature of the wine proteins and their real balanced contribution to protein haze formation in white wines. Particular attention should be given to clarify the role of different protein isoforms towards wine hazing in order to be able to set up better predictive tools for haze formation and to use this knowledge for the development of alternative techniques for white wines stabilization.

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TABLES AND FIGURES

Table 1. Enological parameters of the Chardonnay wine.

Parameters	**Values**
Ethanol (% v/v)	12.3
pH	3.22
Titratable acidity (g H2SO4 L-1)	6
Total SO2 (mg L-1)	88
Free SO2 (mg L-1)	8
Volatile acidity (g L-1)	0.22
Sulfate (mg K2SO4 L-1)	310

Table 2. Experimental variables considered.

Parameters	**VARIABLE A: protein**	**VARIABLE B: treatment**
Concentration (particles/mL)	CHIT C	No addition of PS and PHE (-PS -PHE)
	VVTL1 C	Addition of PS (+PS -PHE)
	VVTL1 D	Addition of PHE (-PS +PHE)
	VVTL1 H	Addition of PS and PHE (+PS +PHE)
	VVTL1 I	
Size (nm)	CHIT C	No addition of PS and PHE (-PS -PHE)
	VVTL1 C	Addition of PS (+PS -PHE)
	VVTL1 D	Addition of PHE (-PS +PHE)
	VVTL1 H	Addition of PS and PHE (+PS +PHE)
	VVTL1 I	

Table 3. Purification steps before Chardonnay wine SCX fractionation. Name, protein concentration (mg L^{-1} *Thaumatococcus daniellii*), volume (L) and total protein content (mg) of fractions collected after Chardonnay wine SCX fractionation.

Purification step	**Fraction**	**Protein concentration**	**Volume**	**Total protein content**
Starting wine	-	79.28	36	2854.38
PVPP treated/filtered wine	-	67.05	36	2414.00
Wine waste after loading SCX column	-	24.52	36	882.75
Flow through SCX	-	230.96	1.7	392.64
SCX fractionation	A	56.34	0.076	4.28
	B	215.50	0.045	9.70
	C	341.60	0.3	102.48
	D	492.31	1	492.31
	E	114.84	0.65	74.65
	F	103.58	0.06	6.21
	G	289.15	0.164	47.42
	H	537.09	0.134	71.97
	I	176.04	0.415	73.06

Table 4. Purified protein characteristics.

		HPLC		SDS-PAGE	X!Tandem			
Protein	**Name**	**RT (min)**	**% purity**	**Apparent MW (kDa)**	**Top ranked protein**	**Unique/Total Peptides matched**	**Log(e)**	**1st homologue**
C 1-2	**CHIT C**	24.84	87	22.5	PREDICTED: Vitis vinifera class IV chitinase (CHI4D), mRNA.	5/5	-71.8	**Class IV chitinase** [Vitis vinifera].
C 4	**VVTL1 C**	12.81	90	22.5	LOC100232841, PREDICTED: Vitis vinifera VVTL1 (LOC100232841), mRNA.	5/7	-57.2	**VVTL1** [Vitis vinifera].
C 6-7		15.24	97					
C 6-7 α				65	LOC100256970, PREDICTED: Vitis vinifera hypothetical protein LOC100256970 (LOC100256970), mRNA.	2/4	-15.8	Vacuolar invertase 1, GIN1 [Vitis vinifera=grape berries, Sultana, berries, Peptide, 642 aa].
C 6-7 β				26	LOC100256970, PREDICTED: Vitis vinifera hypothetical protein LOC100256970 (LOC100256970), mRNA.	4/4	-35.7	Vacuolar invertase 1, GIN1 [Vitis vinifera=grape berries, Sultana, berries, Peptide, 642 aa].
C 6-7 γ				21	PREDICTED: Vitis vinifera thaumatin-like protein (TL3), mRNA.	2/2	-13.3	Thaumatin-like protein [Vitis vinifera].
D 1-2-3-4	**VVTL1 D**	12.76	98	21	LOC100232841, PREDICTED: Vitis vinifera VVTL1 (LOC100232841), mRNA.	5/7	-68.2	**VVTL1** [Vitis vinifera].
E 1-2-3		8.49	94	11	Lipid transfer protein isoform 1 [Vitis vinifera].	1/1	-15	Lipid transfer protein isoform 1 [Vitis vinifera].
H 4	**VVTL1 H**	12.82	>99	21	LOC100232841, PREDICTED: Vitis vinifera VVTL1 (LOC100232841), mRNA.	7/8	-90.5	**VVTL1** [Vitis vinifera].
I 1	**VVTL1 I**	12.98	>99	21	LOC100232841, PREDICTED: Vitis vinifera VVTL1 (LOC100232841), mRNA.	5/8	-63	**VVTL1** [Vitis vinifera].

Table 5. Concentration (particles/mL) of the aggregates formed upon heating of samples containing one of the five proteins tested (CHIT C, VVTL1 C, VVTL1 D, VVTL1 H, VVTL1 I), each one subjected to four different treatments: no addition of PS and PHE (-PS -PHE); addition of PS (+PS -PHE); addition of PHE (-PS +PHE); addition of PS and PHE (+PS +PHE). Data are expressed as mean ± SD (6 replicates).

	Protein				
Treatment	**CHIT C**	**VVTL1 C**	**VVTL1 D**	**VVTL1 H**	**VVTL1 I**
-PS -PHE	4,600,000 ± 141,421	5,350,000 ± 353,553	93,600 ± 70,970	176,667 ± 32,146	7,066,667 ± 763,326
+PS -PHE	7,566,667 ± 472,582	303,333 ± 90,738	650,000 ± 240,416	344,000 ± 182,702	885,000 ± 264,512
-PS +PHE	4,150,000 ± 777,817	5,400,000 ± 2,262,742	1,800,000 ± 707,107	1,105,000 ± 417,193	7,320,000 ± 664,831
+PS +PHE	7,350,000 ± 1,343,503	3,333,333 ± 404,145	203,333 ± 73,711	275,667 ± 219,878	1,500,000 ± 424,264

Table 6. Particle size measurements of aggregates (size range and mean size ± SD based on the number of aggregates detected in 3 replicates for CHIT C and 6 for the other proteins) formed upon heating of samples containing one of the five proteins tested (CHIT C, VVTL1 C, VVTL1 D, VVTL1 H, VVTL1 I), each one subjected to four different treatments: no addition of PS and PHE (-PS -PHE); addition of PS (+PS -PHE); addition of PHE (-PS +PHE); addition of PS and PHE (+PS +PHE).

		Protein: CHIT C	
Treatment	**n. aggregates**	**size range (nm)**	**size (mean ± SD)**
-PS -PHE	514	721.800 – 4050.300	1379.806 ± 351.446
+PS -PHE	1214	749.600 – 3000.900	1414.400 ± 356.148
-PS +PHE	426	847.600 – 2403.400	1363.497 ± 313.695
+PS +PHE	378	731.200 – 3381.200	1483.953 ± 443.825

		Protein: VVTL1 C	
Treatment	**n. aggregates**	**size range (nm)**	**size (mean ± SD)**
-PS -PHE	3528	233.800 – 3419.300	792.607 ± 246.341
+PS -PHE	22	307.800 – 959.300	513.327 ± 183.061
-PS +PHE	4073	294.700 – 1866.300	820.945 ± 249.310
+PS +PHE	587	242.200 – 3863.700	667.496 ± 324.330

		Protein: VVTL1 D	
Treatment	**n. aggregates**	**size range (nm)**	**size (mean ± SD)**
-PS -PHE	106	346.300 – 1850.300	888.017 ± 245.072
+PS -PHE	787	402.800 – 3507.600	941.465 ± 379.160
-PS +PHE	1887	442.800 – 4498.500	807.010 ± 180.857
+PS +PHE	660	466.700 – 3498.100	962.105 ± 372.031

		Protein: VVTL1 H	
Treatment	**n. aggregates**	**size range (nm)**	**size (mean ± SD)**
-PS -PHE	49	330.600 – 2379.400	806.030 ± 362.032
+PS -PHE	171	487.500 – 3534.200	976.812 ± 357.429
-PS +PHE	1019	378.700 – 2446.200	828.816 ± 203.793
+PS +PHE	569	437.000 – 3359.100	861.842 ± 328.455

		Protein: VVTL1 I	
Treatment	**n. aggregates**	**size range (nm)**	**size (mean ± SD)**
-PS -PHE	3188	547.500 – 2255.100	1033.735 ± 239.799
+PS -PHE	1594	512.900 – 3522.700	1077.247 ± 333.423
-PS +PHE	2947	571.900 – 3589.500	1148.243 ± 302.414
+PS +PHE	1579	546.200 – 3715.300	1090.136 ± 352.824

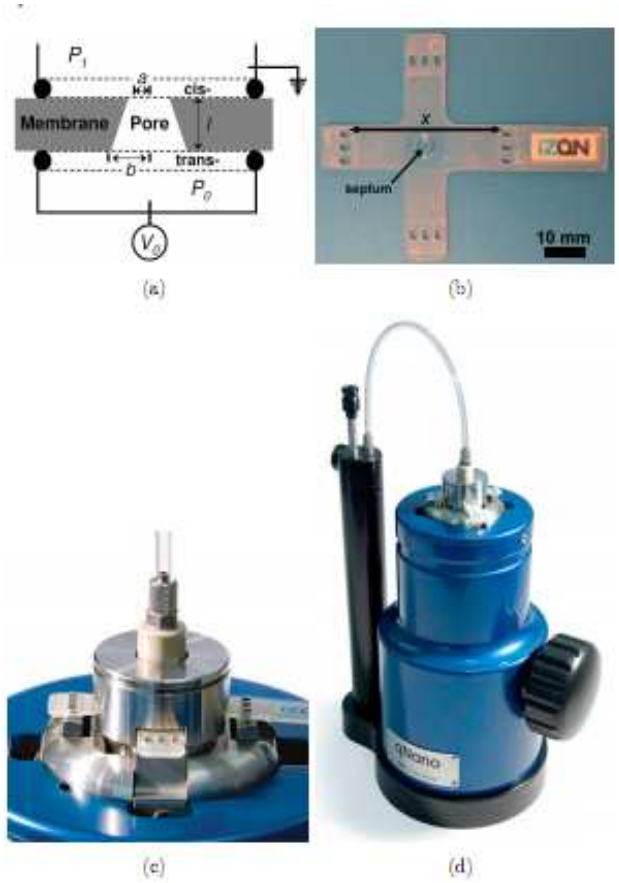

Figure 1. (a) Schematic sectional diagram showing a conical pore between two halves of a fluid cell, symmetric about a cylindrical axis running through the centre of the pore. (b) A thermoplastic polyurethane cruciform of thickness 0.7 mm, increasing up to 1.5 mm around the holes at the ends of the cruciform legs. (c) In use, the cruciform is housed within a fluid cell, with the legs protruding and the holes placed on pegs to enable stretch tuning. (d) The fluid cell is part of the Izon qNano apparatus, which includes customised electronics in the base for precise ionic current measurements; a handle is turned to stretch the cruciform; and a manometer column, which can apply pressure to the upper fluid cell via the transparent polymer tubing (Wilmott *et al*., 2010).

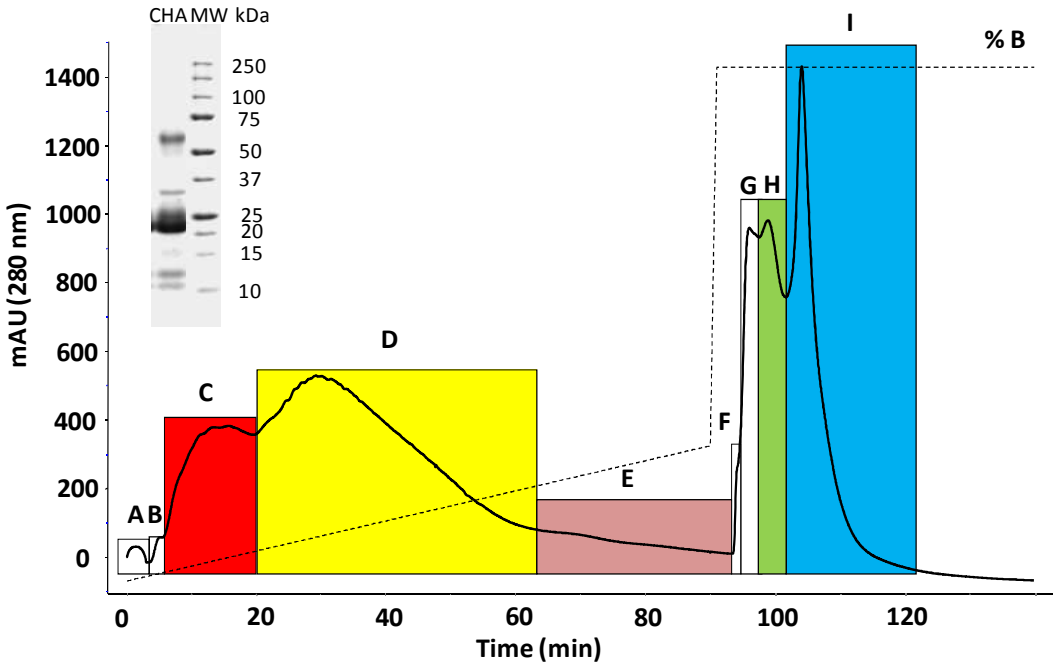

Figure 2. Cation exchange chromatogram (SCX) for Chardonnay wine. The dotted line indicates the salt/pH gradient. Collected fractions (different colours) SCX-C, SCX-D, SCX-E, SCX-H and SCX-I were used for HIC. On the left, SDS-PAGE (in reducing conditions) of the untreated Chardonnay wine (CHA).

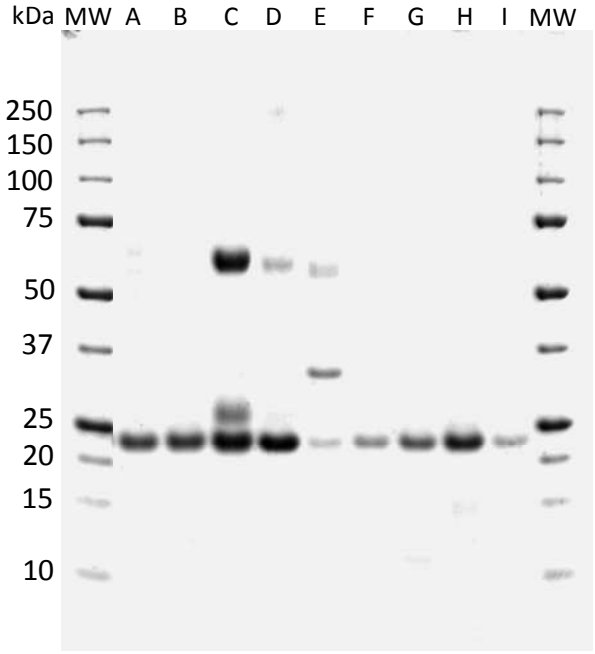

Figure 3. SDS-PAGE (in reducing conditions) of the 9 fractions (A to I) separated by SCX. Three µg of protein were loaded in each lane. MW standard proteins are on the left and on the right of the gel.

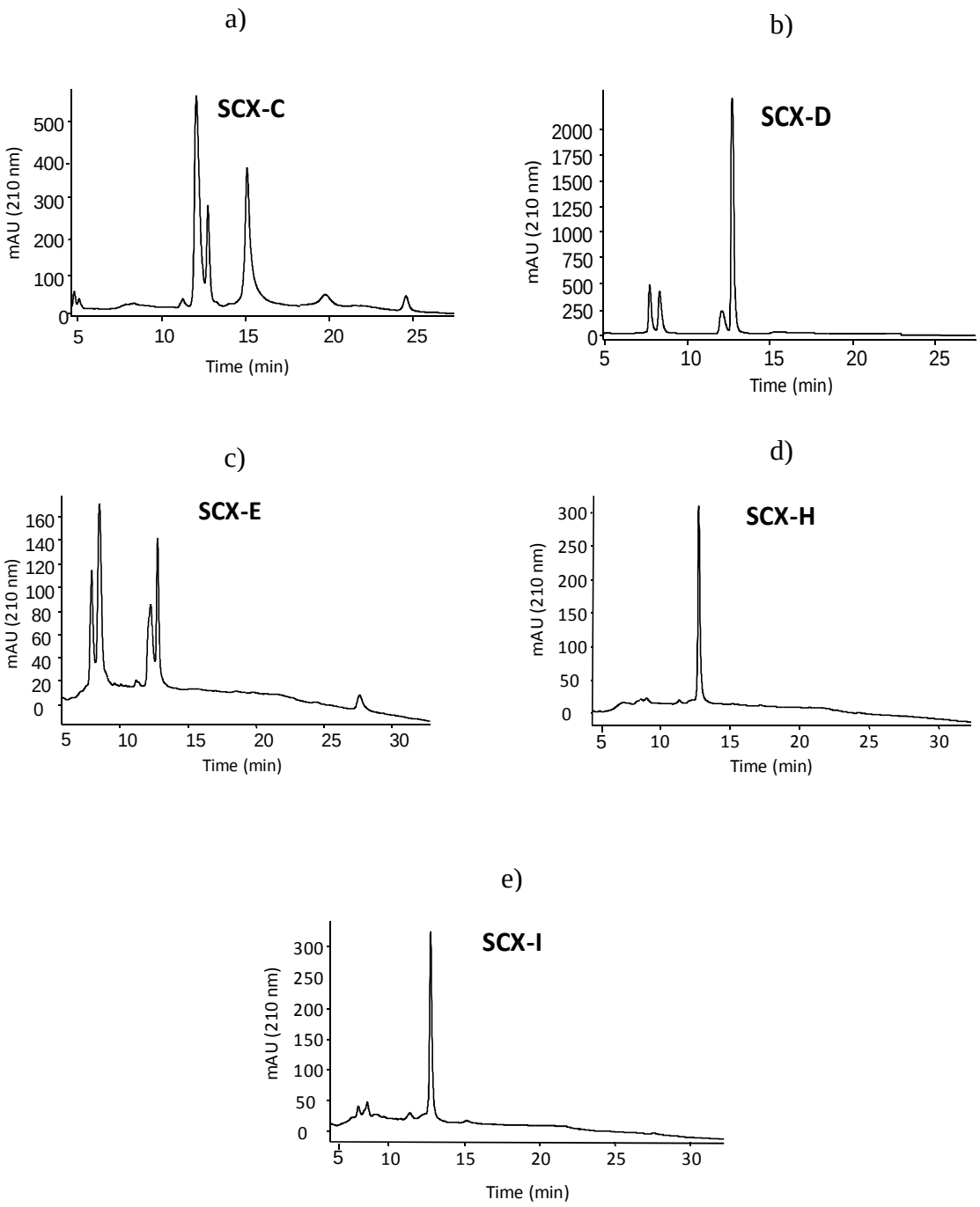

Figure 4. RP-HPLC chromatograms of SCX fractions C (a), D (b), E (c), H (d) and I (e).

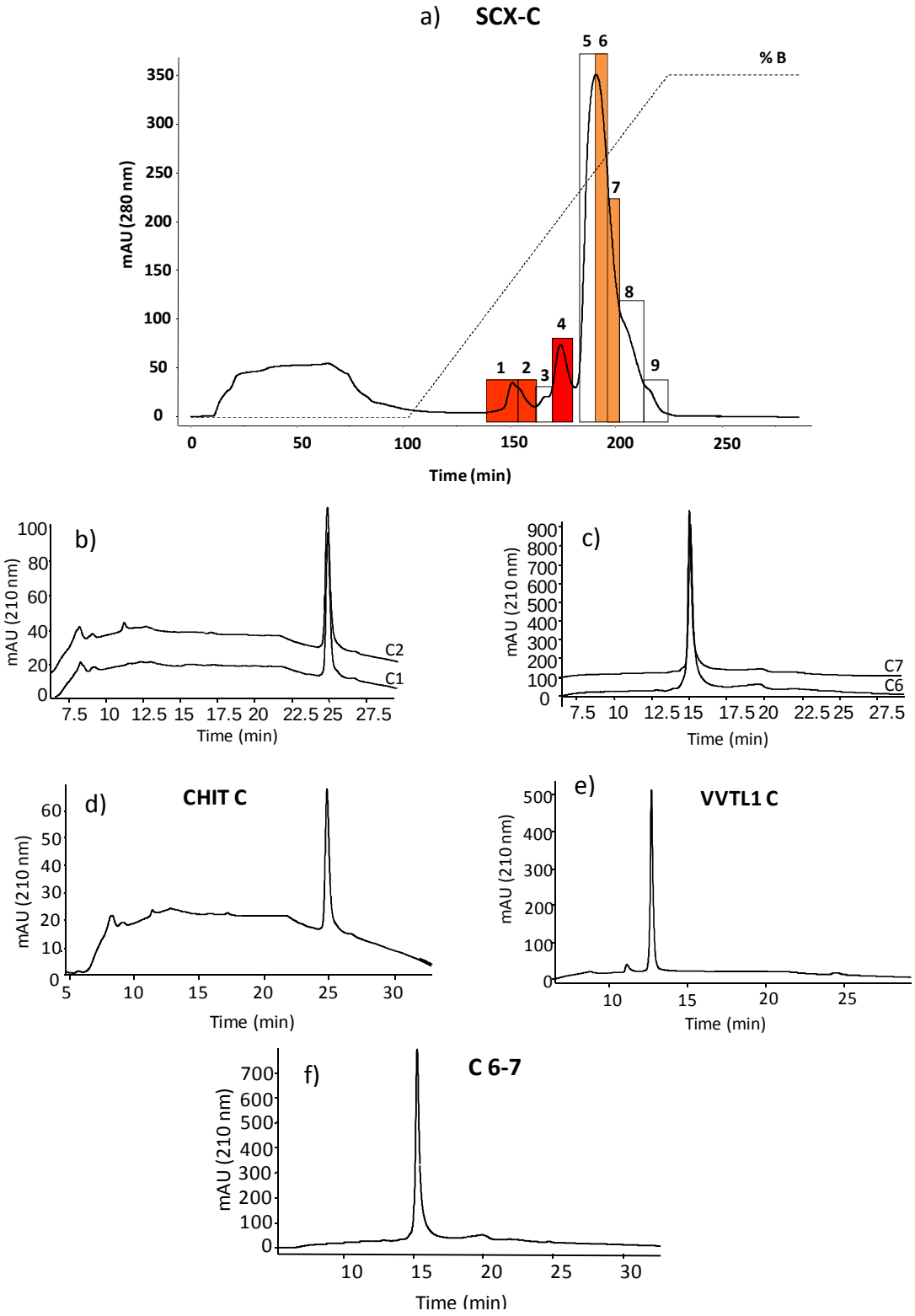

Figure 5. HIC chromatogram for SCX fraction C (a). RP-HPLC chromatograms of HIC single fractions C1 and C2 (b), C6 and C7 (c). RP-HPLC chromatograms of pooled fraction C 1-2 named "CHIT C" (d), C 4 named "VVTL1 C (e) and C 6-7 (f).

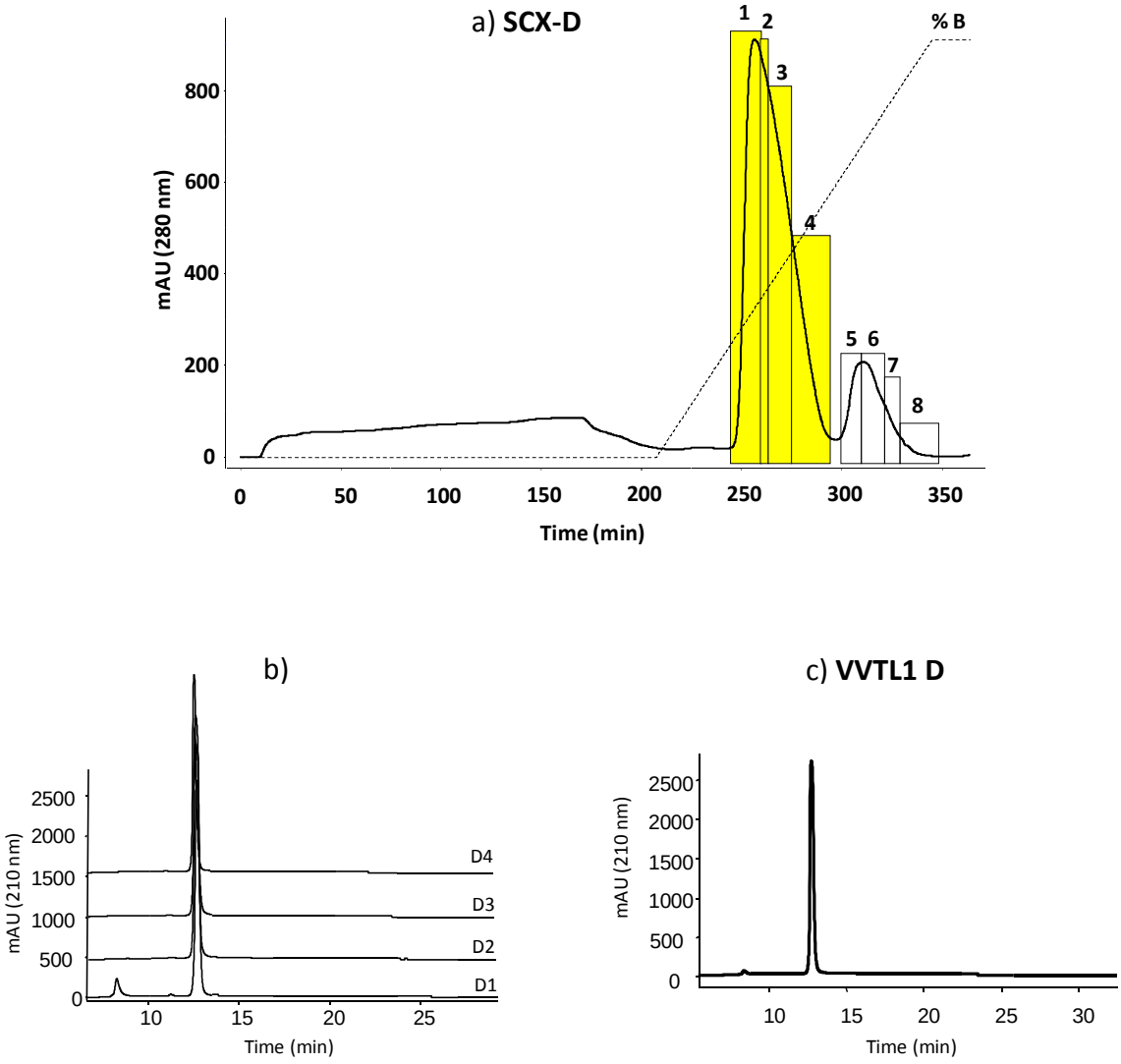

Figure 6. HIC chromatogram for SCX fraction D (a). RP-HPLC chromatograms of HIC single fractions D1, D2, D3 and D4 (b). RP-HPLC chromatogram of collected fraction D 1-2-3-4 named “VVTL1 D” (c).

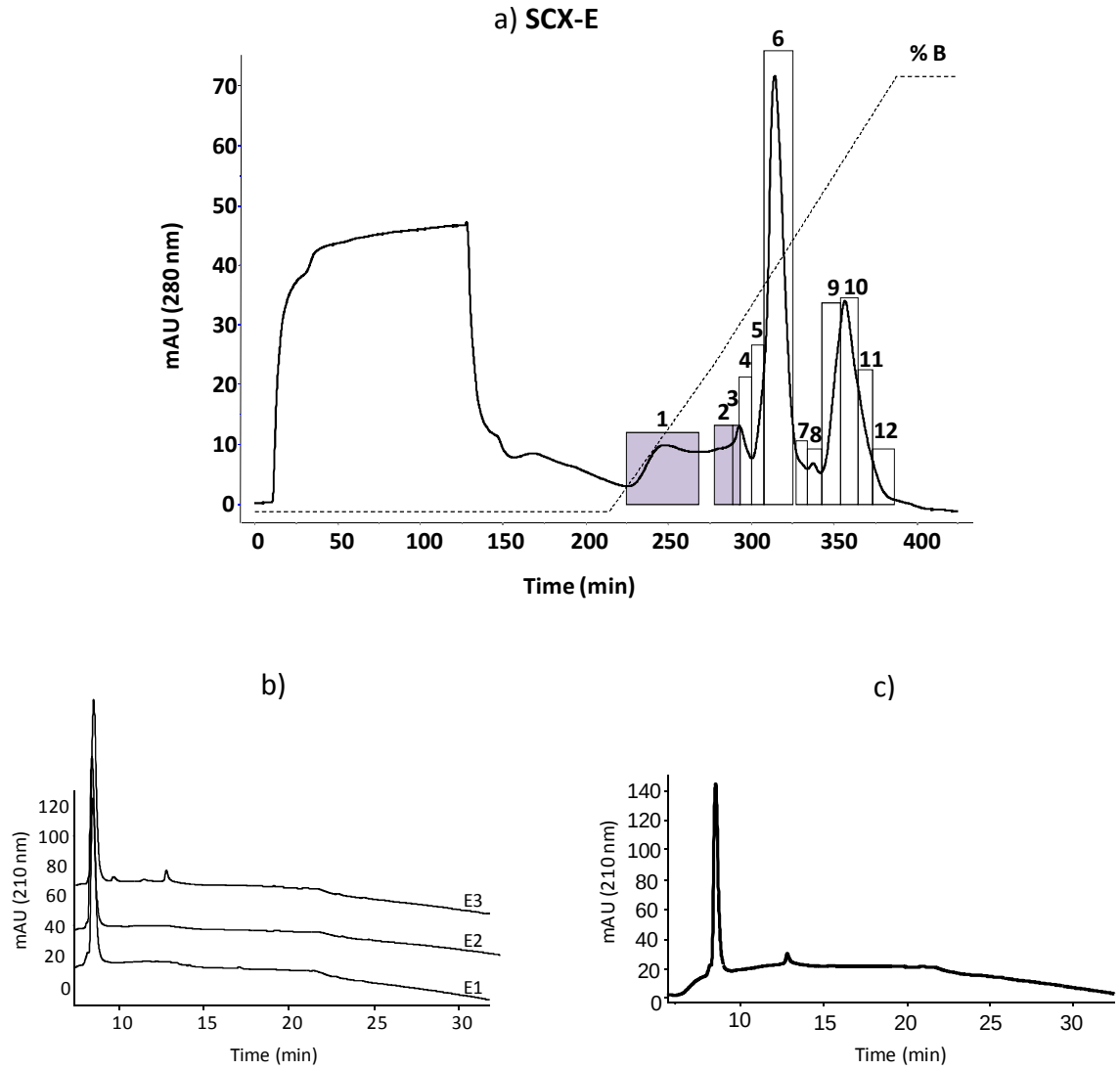

Figure 7. HIC chromatogram for SCX fraction E (a). RP-HPLC chromatograms of HIC single fractions E1, E2 and E3 (b). RP-HPLC chromatogram of collected fraction E 1-2-3 (c).

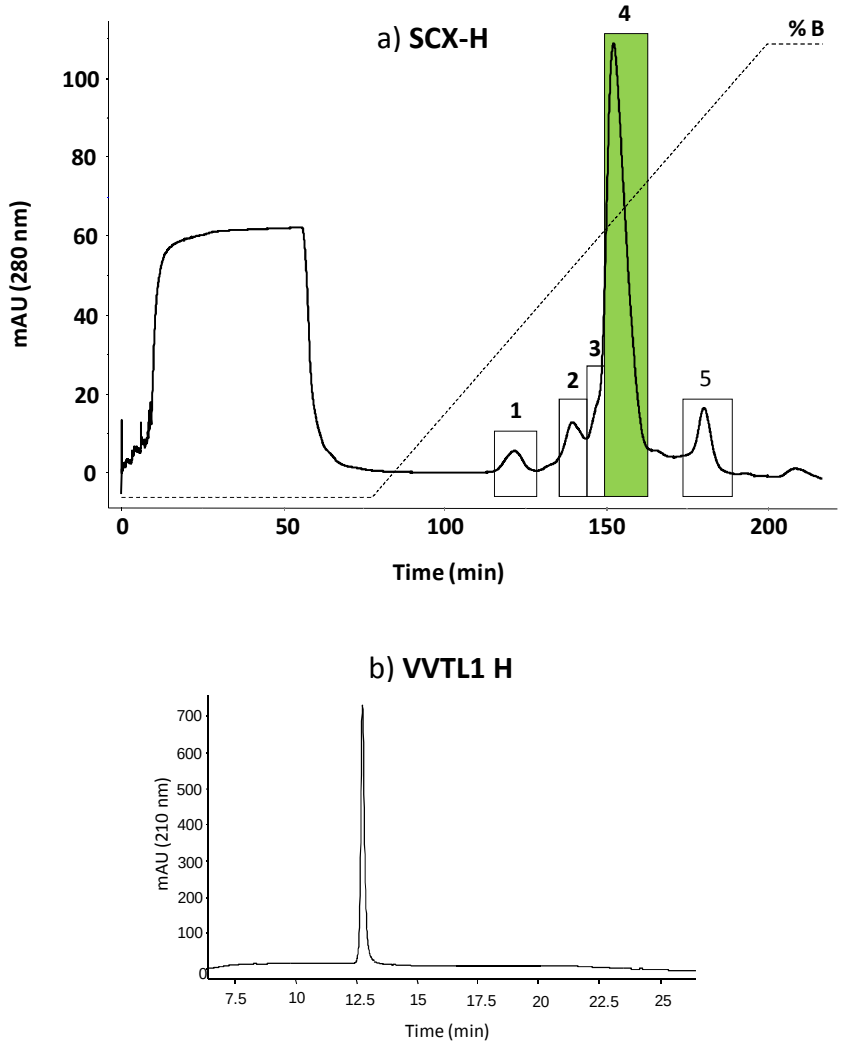

Figure 8. HIC chromatogram for SCX fraction H (a). RP-HPLC chromatogram of HIC fraction H 4 named “VVTL1 H” (b).

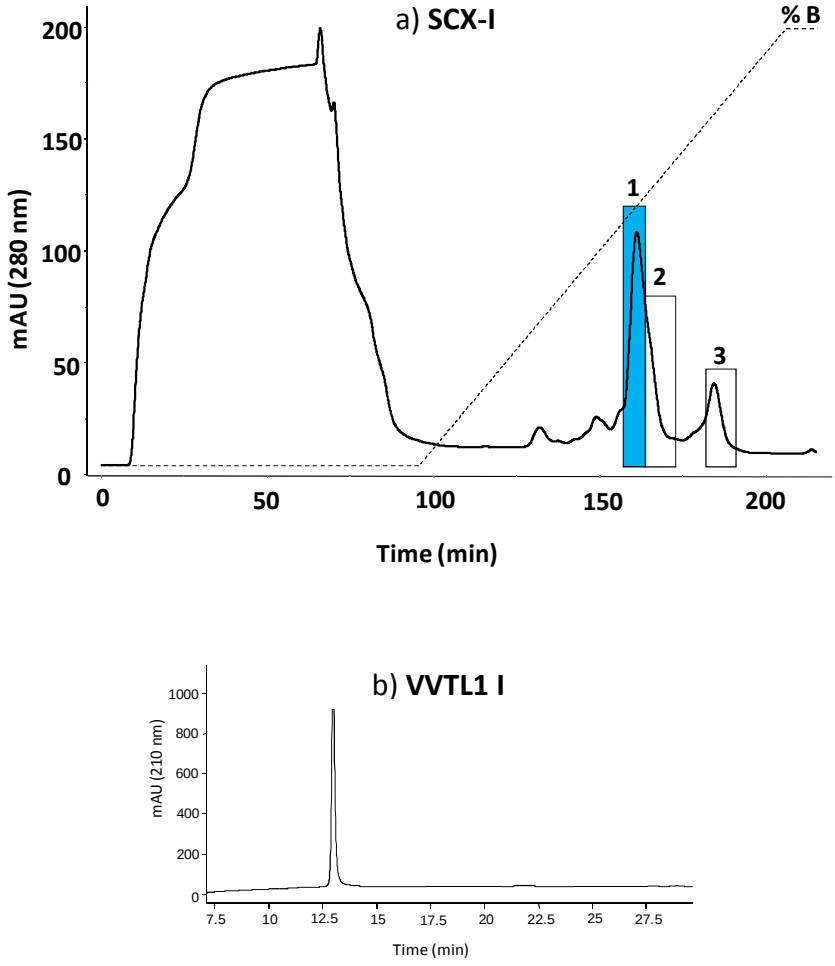

Figure 9. HIC chromatogram for SCX fraction I (a). RP-HPLC chromatograms of HIC fraction I 1 named "VVTL1 I" (b).

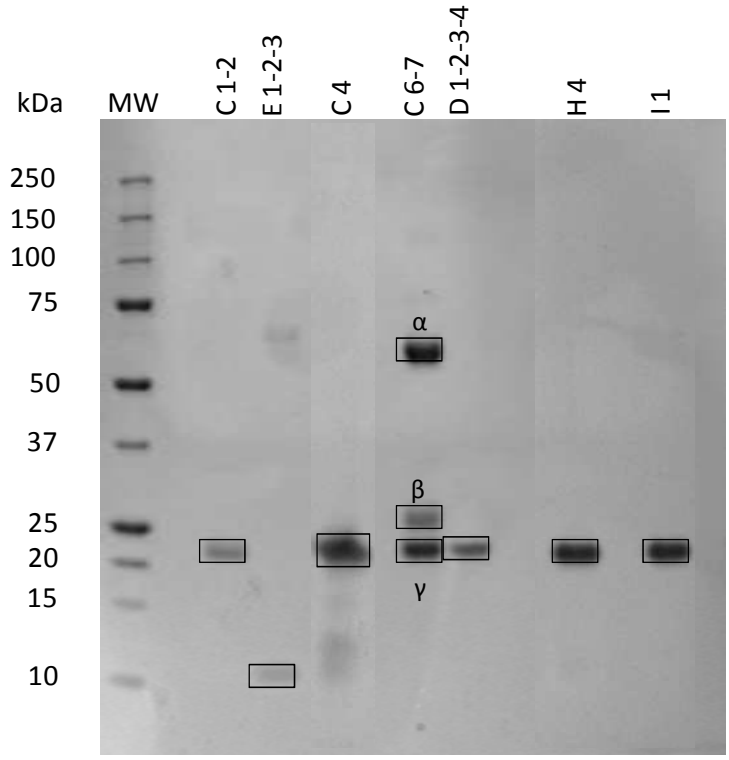

Figure 10. Purified proteins (~3 µg per lane) were reduced, subjected to SDS-PAGE, and stained with Pierce Imperial Protein Stain.

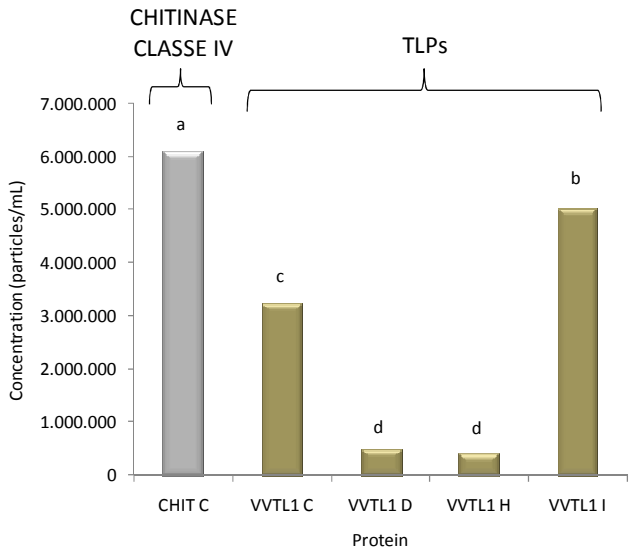

Figure 11. Concentration of the aggregates (particles/mL) formed upon heating of samples containing CHIT C (chitinase class IV), VVTL1 C, VVTL1 D, VVTL1 H, VVTL1 I (TLPs). The main effect "protein" is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tukey's HSD test ($P \leq 0.05$).

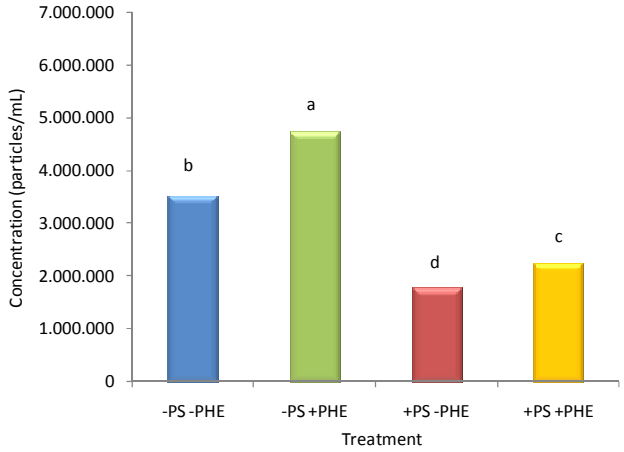

Figure 12. Concentration of the aggregates (particles/mL) formed upon heating of samples subjected to different treatments: no addition of PS and PHE (-PS -PHE); addition of PS (+PS -PHE); addition of PHE (-PS +PHE); addition of PS and PHE (+PS +PHE). The main effect "treatment" is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tukey's HSD test ($P \leq 0.05$).

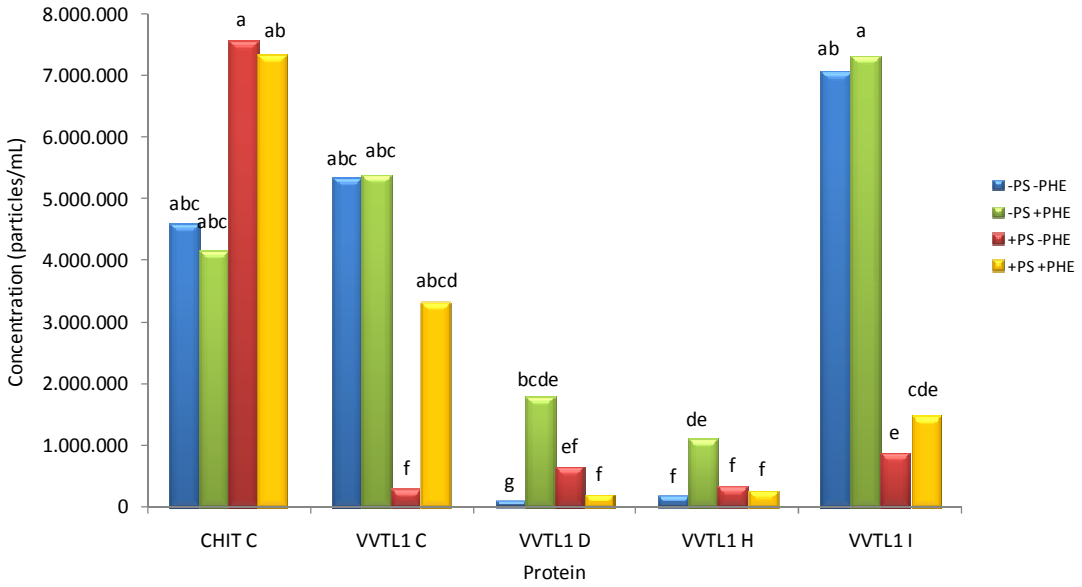

Figure 13. Effect of protein and treatment (interaction "protein x treatment") on concentration of the aggregates (particles/mL) formed upon heating. The interaction effect is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tukey's HSD test ($P \leq 0.05$).

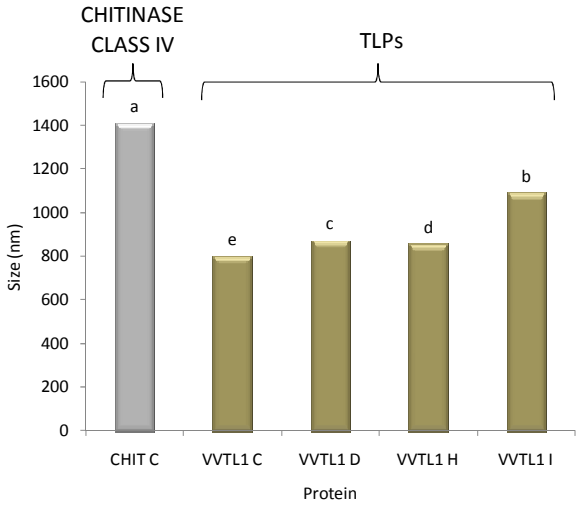

Figure 14. Particle size measurements of aggregates (nm) formed upon heating of samples containing CHIT C (chitinase class IV), VVTL1 C, VVTL1 D, VVTL1 H, VVTL1 I (TLPs) as determined by qNano. The main effect "protein" is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tukey's HSD test ($P \leq 0.05$).

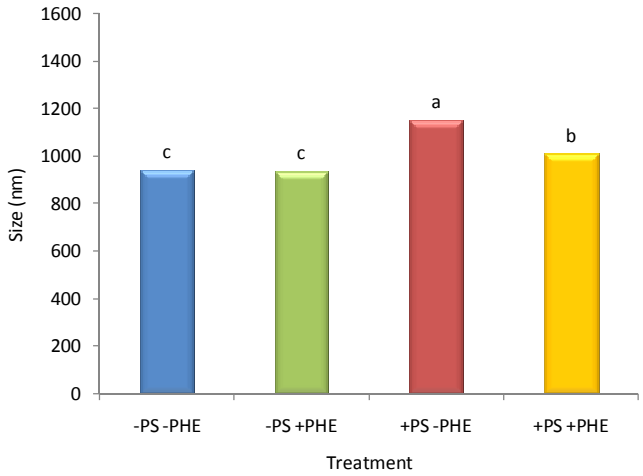

Figure 15. Particle size measurements of aggregates (nm) formed upon heating of samples subjected to different treatments: no addition of PS and PHE (-PS -PHE); addition of PS (+PS -PHE); addition of PHE (-PS +PHE); addition of PS and PHE (+PS +PHE). The main effect "treatment" is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tukey's HSD test ($P \leq 0.05$).

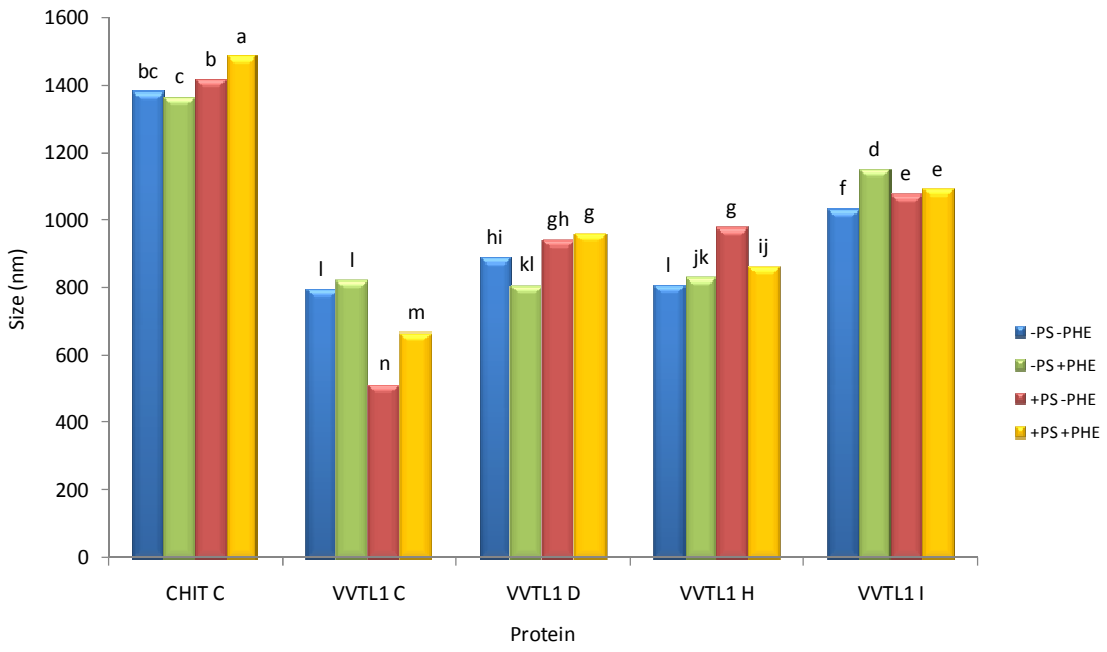

Figure 16. Effect of protein and treatment (interaction "protein x treatment") on size (nm) of the aggregates formed upon heating. The interaction effect is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tukey's HSD test ($P \leq 0.05$).

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CHAPTER 4

Quantification of proteins in grape juice and wine with colorimetric methods

ABSTRACT

Since the research in enology needs to find a precise method that allows an accurate quantification of the protein amount in wines and juices, in this study two colorimetric assays were investigated: the Bradford method (based on the Coomassie Brilliant Blue, CBB) and the potassium dodecyl-sulphate (KDS) protein precipitation followed by BCA assay (KDS/BCA). Some main factors that can potentially affect the quantification of proteins in wine were analysed, including the presence of ethanol, polyphenols and protein glycosylation. Moreover, the response of different proteins towards CBB and BCA reagents was studied.

The Bradford assay did not prove to be accurate for wine protein quantification as it was affected by the presence of interfering substances in the matrices and by the aminoacid composition of the proteins tested. In particular, the presence of ethanol decreased the protein response by 28% and the concentration of 200 mg L^{-1} of polyphenols caused a statistically significant decrease (16%) in the slope of the protein calibration curve. Moreover, lysozyme showed a significantly higher average response than the other proteins, while ovalbumin the lowest. In contrast by applying the KDS/BCA method, the matrix didn't show any statistically significant effect on the slope of the protein calibration curve and there were less differences between the proteins examined. Finally, the BCA method was almost insensitive to the sugars present in glycoproteins and mannoproteins.

In conclusion, as here demonstrated, the KDS/BCA method can be considered superior to the Bradford assay for protein quantification in wines and juices.

Keywords: Bradford, KDS/BCA, protein quantification.

INTRODUCTION

A wide range of techniques are available for protein quantification in general, but three major drawbacks when dealing with wine and grape juice samples impair the use of some of them (Le Bourse *et al*., 2010). Proteins are typically present at very low concentrations (Ferreira *et al*., 2002) in these media, which lead to the use of techniques with very low detection limits. The presence of contaminants, such as phenolic compounds and ethanol, can distort the quantification (Compton and Jones, 1985; Marchal *et al*., 1997). Moreover, the absence of standard grape or wine proteins does not allow the correct protein quantification in a sample (Le Bourse *et al*., 2010).

The employed methods are variable: Kjeldahl (Kjeldahl, 1883), Biuret (Gornall *et al*., 1949), Lowry (1951), Bradford (Bradford, 1976) and Smith (Smith *et al*., 1985).

The Bradford assay, based on Coomassie Blue G-250, is probably the most widely used for protein quantification in wines (Murphey *et al*., 1989; Brissonnet and Maujean, 1991; Waters *et al*., 1991; Boyes *et al*., 1997) its popularity being mainly due to simplicity and speed of execution (Marchal *et al*., 1997; Weiss and Bisson, 2001). In fact it requires the addition of a single reagent to the sample and relatively short incubation before the absorbance is measured (Weiss and Bisson, 2001). However, it was noted that Coomassie Brilliant Blue method has several fundamental limitations. The assay is based on the immediate absorbance shift 470 nm to 595 nm that occurs when the dye binds to protein in acidic solution. The dye is believed to bind to protein via Van der Waals forces and hydrophobic interactions (Compton and Jones, 1985). The mechanism of dye binding can be explained by the dye existing as three absorbing species, a red cationic species (A_{max} 470 nm), a green neutral species (A_{max} 650 nm) and a blue anionic species (A_{max} 595 nm). Color changes are due to successive loss of charge. Prior to protein binding, the dye molecules exist in doubly protonated (the red cationic dye form); upon binding of the dye to protein, the blue anionic dye form is stabilized ad is detected at 595 nm (Compton and Jones, 1985).

Compton and Jones (1985) discussed factors that lead to over- and under-estimation of protein content. The protein content may be overestimated when a non-protein molecule stabilises the neutral species. This species has a very broad absorbance and will contribute to the absorbance at 595 nm. Some of the compounds associated with this phenomenon are detergents, such as sodium dodecyl sulphate (SDS), and flavonoids. Flavonoids broaden the

peak at 650 nm, thereby increasing the absorbance at 595 nm. In some cases, the amount of free dye can become the limiting factor in the assay (Splittgerber and Sohl, 1989). Factors that reduce binding of the dye to protein result in an underestimation of the protein concentration. These non-protein compounds compete with the dye molecule for the protein, reducing the extent of dye binding. In plant products the Bradford assay is limited by interferences from a number of compounds, including polysaccharides (Godshall, 1983) and phenolics (Brenna and De Vecchi, 1990). Marchal *et al*. (1997) noted an increase in A_{595} when this assay was used with pure enological tannins and with protein-free grape skin extract. Waters et al. (1991) found that phenolic compounds could lead to underestimation of the protein content of about 50-80%.

The other colorimetric methods such as Lowry protein assay, Biuret assay or the acid bicinconinic assay, unlike Bradford which is applied directly to wine, are based on the separation of proteins from matrix because they are influenced by interfering substances such as glutathione, phenolics and potassium ions (Godshall, 1983; Brenna and De Vecchi, 1990; Dorrestein *et al*., 1995). Among the mentioned methods, BCA is commonly used (Schoel *et al*., 1995; Bainor *et al*., 2011) since it has many advantages such as sensitivity, simplicity, stability of the chromophor and low protein-to-protein variation. Furthermore, this assay is insensitive to many contaminating substances, such as commonly used detergents (Smith *et al*., 1985). However, other substances, either naturally present in the sample or added during protein purification, have been shown to interfere with the BCA test. Since the assay involves reduction of Cu^{2+} to Cu^{+} by proteins, reducing compounds (such as dithiothreitol, cysteine or reducing sugars) or copper-chelating reagents (such as EDTA) cause interferences (Smith *et al*., 1985).

Vincenzi and co-workers developed a new procedure for the recovery and quantification of wine proteins which involved the precipitation of proteins as potassium dodecyl sulphate (KDS) complexes followed by the bicinchoninic acid (BCA) test (KDS/BCA). This method proved to be less influenced than the Bradford assay by the interfering substances present in wine (Vincenzi *et al*., 2005).

In this study Bradford test was compared with KDS/BCA assay for the quantification of proteins in wine. Some main factors that can potentially affect the quantification of proteins in wine were analysed including the presence of ethanol,

polyphenols and protein glycosylation. Moreover, the response of different proteins towards Coomassie Blue and BCA reagents was studied.

MATERIALS AND METHODS

Materials

Both the wines (Sauvignon Blanc, vintage 2009; Manzoni Bianco, vintage 2009) and the grape juice (Glera, vintage 2009) utilized in this work were kindly supplied by the winery of "Scuola Enologica G.B. Cerletti" of Conegliano (Italy). They were ultrafiltered by means of an Amicon apparatus (RC800) with a regenerated cellulose membrane (Millipore) at nominal cut off of 3000 Da. The ultrafiltered solutions were stored at 4°C until use.

The model wine was prepared with 5 g L^{-1} tartaric acid (Baker), 12% ethanol (Carlo Erba) buffered to pH 3.2 with NaOH (Carlo Erba). Bovine serum albumin (BSA), thaumatin of *Thaumatococcus danielli* (THAU) and ovalbumin (OVA) were purchased from Sigma-Aldrich. Lysozime (LYS) was purified from enological Lysozyme (Oliver Ogar) using S-Sepharose Fast Flow column. Purity was assessed by HPLC on a Vydac column (Waters *et al*., 1995). Polyphenols used in this experiment were isolated from Manzoni Bianco wine (vintage 2008) as described afterwards.

Isolation of wine polyphenols

Polyphenols were extracted from 3 L of Manzoni Bianco ultrafiltered wine (3 kDa cut off) and filtered through 0.2-µm filters (Sartorius). Briefly, a C18 cartridge (Sep-Pak C18, 10 g, Waters) was washed with 50 mL of methanol and equilibrated with 50 mL of deionized water. The white wine was then loaded onto the equilibrated cartridge. The water-soluble compounds were removed by 100 mL of water, while polyphenols were eluted with 30 mL of methanol (Carlo Erba). The methanol phase was collected and the solvent was removed in a rotary evaporator (Buchi Rotavapor R-114***)*** at 35°C. The residue was resuspended in 10 mL of model wine and the concentration of polyphenol was estimated by the Folin-Ciocalteu assay (Singleton and Rossi, 1965).

Analytical methods

Alcohol content and pH were determined following the official methods of analysis proposed by the Office International de la Vigne et du Vin (OIV).

Creation of standard curves

Stock solutions of 10 mg L^{-1} BSA, THAU and LYS and a stock solution of 5 mg L^{-1} OVA, each of them dissolved in distilled water, were stored at 4°C. Standard solutions for calibration curves to be analyzed with Bradford method consisted of 2.5, 5, 10, 15, 20, 30, 40, 60, 80 µg mL^{-1} BSA, THAU, OVA and LYS, instead those to be analyzed with KDS/BCA consisted of 62.5, 125, 250, 500, 1000 µg mL^{-1} of proteins mentioned above. These known amounts of proteins were diluted in matrixes of increasing complexity: water (H_2O), model wine (MW), ultrafiltered Glera grape juice (G J), ultrafiltered Sauvignon wine (SAU W) and ultrafiltered Manzoni Bianco wine (MB W). In a second set of experiments they were diluted in model wine with increasing content of polyphenols (0, 25, 50, 100, 200 mg L^{-1}). Each standard curve was prepared in five separate times. For the glycoproteins calibration curves, the real protein content, as determined by Kjeldahl analysis, was utilized to prepare standards in the same concentration range used for the other proteins.

The absorbance of solutions was determined at 595 nm (Bradford) and at 562 nm (KDS/BCA). The absorbance readings of standard solutions were used to calculate the average response or slope (expressed in A_{595} or A_{562} µg^{-1} proteins) of the linear regression. The *y*-intercept was also computed, along with the coefficient of linear regression (r^2).

Must glycoproteins and wine mannoproteins fractionation

Must glycoproteins and wine mannoproteins were fractionated from must and wine Boschera (vintage 2008). Both the samples were ultrafiltered (MWCO 3000 Da), and the retentate, containing the macromolecules, was dialyzed and freeze-dried. The powder obtained was resuspended in 10 volumes of water and precipitated with 60% ammonium sulfate (Carlo Erba). The obtained supernatant was dialyzed against water and freeze dried.

Kjeldahl digestion method

100 mg of sample was mixed with 4 mL of concentrated sulfuric acid in a 100 mL round-bottomed flask. The mixture was heated to 440 °C for 4 minutes. Then 16 mL of 30% hydrogen peroxide were added and the mixture was heated for 10 min (440 °C). When the digestion flask reached room temperature, the volume was brought to 100 ml with distilled water. A 2.5 mL aliquot of the diluted digest was mixed with 0.1 mL of Nessler'reagent. The absorbance was recorded at 425 nm and it was converted into concentration by means of a linear calibration curve using ammonium sulphate (0.1-10 mg L^{-1}). The protein content was determined multiplying by 6.25 the results.

Protein precipitation with potassium dodecyl sulphate (KDS)

Protein precipitation was achieved by protein denaturation with sodium dodecyl sulfate (SDS) (Bio-Rad) and following precipitation with potassium chloride (KCl) (Carlo Erba) (Vincenzi *et al*., 2005).

5 µL of SDS (stock solution 10% [w/v]) were added to 500 µL of each sample which was heated at 100°C for 5 min. Then 125 µl KCl 1M were added to samples and, after at least two hours of incubation at room temperature, pellets were collected by centrifugation (12.000 rpm for 15 min at 4°C). Further three washes with 1 mL KCl 1M were required to completely eliminate interfering substances from samples. Pellets were then freeze-dried to remove any remaining trace of liquid.

Protein quantification with acid bicinconinic assay (BCA)

It was used the Microwell Plate Protocol BCA-200 Protein Assay kit (Pierce), based on the method described by Smith (1985). After being freeze-dried, samples were resuspended with 500 µl of water and heated for 5 min. Then 50 µl were taken from each sample and placed in 1-cm-path-length cuvette, with 1 ml of BCA reagent, consisting of 50 parts of reagent A and 1 part of reagent B. Cuvettes were incubated at 37°C for 30 minutes. Absorbances of samples were determined with the spectrophotometer (Shimadzu, model UV 6010) at 562 nm wavelength.

Protein quantification with Bradford assay (CBB)

From each standard solution, 400 µl were mixed with 400 µl of deionized water. Finally 200 µl of Bio-Rad Protein assay (Bio-Rad) reagent were added to samples. Then

samples were allowed to react for one hour at room temperature and absorbances were read using the spectrophotometer (Shimadzu, model UV 6010) at 595 nm wavelength (Marchal *et al*., 1997).

Statistical analysis

Statistical analysis was performed with CoHort Software (Costat version 6.4, Monterey, CA). Data were evaluated by two-way completely randomized analysis of variance (ANOVA). The main effects and interactions among effects were tested for each colorimetric assay. The Tukey multiple comparison test was used to compare the means when significant differences were found in the variance analysis ($P < 0.05$ was used as criterion of significance).

Experimental disign

The experimental design has been organized into 4 stages:

i) Evaluation of the reactivity of four proteins (BSA, THAU, OVA and LYS) to CBB and BCA in the presence of water;

ii) Evaluation of the effect of five different matrixes including H_2O, model wine, ultrafiltered grape juice and two ultrafiltered wines on the protein average response to CBB and KDS/BCA assays;

iii) Evaluation of the effect of increasing concentration of polyphenols (0, 25, 50, 100, 200 mg L^{-1}) on the protein average response to CBB and KDS/BCA assays;

iv) Evaluation of the efficiency of CBB and BCA assays for the quantification of juice glycoproteins and wine mannoproteins.

RESULTS AND DISCUSSION

Protein-to-protein variability: evaluation of the average response of four proteins to CBB and BCA in presence of water solution

First, the reactivity of four proteins (BSA, LYS, OVA and THAU) to Bradford and BCA has been evaluated in the presence of water. The reactivity of proteins has been determined calculating the slope of the calibration curves for each protein, in order to take into account also the interaction effect of proteins and dye with the matrix.

Using Bradford colorimetric method, the proteins considered gave average responses significantly different to each other (with the exception of BSA and THAU) (Fig. 1), while, using the BCA test, differences among protein slopes were attenuated (Fig. 2). In the latter case, considering the absence of interfering substances, the BCA assay was performed without prior precipitation with KDS. Regarding the reaction of proteins towards Bradford dye, lysozyme showed an average response (0.04829 A_{595} µg $^{-1}$ protein) significantly higher ($P \leq 0.05$, by 29%) than that given by BSA (0.03554) and THAU (0.03350), which were not differentiated, while ovalbumin gave the lowest response (0.02494) (Fig. 1).

It is well known that the response to Bradford assay is protein dependent and varies with the protein amino acid composition (Tal *et al*., 1985; Compton and Jones, 1985; González-González *et al*., 2011), with lysine, arginine, histine and/or hydrophobic interactions playing an important role in dye binding (Noble *et al*., 2007). Actually, lysozyme has got an unusual aminoacid composition with high percentage of amino acids Arg (8.2%) and His (10.7%) (Table 1) to which Coomassie Brilliant Blue G-250 specificity has already been demonstrated (Compton e Jones, 1985). Ovalbumin, which contains 8.6% aromatic amino acids and 10.9% basic amino acids (Table 1) is less bound by the dye due to its amino acid composition and the presence of glycans (Fountoulakis *et al*., 1992). Even Szöllösi and colleagues (2007) obtained the same results demonstrating that using the Bradford method the protein concentration of ovalbumin standard solution was lower than that of BSA of about 32%. This fact was also confirmed by Antharavally *et al*. (2009). They observed that the colour development was significantly greater with BSA than with most other proteins, included ovalbumin.

The mechanism of interference of carbohydrates with this colorimetric assay is not fully understood. The Coomassie dye binds proteins on the basis of hydrophobic and ionic interactions (Compton e Jones, 1985). The underestimation of glycoproteins can be due to steric hindrance caused by some types of carbohydrates which hinder the binding of dye to hydrophobic and basic residues, or most likely to the fact that the hydrophilic sugar moieties change the hydrophobicity of the glycoproteins so that less dye binds (Fountoulakis *et al*., 1992).

It is interesting to note that THAU (standard thaumatin of *Thaumatococcus daniellii*), a good model for proteins in wine for its high sequence homology to thaumatin-like proteins of *Vitis vinifera* (Edens *et al*., 1982), the main grape proteins together with chitinases (Pocock *et al*., 2000), gave an average response similar to BSA, a widely used protein standard (Noble *et al*., 2009).

In contrast, with the KDS/BCA protocol there were no significant differences between BSA and OVA while LYS and THAU, which were not differentiated, gave a response statistically higher by 32% and 21% respectively than the average slope of the other two proteins (Fig. 2). In fact, the reaction that leads to BCA colour formation, as a result of the reduction of Cu^{2+}, is strongly influenced by the presence of three amino acid residues (tyrosine, tryptophan and cysteine) in the amino acid sequence of the protein. As a matter of fact LYS showed to contain 6.1% Cys and 4.1% Trp, followed by THAU with 7.7% Cys and 3.9% Tyr, while BSA and OVA displayed lower percentages of the amino acids involved in the colour reaction.

Overall, BCA seemed to have the advantage of a low protein-to-protein variability as indicated by the low coefficient of variation (19.27%) for the colour response of 4 different proteins, compared to that obtained using the Coomassie dye (27.44%) (Table 2).

Matrix-to-matrix variability: evaluation of matrix effect on the average response of four proteins to Bradford and BCA

The effect of five different matrixes, including H_2O, model wine, ultrafiltered grape juice and two ultrafiltered wines in which proteins were added at known concentrations was then evaluated for the response to CBB and BCA. In the latter case, for ultrafiltered juice and wines, the BCA quantification was preceded by precipitation with KDS in order to eliminate the interfering substances.

When using Bradford method, the overall effect of the presence of ethanol in model wine was an increase of total absorbance (data not shown), resulting in a overestimation of the protein content. This observation is in agreement with that reported by Lucarini and Kilikian (1999). They found out that substances like ethanol interfered in the Bradford method increasing the experimental value of BSA protein more than 3% compared to the real value. Even Marchal and co-workers (1997) verified that alcohol interfered in the A_{595} nm with CBB causing a significant overestimation of the absorbance. This was

demonstrated by increasing progressively the concentration of ethanol (from 0% to 20%) in aqueous solution. To overcome this problem, they suggested to use a blank standard solution containing the same alcohol content as the studied samples. However, this solution is not completely correct, because the overall results obtained here showed, when comparing the behaviour of proteins in aqueous solution and model wine, that using the Bradford method the presence of ethanol decreased significantly ($P \leq 0.05$) the protein response by 28% (Fig. 3). It means that ethanol not only increased directly the colour of the dye, but also changed the interactions between the CBB and the proteins. As showed by Marchal *et al*. (1997), ethanol interferes with Coomassie ionization, favoring the neutral form of the dye. This results in less ionized form of the dye available to interact with proteins, and this is probably the reason for the lower protein response found in model wine. Some protein response modification may also be accounted for by a small change in the CBB absorbance profile caused by the buffering action of the tartaric acid in model wine, as suggested by Boyes *et al*. (1997).

Proteins in ultrafiltered Glera juice, which we expected to give a slope higher than those in model wine due to the absence of ethanol, gave the same average response probably due to the presence of sugars and polyphenols, other potential interfering substances of CBB (Compton and Jones, 1985). In fact, it is well known that the Bradford assay is limited by interferences from polysaccharides and phenolics (Godshall, 1983; Brenna and De Vecchi, 1990) but these aspects will be better investigated later.

The average slope of all the proteins in model wine (0.02547) was not statistically different from those of the proteins resuspended in the ultrafiltered grape juice (0.02768) or in two different ultrafiltered wines (Manzoni Bianco wine: 0.02309; Sauvignon wine: 0.02738). The average slope of the calibration curve in model wine did not differ statistically from that one of Manzoni Bianco wine, most likely due to the presence in the medium of the same ethanol percentage (Table 3); the average response of proteins in Sauvignon wine, on the other hand, was higher than that given in Manzoni Bianco wine probably for the higher ethanol content in the latter (Table 3).

Finally, just as Marchal who stated that interferences from ethanol and phenolics are not additive (Marchal *et al*., 1997), MW, G J, MB W and SAU W showed the same behaviour to the reagent Coomassie Blue G-250. In fact the interference due to phenolics in

a 12% alcohol wine solution (MB W and SAU W) was statistically comparable to interference due to a 12% alcohol solution (MW) and to interference due to phenolics in a non-alcoholic solution (G J). However, while Marchal came to this conclusion considering the absorbances of interferences, our consideration was deduced by analyzing the average protein response to interfering substances.

In contrast, by applying the KDS/BCA method, the matrix didn't show any statistically significant effect on the slope of the protein calibration curve (Fig. 4). Actually, the potassium dodecyl sulfate (KDS) method described by Vincenzi (2005) allows a rapid protein precipitation from wine by consecutive addition of SDS and potassium chloride (KCl). The KDS-protein complexes so recovered can be precisely quantified by the Smith assay (Smith *et al*., 1985) because they are clearly interference-free. The same procedure of precipitation showed to be incompatible with Bradford determination (Vincenzi *et al*., 2005), probably due to the interferences of residual SDS remaining after centrifugation.

It follows that, using the KDS/BCA method, in the presence of different wine samples, it is possible to quantify the protein content using a calibration curve prepared in water because the slope of proteins dissolved in water did not differ statistically from that of proteins recovered from matrixes different than water (Fig. 4). This cannot be performed using the Bradford method because a calibration curve appropriate for each sample should be made to avoid data overestimations or underestimations.

Evaluation of protein effect on the average response of four proteins to Bradford and BCA.

Taking into account the study of the main effect due to the protein (regardless of the matrix used), using the Bradford method, there was a considerable variability among the protein responses (Fig. 5), as already seen when considering water as matrix (Fig. 1). Comparing the average slopes of the proteins in water (fig. 1) with those in 5 indistinct mediums (fig. 5), the average thaumatin slope decreased significantly by 13% compared to BSA. This difference did not appear in the presence of water where thaumatin and BSA showed to give the same average response; the fact confirms again the strong protein-to-protein variation which is linked to certain features of the matrix.

As confirmation of the fact that the KDS/BCA method is not affected by the matrix but only by aminoacid composition of proteins, fig. 6 shows that LYS and THAU confirm

to have the highest average slope, diversifying from BSA and OVA (the same behaviour seen in fig. 2).

Evaluation of the interaction effect "matrix x protein" on the protein average response to Bradford and BCA

Using the Bradford method, the amino acid composition of each protein showed a significant effect on the slope in the presence of certain matrices (Table 4), which means that there are potential effects on the protein average response due to particular combinations of factors "protein" and "matrix" that could not be explained simply in terms of main effects.

The BSA, a typical model for proteins in wine (Dupin *et al*., 2000, Waters *et al*., 1991), gave the same average slope in all matrices considered, except for water. This means that if the calibration curve is realized using this protein resuspended in model wine, protein quantification results for grape juice or wines should not be overestimated or underestimated. Values statistically similar to those observed for BSA were found using THAU as standard protein which gave an average response in water higher than in other matrices, too. On the other hand, ovalbumin gave always values statistically lower than BSA, except in model wine. On the contrary the lysozyme showed an average slope statistically higher than BSA when resuspended in water and in Glera juice. In this case, to create a calibration curve in model wine would have resulted in a wrong choice since the slope of the protein in Glera juice was statistically higher than that in model wine. From these results the Bradford method showed that not only matrix and protein effects are evident, but also that each protein has a specific behaviour when resuspended in a given medium.

However, in the case of BCA preceded by KDS, the interaction effect was not significant (Table 5), suggesting that the differences among slopes can be explained only by the "main effect protein". In practical terms, if we apply the methods KDS/BCA for wine protein quantification in the laboratory it is necessary to pay attention only to protein variability (which is more attenuated than using the Bradford method) because the analysis is independent of the matrix, while the application of CBB requires more attention both to the choice of protein standard, that should be representative of the protein mixture to be estimated, and to the choice of a suitable solution in which to prepare the calibration curve.

Evaluation of the effect of increasing concentration of polyphenols on the protein average response to Bradford and BCA

Marchal *et al*. (1997) considered the interferences which could falsify the estimation of direct measurement of proteins with the Bradford method in Champagne Pinot Noir and Chardonnay wines and established that exogenous and endogenous phenolic compounds can seriously impair the quantification of the wine protein content. In fact, it was observed that the buffered aqueous solution of phenolics from Pinot Noir and Chardonnay skins gave a blue coloration with CBB equal to that of 16.3 mg L^{-1} and 16.7 eq. BSA, respectively. They, however, measured the response of Bradford to polyphenols in absence of proteins, missing information about the interaction between these two components. Since, as Marchal, other authors found out that CBB react with polyphenolic substances (Compton and Jones, 1985; Siebert and Lynn, 2005; Whiffen *et al*., 2007), an investigation on the effects of increasing concentrations of polyphenolic compounds (0, 25, 50, 100, 200 mg L^{-1}) on the average response of proteins to Bradford and KDS/BCA assays was studied.

A concentrated pool of polyphenols, obtained by C18 cartridge extraction from Manzoni Bianco wine, was diluted in model wine at known concentrations. Since the wines used in this work had a concentration of 173 mg L^{-1} (Manzoni Bianco ultrafiltered wine) and 207 mg L^{-1} (Sauvignon ultrafiltered wine) of polyphenols (Table 3), in the experimental design 200 mg L^{-1} was chosen as the maximum concentration of polyphenols to be considered.

First of all, the addition of polyphenols to model wine caused an increase in the UV absorbance. In fact it has been shown that certain polyphenols give some colour reaction with Bradford, possibly through a direct binding process (Compton and Jones, 1985), that can be mediated by π stacking between the aromatic rings of polyphenols and of CBB.

In addition, considering the main effect “concentration of polyphenols”, using the Bradford method, the concentration of 200 mg L^{-1} of polyphenols caused a statistically significant decrease (16%) in the slope of protein calibration curve, compared to 25, 50, 100 mg L^{-1} concentrations (Fig. 7). As for the ethanol effect, this behavior can be explained by the stabilizing effect of polyphenols towards the neutral form of the dye (Compton and Jones, 1985). Alternatively, the binding of some polyphenols on the protein surface could reduce the binding of CBB by steric hindrance. Another important aspect that can be

extrapolated from the graph in Fig. 7 is that phenolic compounds and alcohol interferences seem to be not additive, as already suggested by Marchal and colleagues (1997). They confirmed this hypothesis verifying that interference due to phenolics in a 12% alcohol solution was less than the interference due to phenolics in aqueous solution.

In the present study it was observed that not only the absorbance increased with increasing polyphenol concentration, but also the protein response modification due to the two interferences (ethanol and polyphenols) was not additive.

Indeed, the slope of the calibration curve realized in model wine did not differ statistically from that of the calibration curves in model wine with polyphenols at increasing concentrations. In other words, the presence of polyphenols, up to the concentration 200 mg L^{-1}, did not change the slope of calibration curves, compared to that one built in model wine. It cannot be excluded that a polyphenol concentration higher than 200 mg L^{-1} can significantly decrease the average response of the proteins.

The quantification using the BCA method was necessarily preceded by precipitation with KDS, since reducing agents (like polyphenols) interfere with the assay during the colour development (Ahmed, 2005). Using KDS/BCA method, there were no significant differences between the protein slopes, even at the highest polyphenol concentration (200 mg L^{-1}) (Fig. 8).

These results are in agreement with those emerged previously, which showed that the BCA assay coupled with KDS is not influenced by specific interfering substances in the medium.

Protein determination of must glycosylated proteins and wine mannoproteins

In this part of the research it was evaluated which method was more appropriate in estimating the protein content in glycosylated proteins and mannoproteins in must and wine, respectively.

Glycoproteins and mannoproteins, extracted by precipitation with 60% ammonium sulfate, were analyzed using the Kjeldahl method to quantify the protein content. The glycosylated proteins of must (especially arabinogalactan-proteins) contained approximately 13.8% of proteins while those of wine (particularly mannoproteins) about 12.9%. These data are in good agreement with previous works, in which it was demonstrated that in both arabinogalactan proteins (Saulnier and Brillouet, 1989) and yeast

mannoproteins (Waters *et al*, 1994) the protein moiety represent less than 10% of the molecule. The percentages were utilized to set up the calibration curves in water at the same protein concentration used for the other proteins.

Taking into account the real protein content of mannoproteins and glycosylated proteins, using the Bradford method wine mannoproteins showed an average response reduced by 76% compared to the average response of the other four proteins considered. The decrease of the slope for grape juice glycosylated proteins was even higher (85%) (Fig. 9).

According to these results, the CBB was not considered a reliable method for the quantification of glycosylated proteins. Fountoulakis *et al*. (1992) suggested that the underestimation of the glycoproteins could be due to steric hindrance caused by some types of carbohydrates which hinder the binding of dye to hydrophobic and basic residues, or most likely to the fact that the hydrophilic sugar moieties change the hydrophobicity of the glycoproteins so that less dye binds.

Precipitation following KDS protocol did not give acceptable results (data not shown), probably because the high glycosylation of proteins interfered with the binding of SDS and it prevented precipitation. The ineffectiveness of the KDS for glycoproteins has been observed previously (Fusi *et al*., 2010). For this reason the BCA method was applied directly on samples dissolved in water, to compare the effectiveness of this method with that of the Bradford assay. The slope of the glycosylated proteins of juice (0.0009 A_{562} μg^{-1}) was quite comparable to the average slope of the other proteins (0.00088), while the calibration curve of wine mannoproteins presented a slope lower of 32% (Fig. 10).

The results suggest that the BCA assay is less sensitive to the sugars present in the protein and they are in disagreement with those found in the literature (Fountoulakis *et al*., 1992) showing the overestimation of glycosylated proteins, probably due to a limited reduction of Cu^{2+} caused by some protein carbohydrates.

CONCLUSIONS

Evaluating the average response of four proteins to Bradford and KDS/BCA in presence of water solution, it is clear that there is greater variability among proteins using Bradford compared to KDS/BCA confirming that Coomassie G-250 dye is more sensitive

to aminoacid composition of proteins. Evaluating the matrix effect on the average response of different proteins to Bradford and BCA, it was seen that applying KDS/BCA protein quantification it is possible to use a calibration curve prepared in water because in this case the slope does not differ statistically from those using other matrixes. This can't be done applying the Bradford method because it's necessary to prepare a specific calibration curve, (i.e. in ultrafiltered sample) for each juice/wine to eliminate the significant variability between matrixes. In addition, it is not sufficient to prepare a blank with the ultrafiltered sample as previously suggested (Marchal et al, 1997), because it has been demonstrated that the presence of interfering substances is able to change also the specific response of protein to the dye. In particular, it was verified that the presence of ethanol can decrease the average response of the calibration curve by 28%, as well as a polyphenol concentration of 200 mg L^{-1} can cause a statistically significant decrease of 16% on the slope of the calibration curve. However it was demonstrated that phenolic compounds and alcohol interferences are not additive.

Moreover, Bradford is not a reliable method to quantify glycosylated proteins. Probably Coomassie G-250 dye, with a significant steric hindrance, is not able to bind proteins effectively because it is hindered by the presence of glycosylated parts in the macromolecules, confirming previous reports, while BCA was less influenced by the presence of sugar moieties on the proteins.

In conclusion, as here demonstrated, the KDS/BCA method can be considered superior to the Bradford assay for protein quantification in wines.

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TABLES AND FIGURES

Table 1. Amino acid profiles of selected standard proteins, number of amino acids in the proteins (ProtParam, Expasy Bioinformatics Resource Portal) and the calculated percentages in brackets. BSA = Bovine Serum Albumin; LYS = Lysozyme (Egg lysozyme); OVA = Ovalbumin (Egg albumin); THAU = Thaumatin I (*Thaumatococcus daniellii*).

Amino acids	BSA	LYS	OVA	THAU
Ala	48 (7.9%)	14 (9.5%)	35 (9.1%)	16 (7.7%)
Arg	26 (4.3%)	12 (8.2%)	15 (3.9%)	12 (5.8%)
Asn	14 (2.3%)	14 (9.5%)	17 (4.4%)	10 (4.8%)
Asp	40 (6.6%)	7 (4.8%)	14 (3.6%)	12 (5.8%)
Cys	35 (5.8%)	9 (6.1%)	6 (1.6%)	16 (7.7%)
Gln	20 (3.3%)	3 (2.0%)	15 (3.9%)	4 (1.9%)
Glu	59 (9.7%)	2 (1.4%)	33 (8.5%)	6 (2.9%)
Gly	17 (2.8%)	13 (8.8%)	19 (4.9%)	24 (11.6%)
His	17 (2.8%)	1 (0.7%)	7 (1.8%)	0 (0.0%)
Ile	15 (2.5%)	7 (4.8%)	25 (6.5%)	8 (3.9%)
Leu	15 (2.5%)	15 (10.2%)	32 (8.3%)	9 (4.3%)
Lys	60 (9.9%)	6 (4.1%)	20 (5.2%)	11 (5.3%)
Met	5 (0.8%)	3 (2.0%)	17 (4.4%)	1 (0.5%)
Phe	30 (4.9%)	4 (2.7%)	20 (5.2%)	11 (5.3%)
Pro	28 (4.6%)	3 (2.0%)	14 (3.6%)	12 (5.8%)
Ser	32 (5.3%)	11 (7.5%)	38 (9.8%)	14 (6.8%)
Thr	34 (5.6%)	7 (4.8%)	15 (3.9%)	20 (9.7%)
Trp	3 (0.5%)	6 (4.1%)	3 (0.8%)	3 (1.4%)
Tyr	21 (3.5%)	3 (2.0%)	10 (2.6%)	8 (3.9%)
Val	38 (6.3%)	7 (4.8%)	31 (8.0%)	10 (4.8%)
Pyl	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Sec	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total	607	147	386	207

Table 2. Protein-to-protein variation. The average response for BSA (A_{595} μg^{-1} protein for Bradford method or A_{562} μg^{-1} protein for Bicinchoninic Acid assay) was normalized to 1 and the average response of the other proteins was expressed as a ratio to the response with BSA.

Protein tested	Coomassie Blue (Bradford) Protein Assay	Bicinchoninic Acid (BCA) Assay
BSA	1.00	1.00
LYS	1.38	1.51
OVA	0.70	1.05
THAU	0.95	1.29
Average ratio	1.01	1.21
Standard deviation	0.28	0.23
Coefficient of variation	27.44%	19.27%

Table 3. Enological parameters of Prosecco grape juice, Sauvignon and Manzoni Bianco wines.

Matrix	pH	Ethanol (% v/v)	Polyphenols (mg L^{-1})
Glera grape juice	3.2	-	230.84
Sauvignon wine	3.4	10.56	207.77
Manzoni Bianco wine	3.6	12.2	173.13

Table 4. Effect of five different matrixes on the individual proteins (interaction effect "matrix x protein") on the protein slope, expressed in A_{595} μg^{-1} of protein ± SD, using the Bradford method. The interaction effect "matrix x protein" is significant at $P \leq 0.01$ according to ANOVA. Values with different letters are significantly different according to Tuckey's HSD test ($P \leq 0.05$). The goodness of fit (r^2 value) is in all cases > 0.99.

Matrix	Protein			
	BSA	**LYS**	**OVA**	**THAU**
H2O	0.03554 ± 0.00655 bc	0.04829 ± 0.00643 a	0.02494 ± 0.00410 fgh	0.03350 ± 0.00509 bcd
Model wine	0.02751 ± 0.00361 defg	0.02577 ± 0.00904 efgh	0.02337 ± 0.01011 fghi	0.02493 ± 0.00369 fgh
Glera juice	0.02882 ± 0.00320 defg	0.03693 ± 0.00504 b	0.01832 ± 0.00321 hi	0.02477 ± 0.00321 fgh
Manzoni Bianco wine	0.02626 ± 0.00596 efg	0.02725 ± 0.00326 defg	0.01678 ± 0.00305 i	0.02290 ± 0.00211 ghi
Sauvignon wine	0.03254 ± 0.00441 bcde	0.02962 ± 0.00699 cdef	0.01953 ± 0.00288 hi	0.02628 ± 0.00217 efg

Table 5. Effect of five different matrixes on the individual proteins (interaction "matrix x protein") on the protein slope, expressed in A_{562} μg^{-1} of protein ± SD, using the KDS/BCA method. The interaction effect "matrix x protein" is not significant according to ANOVA. The goodness of fit (r^2 value) is in all cases > 0.99.

Matrix	Protein			
	BSA	**LYS**	**OVA**	**THAU**
H2O	0.00073 ± 0.00007	0.00111 ± 0.00014	0.00077 ± 0.00011	0.00095 ± 0.00012
Model wine	0.00073 ± 0.00005	0.00102 ± 0.00011	0.00074 ± 0.00008	0.00106 ± 0.00007
Glera juice	0.00092 ± 0.00018	0.00106 ± 0.00022	0.00073 ± 0.0007	0.00104 ± 0.00021
Manzoni Bianco wine	0.00071 ± 0.00014	0.00084 ± 0.00029	0.00078 ± 0.0009	0.00104 ± 0.00007
Sauvignon wine	0.00079 ± 0.00015	0.00094 ± 0.00013	0.00096 ± 0.00032	0.00107 ± 0.00009

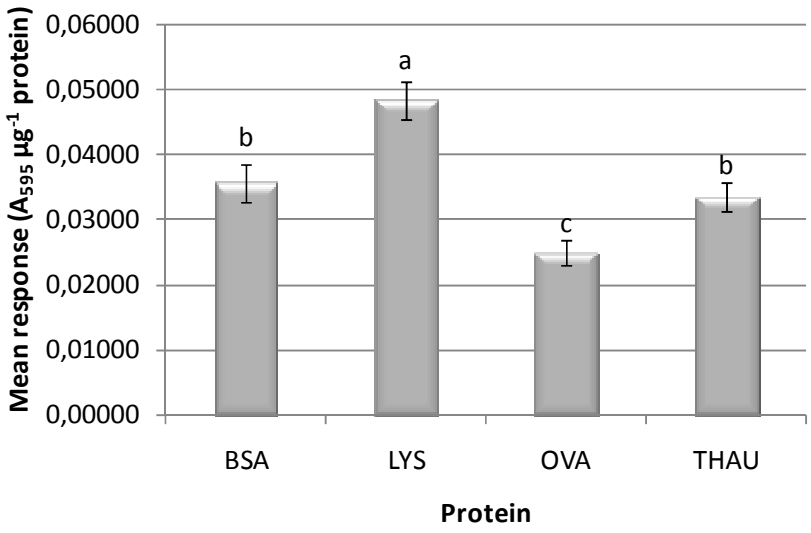

Figure 1. Effect of the amino acid composition on the protein average response (expressed in A_{595} μg^{-1} of protein) in the presence of water using the Bradford method. The main effect "protein" is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tuckey's HSD test ($P \leq 0.05$). Data are represented as means ± SEM of 5 determinations.

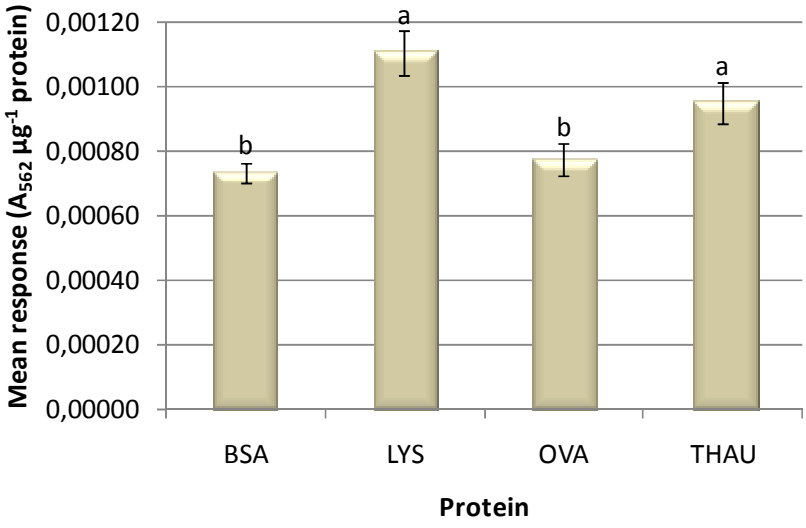

Figure 2. Effect of the amino acid composition on the protein average response (expressed in A_{562} μg^{-1} of protein) in the presence of water using the BCA method. The main effect "protein" is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tuckey's HSD test ($P \leq 0.05$). Data are represented as means ± SEM of 5 determinations.

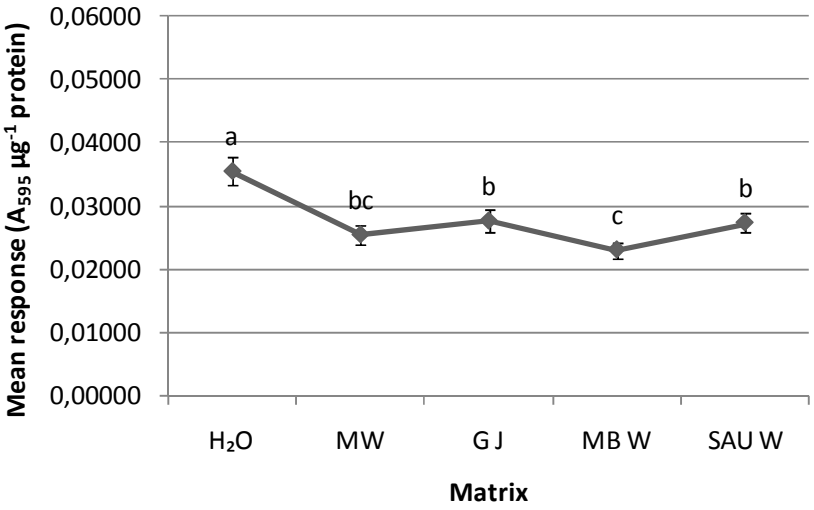

Figure 3. Matrix effect on the protein average response (expressed in A595 µg-1 of protein), using the Bradford method. The main effect "matrix" is significant at $P \leq 0.001$ according to ANOVA. Samples with different letters are significantly different according to Tuckey's HSD test ($P \leq 0.05$). Data are represented as means ± SEM of 5 determinations.

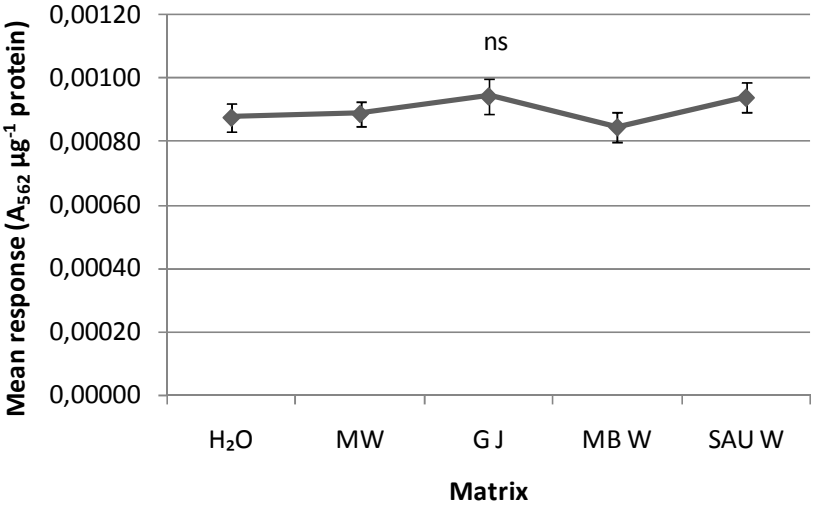

Figure 4. Matrix effect on the protein average response (expressed in A562 µg-1 of protein), using the KDS/BCA method. The main effect "matrix" is not significant according to ANOVA. Data are represented as means ± SEM of 5 determinations.

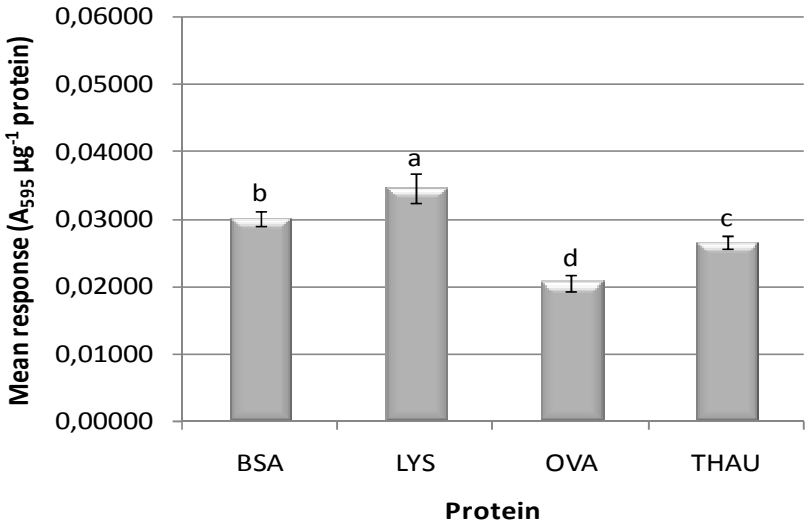

Figure 5. Effect of the amino acid composition on the average response (expressed in A595 µg-1 of protein) to the Bradford method regardless of the matrix used. The main effect "protein" is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tuckey's HSD test ($P \leq 0.05$). Data are represented as means ± SEM of 5 determinations.

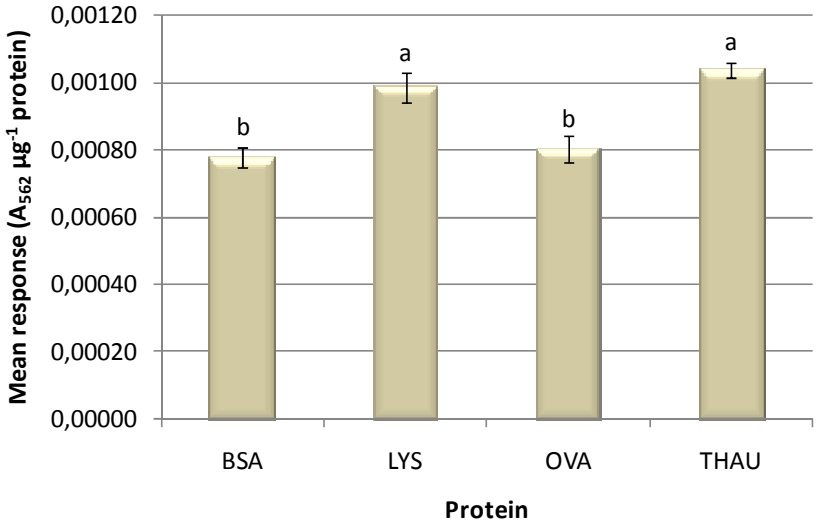

Figure 6. Effect of the amino acid composition on the average response (expressed in A562 µg-1 of protein) to the KDS/BCA method regardless of the matrix used. The main effect "protein" is significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to Tuckey's HSD test ($P \leq 0.05$). Data are represented as means ± SEM of 5 determinations.

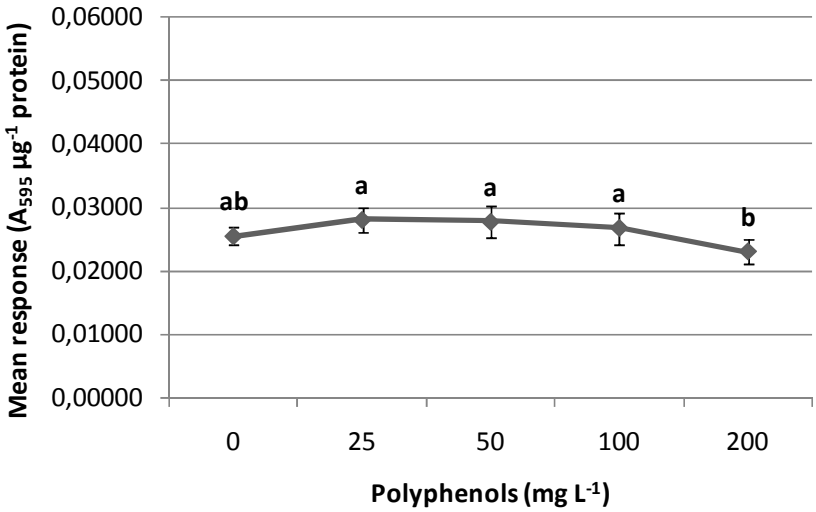

Figure 7. Effect of the increasing concentration of polyphenols on the protein average response (expressed in A595 µg-1 of protein), using the Bradford method. The main effect "polyphenol concentration" is significant at $P \leq 0.01$ according to ANOVA. Samples with different letters are significantly different according to Tuckey's HSD test ($P \leq 0.05$). Data are represented as means ± SEM of 3 determinations.

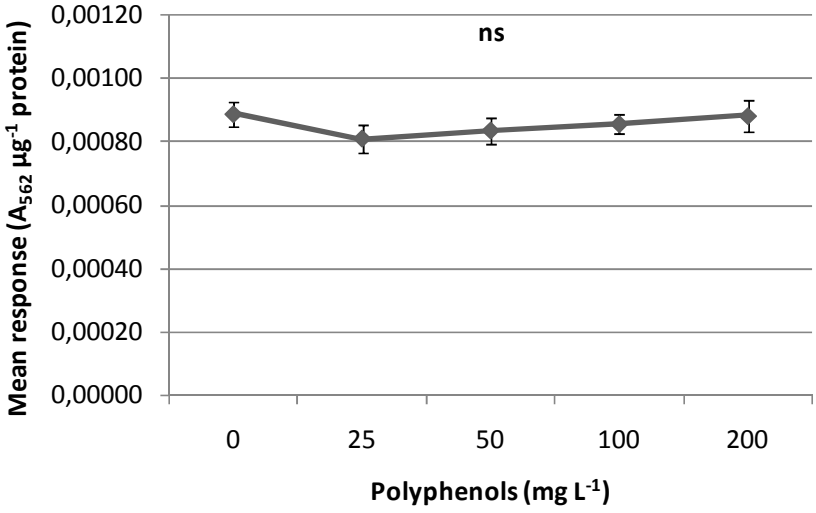

Figure 8. Effect of the increasing concentration of polyphenols on the protein average response (expressed in A562 µg-1 of protein), using the KDS/BCA method. The main effect "polyphenol concentration" is not significant according to ANOVA. Data are represented as means ± SEM of 3 determinations.

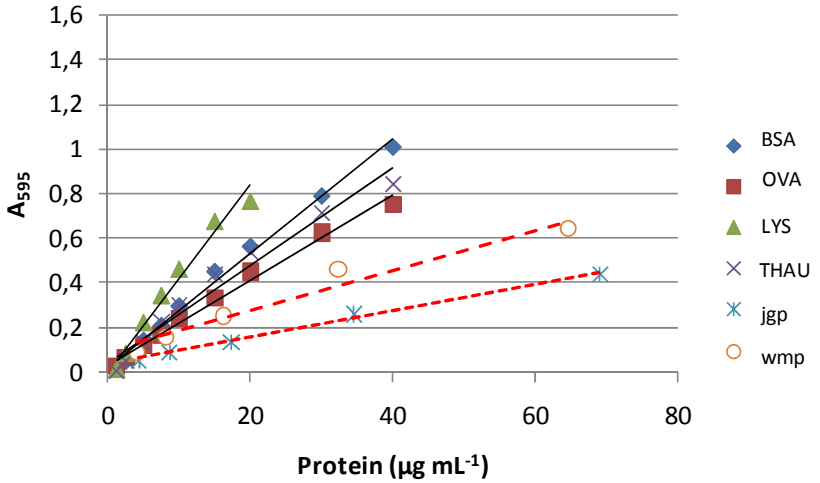

Figure 9. Comparison between calibration curves realized with known concentrations of BSA, ovalbumin (OVA), lysozyme (LYS), thaumatin (THAU), juice glycosilated proteins (jgp) and wine mannoproteins (wmp) resuspended in water. Protein quantification was assessed with Bradford method.

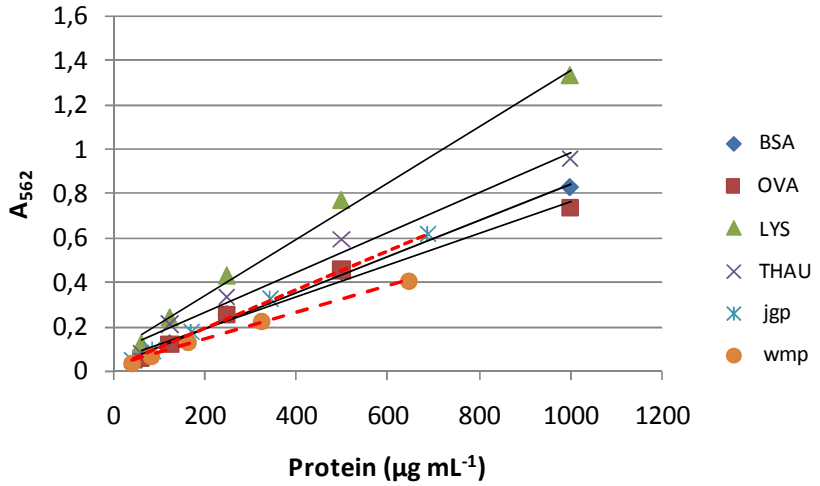

Figure 10. Comparison between calibration curves realized with known concentrations of BSA, ovalbumin (OVA), lysozyme (LYS), thaumatin (THAU), juice glycosilated proteins (jgp) and wine mannoproteins (wmp) resuspended in water. Protein quantification was assessed with BCA method.

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CHAPTER 5

Purification and characterization of grape seed proteins from different *Vitis vinifera* varieties

ABSTRACT

According to protein sequential extraction based on the solubility criteria proposed by Osborne, the proteins mostly expressed in seeds of *Vitis vinifera* L. cv. Glera were albumins (29.6%) and globulins (30.8%), while the insoluble proteins comprised the 35% of total protein.

The protein extraction was not performed on the whole ground seeds but on the manually extracted endosperms, thus avoiding possible interferences by cellulose and polyphenols that can bind proteins making them insoluble. Many protein bands were detected by SDS-PAGE and identified by means of LC-MS/MS.

In particular, the 65 kDa protein, already reported as the major storage protein in grape seeds, resolved into two subunits of 40 and 25 kDa in SDS-PAGE under reducing conditions. Each of these subunits showed a double band in SDS-PAGE and revealed high sequence homology with the family of 11S globulin-like proteins of other plant species. The 35 kDa protein generated, in SDS-PAGE under reducing conditions, two subunits of 25 and 15 kDa and proved to be a 11S globulin-like protein. This storage protein has never been identified before.

The 40 kDa MW protein, which increased its apparent MW up to 43 kDa in SDS-PAGE upon reduction, presented high homology with the precursor of the 7S globulin of *Glycine max* or the gamma conglutin of *Lupinus albus*. No carbohydrate groups were detected using the PAS staining. Even in this case, it is the first time that a putative 7S seed globulin precursor is found in *Vitis vinifera* seeds. Other two low molecur weight proteins were identified: from the globulin fraction, a putative 11S globulin-like protein which did not change its 8 kDa MW after reduction and, from glutelin fraction, a nonspecific lipid-transfer protein.

Furthermore, a comparison was made between the protein patterns of different Italian varieties of *Vitis vinifera*. It was found that the 40 kDa band, subunit of the protein at 65 kDa, according to its pronounced polymorphism, could be used as a molecular marker.

Keywords: globulin; 11S; 7S; grape seed endosperm; LC-MS/MS; storage proteins; variety characterization.

INTRODUCTION

The grape vine (*Vitis vinifera* L.) is one of the most economically important fruit crops in the world (Zhou *et al*., 2010a) with about 5000 varieties identified (Alleweldt, 1992). In viticulture varietal identification and characterization have always played a key role (Moreno-Arribas *et al*., 1999). The great varietal diversity of *Vitis vinifera* L. species has led, over the centuries, to the development of useful descriptive criteria for the identification of grapevine cultivars. Traditionally ampelographic methods have been carried out to differentiate grapevine varieties (Moreno-Arribas *et al*., 1999). These methods, with the use of ampelographic descriptors, are based only on morphology and morphometry, leading some authors to state that they are outdated (Dettweiller, 1993). In recent years, numerous techniques for the varietal characterisation have been developed that rely on molecular markers. Nowadays, biochemical systems, such as the study of isoenzymes, and biogenetic systems, which are based on DNA analysis, are generally used for grapevine variety characterisation. In particular, isozymes (Wolf, 1976) and restriction fragment length polymorphisms (RFLPs) (Bowers and Meredith, 1996), as well as random amplified polymorphic DNAs (RAPDs) (Moreno *et al*., 1995) and microsatellites (Baleiras-Couto and Eiras-Dias, 2006) have been widely used for identifying grapevine varieties, but such studies do not focus on an important problem, that is the cost of analysis, (i.e. the number of amplifications, and thus the number of primers) (Tessier *et al*., 1999). These latter systems are very effective compared to ampelography and ampelometry because they do not consider the morphological features of the plants and this allows them not to be influenced by environmental factors (Hayasaka *et al*., 2001).

A method more recently used as tool for varietal differentiation is proteomics. In fact the proteome is the set of proteins expressed and encoded within the genome of an organism in a very specific district and at a specific time in the life cycle. It can therefore be used primarily in the identification of varieties because genomes of different plant species are translated into different protein profiles (Bradshaw *et al*., 2008; Bertazzo *et al*., 2010). In the proteomic approach to systematics, seeds have long been recognized as the most suitable material. In fact they correspond to a well defined step in the vegetable cycle (in contrast to a continuum of transient growth states for other parts of the plant, leaves for instance) and over and over their composition has been shown to be species-specific and invariant under different growing conditions (Gianazza *et al*., 1989). Seed storage proteins are deposited in relatively large quantities in mature seeds and typically remain stable until the seed germinates. Their purpose is to provide the germinating seedling with a source of amino acids until the sprout is able to begin photosynthesis and synthesize its own amino acids from photosynthate precursors. Since seed storage proteins are an amino acid source, rather than enzymes or structural proteins, alterations in their amino acid sequences are not as critical to the plant. Thus, they are not as subject to natural selection as their isozyme counterparts and may have higher levels of genetic polymorphism. Seed storage proteins can be easily extracted from seeds and analyzed electrophoretically using polyacrylamide gel electrophoresis. Polymorphisms are detected as differences in proteins electrophoretic mobilities and can be analyzed statistically just as isozymes are (Fairbanks and Andersen, 1996).

To date, the majority of studies on grape seed proteins has focused on the optimisation of protein extraction, the protein composition of grape seeds (Gianazza *et al*., 1989; Zhou *et al*., 2010b) and grape varieties differentiation by seed protein compositions (Pesavento *et al*., 2008; Bertazzo *et al*., 2010). Gianazza and coworkers (1989) discovered that the major protein of the grape endosperm is a globulin with $Mr \approx 65$ kDa, which in turn is composed of disulfide-bridged peptides with, *Mrs* of 19-21 kDa and 38-44 kDa. In the study carried out by Zhou *et al.* (2010b), a 11S globulin-like protein was isolated and purified from grape (*Vitis vinifera* L.) seeds by consecutive cation exchange and size exclusion chromatography. The protein consisted of two subunits with molecular masses of 25.5 and 40.0 kDa, respectively.

Pesavento and co-workers (2008) proposed a method potentially suitable for the grape varieties differentiation based on the matrix-assisted laser desorption/ionization (MALDI)/MS of the grape seed proteins. The results obtained seemed to prove that MALDI/MS can well characterize different grape varieties on the basis of the protein profile contained in the grape seeds. The general pattern is maintained and in particular, the species at *m*/*z* 6113 characteristic of the variety Raboso Piave is well detectable in the spectra of all the samples, irrespective of the harvest year, area, and plant treatments.

Bertazzo and co-workers (2010) evaluated the power of seed protein profiles obtained by MALDI/MS for parentage investigation. The three cultivars considered lead to very similar spectra with differences in the relative intensity of the most abundant species. The results provided evidence for the ability of MALDI/MS to individuate minor differences in protein profiles of complex protein mixtures.

In this work the major storage proteins expressed in the seed endosperms have been identified. At first, their characterization was performed by electrophoretic techniques and mass spectrometry. The electrophoretic analysis of seed storage proteins has the advantage over traditional identification techniques that it offers a rapid and reliable evaluation of genotypic differences among most of the cultivars. After that, it has been tried to assess if some of these macromolecules could be identified as “molecular markers”, belonging only to certain cultivars of *Vitis vinifera*.

MATHERIALS AND METHODS

Plant materials

Seeds were manually extracted from grapes belonging to some cultivars (Glera, Trebbiano, Durella, Cortese, Moscato Colli Euganei, Manzoni Bianco, Moscato Fiori d’Arancio, Raboso, Garganega, Prosecco Serprino, Corvina) of *Vitis vinifera* L. Wine grapes were harvested in the experimental vineyard of “Scuola Enologica G.B. Cerletti” of Conegliano (Italy) (vintage 2009).

Seeds were washed with distilled water and dried. By means of a razor blade, seeds were dissected; the integuments were removed, while the endosperm was recovered and immediately frozen for storage.

Lipids extraction

Approximately 2 g of seed endosperm (cv. Glera) was ground to a fine flour in a mortar in the presence of *n*-hexane (Carlo Erba). To recover all the material carefully, the mortar was washed twice with *n*-hexane. The liquid was filtered at 0.45 µm with filters for organic solvents (polyethersulfone, PESU, Sartorius). The remaining flour on the filter was recovered and weighed together with the flour obtained previously. The same procedure was also applied to seed endosperms of the other varieties (≈ 400 mg).

Protein sequential fractionation based on solubility criteria

Albumin, globulin, prolamin and glutelin fractions were sequentially fractionated according to the Osborne method (Osborne, 1924) using appropriate extraction solutions as follows (Fig. 1). The soluble fraction (A) was extracted from the defatted flour (≈ 1 g for cv. Glera and ≈100 mg for the other varieties) with 0.5 M sodium chloride (Carlo Erba) (1:10, w/v) at constant stirring, for 30 minutes, at room temperature. The slurry was centrifuged at 14000g for 5 minutes. The supernatant was recovered and a second extraction with an equal volume of buffer was performed on the water-insoluble pellet obtained. The dispersion was stirred again for 15 minutes. After centrifugation, the procedure was repeated a third time. The supernatants were pooled, filtered at 0.45 µm and dialyzed (3 kDa) extensively against distilled water. After dialysis, the suspension was centrifuged (14000g for 5 minutes). The precipitate (globulins) and the supernatant (albumins) were separated and freeze-dried.

The residual pellet from the first centrifugation was used for the following extraction step. The pellet was extracted with 70% v/v aqueous ethanol (Carlo Erba) (pellet to solvent ratio 1:10 w/v) and centrifuged (14000 g, 5 minutes). After two more extractions, the supernatants were diluted with an equal volume of water to halve the ethanol concentration, then pooled, filtered at 0.45 µm and dialyzed against distilled water. Finally, this fraction that should have contained prolamins was directly freeze-dried. The glutelin fraction was obtained by treating the insoluble pellet with 0.05 M acetic acid (Baker) (1:10 w/v). The suspension was stirred for 30 minutes and centrifuged at 14000g for 5 minutes. After dialysis against distilled water, the fraction was then freeze-dried.

The insoluble pellet, obtained previously after centrifugation, was finally extracted using a solution containing 2% SDS (Bio-Rad) and 60 mM DTT (Fluka) (1:10 w/v). After boiling at 100°C for 10 minutes and stirring for 20 minutes, the sample was centrifuged and the pellet was extracted again with the same buffer. Supernatants were then dialyzed against water and freeze-dried.

Protein content determination

Total nitrogen of the defatted grape seed flour (*Vitis vinifera* L. cv. Glera) and of the fractions obtained after protein sequential fractionation according to the Osborne method was determined by the Kjeldahl method (TKN) (AOAC, 1985) using a Digesdahl digestion apparatus (Hach, Loveland, CO).

An accurately weighted amount of sample was mixed with 4 mL of 96% sulfuric acid (Carlo Erba) in a 100 mL round-bottomed flask. The mixture was heated to 440 °C with the conventional convective-conductive heating system and digested for 5 minutes until the solution refluxed to the top of the head. The flask was then removed from heat, allowed to cool for ≈ 2 minutes and 16 mL of 30% hydrogen peroxide (Carlo Erba) were added cautiously. The H_2O_2 oxidized the carbon to CO_2 and the hydrogen to water and converted amine nitrogen to ammonium ions. The flask was returned to the heater for further digestion and the solution was left to boil for 1 minute to remove excess H_2O_2. The digested sample was then removed from the heater, cooled and diluted to 100 mL with deionized water.

A 2.5 mL aliquot of the diluted sample was mixed with 0.1 mL of Nessler's reagent (Fluka). After mixing, the absorbance of the reacted samples was measured with a spectrophotometer (Shimadzu UV 6010) set at a wavelength of 425 nm. To determine the nitrogen level, a standard calibration curve was prepared by serial dilutions of ammonium sulphate (Carlo Erba) (0.1-10 mg L^{-1} range). The conversion factor of 6.25 was used to calculate the protein content.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoretic analyses were performed according to Laemmli (1970) in a Mini-Protean III apparatus (Bio-Rad). Aliquots of albumin, globulin, prolamin and glutelin fractions were solubilized in 10 µL of 0.5 M Tris-HCl buffer, pH 6.8, containing 15% (w/v)

glycerol (Sigma) and 1.5 % (w/v) SDS (Bio-Rad). Samples were heated at 100°C for 5 minutes before loading. For SDS-PAGE analyses under reducing conditions 4% (v/v) β-mercaptoethanol (Sigma) was added to the loading buffer. Electrophoresis was carried out at 25 mA constant current until the tracking dye Bromophenol Blue ran off the gel. The molecular weight standard proteins were: Myosin (200,000 Da), β-galactosidase (116,250 Da), Phosphorylase b (97,400 Da), Bovine Serum Albumin (66,200 Da), Ovalbumin (45,000), Carbonic anhydrase (31,000 Da), Trypsin inhibitor (21,500 Da), Lysozyme (14,400 Da) and Aprotinin (6,500 Da) (Broad Range Molecular Weight Markers, Bio-Rad). 1.5 mm thick gels were prepared with T = 16% (acrylamide-N, N' metilenbisacrylamide 29:1; Fluka) and stained with Colloidal Coomassie Brilliant Blue G-250 (Sigma) (20% ethanol, 1.6% phosphoric acid, 8% ammonium sulfate, 0.08% Coomassie Brilliant Blue G-250), wich is compatible with MS analysis. Gels were destained with deionized water until background was low (≈ 6 hours).

Trasversal SDS-PAGE (unreducing X reducing)

A standard SDS-PAGE (first dimension), in the absence of reducing agent in the sample, was performed. The gel lane of interest was cut using a sharp razor blade, placed in a polypropylene tube with 5 mL of loading buffer (0.5 M Tris-HCl buffer, pH 6.8, containing 15% glycerol and 1.5 % SDS) containing 4% (v/v) β-mercaptoethanol and heated for 5 minutes at 100 °C. Immediately, this reduced gel lane was placed on the top of a second gel (second dimension), consisting of a common running gel and a thin layer (1 mm) of stacking gel. After placing the sample, the strip of gel was fixed seeping among the two glasses a 0.5% (w/v) agarose solution. Runs were performed under the same conditions previously mentioned for SDS-PAGE.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses

The selected bands were excided from the gel, dehydrated with acetonitrile for 10 minutes and dried in Speed Vac concentrator. The obtained pellets were dissolved in 50 mM NH_4HCO_3 containing 8 M urea. Cysteines were reduced with 10 mM dithiotreitol (DTT) (1 h, 37 °C, in the dark) and alkylated with 30 mM iodoacetamide (1 h, at room temperature, in the dark). 1 μg of sequencing grade modified trypsin (Promega, Madison, WI, USA) was added after 1:10 dilution of the samples with 50 mM NH_4HCO_3 to reach a

final concentration of 0.8 M urea and digestion was carried out overnight at 37 °C. Samples were desalted with C18 cartridges (Strata C18-E, 50 mg mL^{-1}, Phenomenex). Extracted peptides were dissolved in 50 μL of 0.1% formic acid. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed with a 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled to a chip-based chromatographic interface. A Large Capacity Chip was used and 2 μL of samples were injected into the enrichment column (C18, 9 mm, 160 nL volume) at a flow rate of 4 μL/min and peptides were separated in the C18 nano-column (150 mm × 75 μm) at a flow rate of 0.5 μL/min. Water/formic acid 0.1% and acetonitrile/formic acid 0.1% were used as eluents A and B, respectively. The chromatographic separation was achieved by using a gradient of eluent B from 3% to 50% in 50 min. Mass spectra were acquired in a data dependent mode: MS/MS spectra of the 3 most intense ions were acquired for each MS scan in the range of 350–2400 Da. Scan speed was set to 4 MS spectra/sec and 3 MS/MS spectra/sec. Capillary voltage was set to 1750 V and drying gas to 5 L/sec. Raw data files were converted into Mascot Generic Format (MGF) files with MassHunter Qualitative Analysis Software version B.03.01 (Agilent Technologies) and analyzed using Proteome Discoverer Software (version 1.2, ThermoFisher Scientific). The software was connected to a Mascot Search Engine server version 2.2.4 (Matrix Science, London, UK). Spectra were searched against UniRef100 database (version 2010, 10573053 sequences, 3710354253 residues). Enzyme specificity was set to trypsin with 2 missed cleavages, precursor and fragment ions tolerance was set to 10 ppm and 0.05 Da, respectively. Carbamidomethylcysteine was selected as fixed modification and oxidation of methionine as variable modification. A False Discovery Rate (FDR) of 0.05 and 0.01 was calculated by Proteome Discoverer based on the search against the corresponding randomized database. Before the search, data were filtered to exclude MS/MS spectra containing less than 5 peaks and with a total ion count lower than 50. Identified peptides were classified as high (99%) and medium (95%) confidence, according to the corresponding FDR. Proteins were considered as positive hits if at least 1 peptide was identified with high confidence. In order to obtain more information about the function of the identified protein, a BLAST search was carried out.

RESULTS AND DISCUSSION

Protein sequential fractionation based on solubility criteria

With the aim of characterizing the seed storage proteins belonging to a species that has never been studied in detail, as in the case of *V. vinifera* L. seeds, the first approach to follow is to extract as much proteins as possible from the tissue to obtain a protein profile representative of the seed proteome. For this reason, the first experiment to perform was the sequential protein extraction with appropriate solutions, following the fractionation proposed by Osborne that divides the seed storage proteins according to their solubility characteristics into: i) albumins (water-soluble), ii) globulins (salt-soluble), iii) prolamins (alcohol-soluble) and iv) glutelins (alkali-soluble). The final extraction of the proteins bound to the tissue was performed using a solution containing sodium dodecyl sulfate (SDS) and dithiothreitol (DTT).

This protein fractionation is an ambiguous system because not all the proteins fulfill strictly the solubility criteria and the extraction method has many inaccuracies that have been widely discussed in the literature (Ribeiro *et al*., 2004). However, this approach is a convenient procedure to start the characterization of seed storage proteins to proceed afterwards with electrophoretic analyses and the potential purification.

To our knowledge, only other two works (Castriotta and Canella, 1978; Fazio *et al*., 1983) proposed Osborne's protocol as the first screening step for the grape seed storage proteins. In addition, this work together with that of Gianazza (1989), seems to be the only one reporting the protein extraction on the seed endosperm and not on the whole grape seed. Excluding the seed internal and external epiderm improves the efficiency of protein extraction. In fact, the high amount of polyphenols, especially tannins, in the epiderm (Fantozzi et al., 1981) can interfere with the extraction analysis because tannins could bind and precipitate some proteins, which are present within the lipidic endosperm, thus removing specific proteins from the extract. Gianazza and co-workers (1989) observed that applying the extraction directly to the endosperm deprived of the epiderm, solved the problem of tannin-protein interactions, obtaining a flour mixture suitable for SDS-PAGE analysis.

In this work the extraction was performed on the defatted endosperm to eliminate interferences due to lipids that may affect protein extraibility (Byers *et al*., 1983). The

protein content of the defatted flour was found to be ≈ 40% by weight, as determined by the Kjeldahl method. This percentage is much higher than that found in previous works probably due to the fact that the endosperm rich in protein was separated from the seed coat consisting mainly of fibers (which represent 57% of the defatted grape seed meal according to Castriotta e Canella, 1978). Castriotta and Canella (1978) found a protein content of 10.1% by analyzing defatted grape seed meal, whereas Fazio and colleagues (1983) found a protein percentage ranging from 11.3% (from grape pomace seeds) to 25.9% (from seeds where fibers were in part removed) and Igartuburu *et al*. (1991) observed that the protein content of whole seeds was around 8%.

The results of Osborne extraction applied on Glera grape seed endosperms are presented in Table 1. The nitrogen in each fraction (expressed in %) was determined by the Kjeldahl method, then the protein content (%) was estimated by multiplying the nitrogen value by the factor 6.25. The protein content (mg) in each fraction was then estimated multiplying the protein value expressed in % by the extraction yield (mg).

Several observations can be done looking at the data of Table 1: i) globulins (comprising 29.6% of the total seed protein) and albumins (comprising 30.8% of the total seed protein) constituted the vast majority of the seed endosperm protein. These data seem to be in conflict with those detected by Castriotta and Canella (1978) who found that albumin accounted for 5.7% of total protein content, while the globulin amount was negligible. Fazio and colleagues (1983) reported that the albumins accounted for 40.3% while globulins for 4.2% only. It's evident that extraction of separated endosperms allowed to obtain a higher yield of extraction for albumins and globulins. ii) The denaturing activity of SDS on non-covalent protein bonds (hydrophobic interactions and hydrogen bondings) and at the same time the reducing activity of DTT, allowed to extract a protein fraction which represented the 19.7% of total protein; this latter percentage is higher than that obtained for prolamins (1.3%) and glutelins (2.9%). Both research groups mentioned before (Castriotta and Canella, 1978; Fazio *et al*., 1983) found a higher content of glutelin. It is worth noting that in both works the fraction of glutelin was extracted in a basic environment which is unfavourable to interaction between proteins and tannins (Castriotta and Canella, 1978; Fazio *et al*., 1983; Igartuburu *et al*., 1991). iii) 15.7% of endosperm proteins could not be extracted with any solutions. While in previous works the

unextractable residue ranged from 80% (Castriotta and Canella, 1978) to 40% (Fazio *et al*., 1983), in this work, considering the material insoluble in the solutions used to extract proteins and that one unbound with SDS-DTT solution, only 35% of total protein was not extractable.

By SDS-PAGE analyses (under non-reducing and reducing conditions) showed that the different protein fractions comprised distinct sets of polypeptides (Figure 2). The albumins (fraction named A) were composed of many different polypeptides (in non-reducing conditions) covering a wide range of molecular masses (25-65 kDa). In particular, bands are visible at 35, 40, 44, 50, 51, 52 and 65 kDa. In contrast, the globulin fraction (named G) showed to have four major polypeptides with molecular masses of about 8, 35, 40 and 65 kDa in non-reducing conditions. In detail, the band at 65 kDa (lane G) might be the 11S globulin-like protein already described in the literature (Gianazza *et al*., 1989; Zhou *et al*., 2010b; Zhou *et al*., 2011) according to its molecular weight and to the fact that it separates into two subunits of 40 and 25 kDa, as can be seen in the protein pattern of globulin under reducing conditions). According to solubility criteria, this band should not be also present in the albumin fraction. For this reason it could be assumed that the protein may be partially water-soluble.

The protein pattern of the fraction extracted with SDS-DTT (lane S) paralleled that achieved for the globulin fraction (lane G). This suggests that the Osborne's method presents some degree of inefficiency towards the extraction of globulins, implying that the total amount of globulins would be higher than that estimated using Osborne's method. On the other hand it is also possible that part of the proteins present in the globulin fraction bound in some way with the insoluble material through interactions that are sensitive to the presence of SDS and /or DTT in the extraction medium.

The fractions extracted with 70% ethanol (lane P) and 0.05 M acetic acid (lane Gl) showed bands of low molecular weight, between 3.5 and 10 kDa. Especially in SDS-PAGE under reducing conditions two bands are evident: one in lane P of 3.5 kDa (with low resolution, very large) and the other of 10 kDa in lane Gl.

The comparison between the unreduced and reduced samples showed a considerable variation in the migration pattern of the bands. This indicates that most of the grape seed proteins are organized in aggregates held together by disulfide bonds. In fact, data in the

literature claim that seed proteins can be composed of dimers linked together by disulfide bonds, which in turn aggregated to form trimeric and hexameric structures (Shewry, 1995).

Trasversal gel electrophoresis of the globulin fraction

Since the protein pattern profile of globulins in SDS-PAGE under non-reducing conditions contained several bands and therefore it was difficult to find a correspondence between polypeptides present in SDS-PAGE under non-reducing conditions and their subunits appearing after reduction of the sample, trasversal (two-dimensional: unreduced X reduced) gel electrophoresis was used to detect disulphide linked protein aggregates.

The following considerations obtained from the trasversal gel electrophoresis results (Fig. 3) can explain the globulin protein profile observed in one-dimensional-SDS-PAGE in reducing conditions (Fig. 2). The band at 65 kDa of the SDS-PAGE in non-reducing conditions split into two dimers, probably those of 40 and 25 kDa detectable in reducing conditions (see Fig. 2), which in non-reducing conditions were linked by disulfide bonds. Even Zhou and colleagues (2010b) analyzed by SDS-PAGE the 11S globulin-like protein from grape seeds, purified by cation exchange and size exclusion chromatography. They found that this protein consisted of two subunits with molecular masses of 25.5 and 40 kDa. Similar results were obtained in this experiment. Dimeric polypeptides linked by disulfide bonds are widespread in plant seed tissue and they were also found in soybeans (22 and 35 kDa) (Nielsen, 1985), beans (24 and 40 kDa) (Tecson-Mendoza *et al*., 2001) and coconut (34 and 24 kDa) (Garcia *et al*., 2005).

The 40 kDa band detected in SDS-PAGE in non-reducing conditions (Fig. 2) appeared above the diagonal of trasversal gel (Fig. 3), revealing to correspond to the 43 kDa band in SDS-PAGE under reducing conditions (Fig. 2). This behaviour indicates that the 40 kDa unreduced polypeptide is a monomer having intramolecular disulphide bonds which, if not broken, make the structure of the protein more compact than that of the reduced form, thus lowering its hydrodynamic volume and apparent MW in SDS-PAGE. The most represented band was that at 35 kDa in SDS-PAGE under non-reducing conditions (Fig. 2). According to the trasversal gel electrophoresis results, this band seems to be composed by two disulphide linked subunits of 25 and 15 kDa (Fig. 3).

Grape seed proteins detection and identification

A first step for the identification of the grape seed proteins by MS was performed.

The following bands were selected from the globulin fraction in the gel run under non-reducing conditions (Fig. 4A): band 1 (65 kDa) which split in two major subunits in trasversal SDS-PAGE (Fig. 3); band 2 (40 kDa), a monomer which showed to be delayed to 43 kDa under reducing conditions; band 3 (35 kDa) which also split in two major subunits of 25 and 15 kDa in trasversal SDS-PAGE (Fig. 3); band 4 (8 kDa) which did not change its MW in reducing or unreducing conditions gel. From prolamin fraction, band 5 (10 kDa) and from the glutelin fraction (gel non-reducing conditions) band 6 (3.5 kDa, not well focused on the gel) were selected (Fig. 4C). These bands were excised from the gel and, with band 4, directly submitted to liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

In contrast, bands 1, 2 and 3 of the globulin fraction were excised, extracted and resuspended in β-mercaptoethanol; then samples were loaded again in SDS-PAGE (Fig. 4B).

It was confirmed that band 1 (65 kDa), in the presence of a reducing agent, was split into two major subunits (40 and 25 kDa), each one represented by a double band (1A and 1B, 1C and 1D); band 2 was confirmed to be a monomer and to change its MW from 40 to 43 kDa, when reduced; band 3 was split into two subunits of 15 (named 3C) and 25 kDa with the latter consisting of a double band (3A and 3B).

Bands 1A, 1B, 1C, 1D, 2, 3A, 3B, 3C were then excised and submitted to LC-MS/MS.

Bands 1A and 1B can be ascribed to the same protein identified in the Mascot database as "*hypothetical protein isoform 2 (Vitis vinifera)*". Therefore they might be two isoforms of the same protein with a slight difference in the electrophoretic mobility. Similar to this protein appeared to be also band 1C wich showed the same accession number (even though its electrophoretic mobility was very different from that of bands 1A and 1B), while the band 1D had an accession number different from the other bands.

Introducing the identified protein sequences into the BLAST analysis, bands 1A, 1B and 1C showed high sequence homology with putative legumin A precursor (*Ricinus communis*), 11S legumin protein (*Carya illinoinensis*), 11S globulin-like protein (*Actinidia*

chinensis), 11S globulin seed storage protein (*Amaranthus hypochondriacus*). Band 1D was matched with the 11S globulin seed storage protein (*Amaranthus hypochondriacus*) and 11S seed storage globulin (*Chenopodium quinoa*) (Table 2). 1A, 1B and 1C also showed high sequence homology with 11S globulin-like proteins of *Ficus pumila* var. *Awkeotsang*, *Sesamum indicum* and *Glycine max.* It must be underlined that the 11S seed storage proteins are also called legumins.

Zhou and colleagues (2010a) purified a grape seed protein that they named "11S globulin-like protein" corresponding to the 11S globulin of *Ficus pumila* var. *Awkeotsang* for sequence similarity. The 11S globulin, when analyzed in SDS-PAGE under reducing conditions, showed to be composed of two subunits with molecular masses of 25.5 and 40.0 kDa encoded by different genes according to MALDI-TOF-MS spectra. The same author (2011) confirmed again that the size distribution of the subunits forming the major grape seed protein (65 kDa MW) fell within two MW ranges, i.e. 31.0-43.0 and 20.1-31.0 kDa. These two subunitss showed striking similarity to acidic and basic subunits existing in seed globulins (Neilsen, 1985). Therefore, the results showed by Zhou and colleagues (2010a, 2011) and Gianazza (1989) have been extensively confirmed in our work by analyzing the band 1 at first by trasversal electrophoresis, then in LC-MS/MS.

Protein band 2 was identified as "Putative uncharacterized protein" (*Vitis vinifera*) and showed high sequence similarity to 7S seed globulin precursor (*Glycine max*) and conglutin gamma (*Lupinus albus*) (Table 2). It is the first time that a 7S globulin-like protein is found in *Vitis vinifera* seeds. A quite similar polypeptide pattern emerged for the 40 kDa MW protein treated with or without the reducing agent 2-ME and this fact indicates that the protein does not have disulphide bonds holding the polypeptides together. The findings are supported by the knowledge that purified 7S globulins are recognised as being stabilized by intramolecular disulphide bonds (Shewry *et al*., 1995).

The globulin fraction was subjected to SDS-PAGE analysis with PAS staining, in order to detect the presence of sugars associated with the different protein bands. No bands were observed to have carbohydrate moieties (data not shown). While glycosilation is not commonly observed among 11S, it is rare to find 7S globulins not glycosylated (Bewley *et al*., 2006). However, to our knowledge there is at least another reported case of a 7S globulin having no carbohydrate moiety attached to the protein (Garcia *et al*., 2005).

Bands 3A, 3B and 3C were identified by MS analysis as *"Putative uncharacterized protein", "hypothetical protein isoform 2"* and *"Whole genome shotgun sequence of line PN40024, scaffold_5.assembly12x",* respectively. Bands 3B and 3C showed sequence similarity with both 11S globulin-like protein (*Actinidia chinensis*) and 11S globulin seed storage protein (*Amaranthus hypochondriacus*) (Table 2). Band 3A was found to have high homology sequence with basic 7S globulin 2 (*Glycine max*) and conglutin gamma (*Lupinus albus*). It seems that bands 3B and 3C could correspond to the subunits of 25 and 15 kDa obtained in SDS-PAGE under reducing conditions (Fig. 4B) which constitute the 35 kDa dimer displayed in the SDS-PAGE in the absence of reducing agents (Fig. 4A). This 35 kDa dimer has never been found *Vitis vinifera* seeds, although 11S globulin subunits of 25 and 15 were found in amaranth, alfalfa, cumin and mustard (Marcone *et al*., 1999).

The band named 3A was identified as *"Putative uncharacterized protein" (Vitis vinifera)* and showed sequence similarity to 7S seed globulin precursor (*Glycine max*) and conglutin gamma (*Lupinus albus*) (Table 2), similarly to band 2.

Band 4, which did not change its 8 kDa MW after reduction, was identified as being *"hypothetical protein isoform" (Vitis vinifera)* with high sequence homology with 11S seed storage globulin B (*Chenopodium quinoa*) and globulin seed storage protein (*Amaranthus hypochondriacus*) (Table 2). Subunits of low molecular weight in the range 4-15 kDa were detected by Pesavento *et al*. (2008) on a grape seed sample (cv. Prosecco) using MALDI/MS but they were not identified.

Band 5 from the glutelin fraction was identified as *"hypothetical protein"* of *Vitis vinifera* showing high homology with a putative nonspecific lipid-transfer protein (nsLTP) A (*Ricinus communis*) and lipid transfer protein 2 (*Euphorbia lagascae*). It is the second time that the presence of a LTP is reported in grape seeds. A LTP was detected in medieval grape seeds (*Vitis vinifera* L.) preserved by anoxic waterlogging from an early medieval (seventh–eighth century) Byzantine rural settlement in the Salento area (Lecce, Italy) and a late (fourteenth–fifteenth century) medieval site in York (England).

Band 6 was not identified due to its lack of resolution in the gel (Fig. 4C).

Storage proteins as molecular markers for the varietal characterization of grapes (*Vitis vinifera* L.)

In the chemical approach to systematics, seeds have long been recognized as the most suitable material. In fact they correspond to a well defined step in the vegetable cycle (in contrast to a constant growth state for other parts of the plant, i.e. leaves) and their composition has been shown to be species-specific and invariant under different growing conditions (Ladizinsky *et al*., 1983).

Seed endosperms were manually extracted from 11 grape varieties (Glera, Trebbiano, Durella, Cortese, Moscato Colli Euganei, Moscato Fiori d'Arancio, Manzoni Bianco, Raboso, Garganega, Prosecco Serprino, Corvina) collected in the experimental vineyard of "Scuola Enologica G.B. Cerletti" (Conegliano, Italy) in 2009. The aim of the second part of this work was to compare the seed protein profiles of different varieties of *Vitis vinifera* and to check if some of the proteins identified by LC-MS/MS could be used as molecular markers to identify and characterize the cultivars.

Comparing the albumin fractions belonging to the different varieties, the putative 11S globulin-like protein (65 kDa) was the most represented protein (Fig. 5A). Moreover, while in the case of Glera this protein is distributed equally between the albumin and globulin fractions, in many varieties it is almost completely extracted only in the albumin fraction (Compare Fig. 5A to Fig. 6A). This fact confirms that seed protein fractionation based on solubility criteria proposed by Osborne is an ambiguous system, resulting in many inaccuracies (Ribeiro *et al*., 2004).

The band at 65 kDa in the gel under non-reducing conditions (Fig. 5A) did not show any differences among the grape varieties examined. The only exception is represented by cv. Manzoni Bianco, whose 65 kDa 11S globulin-like protein seems to have a relative intensity in SDS-PAGE lower than the other varieties. The 25 kDa subunit consisted of a double band that did not change its electrophoretic mobility in the different varieties (Fig. 5B). The intensity of the 25 kDa bands did not change among the varieties, and the relative intensity of the two bands belonging to this subunit remained the same within each variety. The 40 kDa subunit, appeared after reduction, showed an increased polymorphism since there are differences among the varieties for the number of isoforms involved, their electrophoretic mobility and their relative intensity (Fig. 5B). For example, Glera and

Cortese varieties showed a double band with an apparent MW slightly greater than Corvina and Trebbiano, while Raboso, as well as Durella, Moscato Colli Euganei and Moscato Fiori d'Arancio differed from the others because 3 bands were detected for the 40 kDa subunit. Therefore, the 40 kDa subunit could be used as varietal marker due to its pronounced polymorphism.

The globulin fraction was mainly represented by the band at 40 kDa in almost all varieties (Fig 6A). Under reducing conditions (Fig. 6B), it showed the already mentioned electrophoretic behaviour observed in previous results. This 40 kDa band (putative 7S globulin-like protein) cannot be taken into account for its potential role as molecular marker since it was found in all varieties, changing only in relative intensity. For instance it is more pronounced in Durella, Cortese, Moscato Colli Euganei, Moscato Fiori d'Arancio e Garganega.

In the globulin fraction, the band of 35 kDa is more represented in Glera and Manzoni Bianco (Fig. 6A); in the gel under reducing conditions (Fig. 6B), its 25 kDa and 15 kDa subunits are visible in Glera, Manzoni Bianco and, although with less relative intensity, in Trebbiano, Durella, Cortese, Moscato Colli Euganei, Moscato Fiori d'Arancio. In contrast, these subunits were not found in Raboso, Garganega, Prosecco Serprino and Corvina. However, this result could be due also to inaccuracies during the globulin extraction rather than lack of polymorphism for that band. For this reason the 25 kDa and 15 kDa subunits could not be considered as a useful tool for varietal identification. The band of 8 kDa seems to be common to all varieties, therefore, it is not functional for the purpose of the second part of this work.

CONCLUSIONS

In this work the characterization of seed storage proteins of *Vitis vinifera* cv. Glera was initiated. An effective dehulling of the seeds, removing part of the fibers and the polyphenolic constituents, improved the protein extraction that was thus performed on the defatted endosperms. In fact, the protein content of the defatted flour (≈ 40%) was the highest ever obtained.

The seed storage proteins were fractionated by applying the sequencial extraction based on solubility criteria proposed by Osborne. Albumins and globulins were the most

represented fractions in seed endosperms, representing respectively 29.6% and 30.8% of total protein. Even if the Osborne's method presented some degree of inefficiency towards the extraction of globulins, implying that the total amount of globulins would be higher than that estimated, the approach followed was a convenient step to start the characterization of the grape seed storage proteins.

Using a combination of electrophoretic techniques (SDS-PAGE under reducing and non-reducing conditions, trasversal electrophoresis) coupled with Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses, two 11S globulin-like proteins of 65 and 35 kDa respectively were isolated from seed endosperms (Vitis vinifera L. cv. Glera) and identified. The first one, consisting of two subunits with MW of 40 and 25 kDa has already been discovered in recent studies while this is the first report on the identification of a 11S globulin-like protein which resolves into a major band of 35 kDa on SDS-PAGE under non-reducing conditions and two subunits of 25 and 15 kDa on SDS-PAGE under reducing conditions.

It is also the first time that a putative 7S seed globulin precursor is found in *Vitis vinifera* seeds. It migrated as a single band with a slight delay on SDS-PAGE under reducing conditions, suggesting to be a monomer stabilized by intramolecular disulphide bonds. Surprisingly no carbohydrates were detected on the grape seed proteins using the PAS test.

Other two low molecular weight proteins were identified: from the globulin fraction, a putative globulin-like protein which did not change its 8 kDa MW in the presence of reducing conditions and from glutelin fraction a nonspecific lipid-transfer protein.

The results could be useful for providing knowledge about the identity of grape storage proteins, their structure and subunit composition, thus facilitating the utilisation of these proteins in pharmaceutical and nutraceutical/functional food applications for humans.

Comparing the protein patterns of different varieties of *Vitis vinifera* belonging to the Italian territory, it was seen that the doublet at 40 kDa (subunit of the 65 kDa protein), can be used as a potential molecular marker because there are differences attributable to this band in the number of isoforms, electrophoretic mobility and relative intensity of the bands.

Seed protein profiles have been shown to be a discriminating tool for the identification of *Vitis vinifera* varieties. SDS-PAGE banding patterns of grape seed proteins can serve as a “fingerprint” for the purpose of seed identification, providing evidence of origin and genetic relationships of the grape cultivars. SDS-PAGE of proteins from grape seed endosperms can be considered a rapid and reliable tool for varietal identification.

TABLES AND FIGURES

Table 1. Extraction yield (mg), amount of protein in each fraction expressed in mg and percentage of each fraction on the total amount of protein.

	Yield of extracted protein N (mg)	Protein (mg)	% Protein (w/w)
Albumins	140,0	105,6	29,6
Globulins	117,5	109,8	30,8
Prolamins	4.5	4,5	1,3
Glutelins	10,4	10,4	2,9
Soluble with SDS-DTT	221,0	70,3	19,7
Unextractable protein	425,3	55,9	15,7
Total	914,2	356,4	100,0

Table 2. List of identified bands by LC-MS/MS and software Mascot.

Gel band	Protein accession	Protein identification name	Mascot score	# Peptides	BLAST homology attribution
1A	UPI00019839EA	PREDICTED: hypothetical protein isoform 2 [Vitis vinifera]	363	4	legumin A precursor, putative [Ricinus communis] 11S legumin protein [Carya illinoinensis]
	UPI00019839EE	PREDICTED: hypothetical protein isoform 2 [Vitis vinifera]	261	4	11S globulin-like protein [Actinidia chinensis] 11S globulin seed storage protein [Amaranthus hypochondriacus]
1B	UPI00019839EE	PREDICTED: hypothetical protein isoform 2 [Vitis vinifera]	1251	6	11S globulin-like protein [Actinidia chinensis] 11S globulin seed storage protein [Amaranthus
	UPI00019839EA	PREDICTED: hypothetical protein isoform 2 [Vitis vinifera]	974	4	legumin A precursor, putative [Ricinus communis] 11S legumin protein [Carya illinoinensis]
1C	UPI00019839EE	PREDICTED: hypothetical protein isoform 2 [Vitis vinifera]	608	4	11S globulin-like protein [Actinidia chinensis] 11S globulin seed storage protein [Amaranthus hypochondriacus]
	D7U304	Whole genome shotgun sequence of line PN40024, scaffold_5.assembly12x [Vitis vinifera]	440	4	-
1D	UPI00019839EF	PREDICTED: hypothetical protein isoform 3 [Vitis vinifera]	471	3	11S globulin seed storage protein [Amaranthus hypochondriacus] 11S seed storage globulin [Chenopodium quinoa]
	UPI00019839EC	PREDICTED: hypothetical protein isoform 4 [Vitis vinifera]	265	2	11S globulin seed storage protein [Amaranthus hypochondriacus] 11S globulin-like protein [Actinidia chinensis]

2	A5C7L5	Putative uncharacterized protein [Vitis vinifera]	648	7	7S seed globulin precursor [Glycine max] conglutin gamma [Lupinus albus]
3A	A5C7L5	Putative uncharacterized protein [Vitis vinifera]	375	2	basic 7S globulin 2 [Glycine max] conglutin gamma [Lupinus albus]
3B	UPI00019839EE	PREDICTED: hypothetical protein isoform 2 [Vitis vinifera]	322	4	11S globulin-like protein [Actinidia chinensis] 11S globulin seed storage protein [Amaranthus hypochondriacus]
	UPI00019839EC	PREDICTED: hypothetical protein isoform 4 [Vitis vinifera]	251	2	11S globulin seed storage protein [Amaranthus hypochondriacus] 11S globulin-like protein [Actinidia chinensis]
3C	D7U302	Whole genome shotgun sequence of line PN40024, scaffold_5 assembly 12x [Vitis vinifera]	144	2	11S globulin-like protein [Actinidia chinensis] 11S globulin seed storage protein [Amaranthus hypochondriacus]
4	UPI00019839D3	Hypothetical protein isoform 1[Vitis vinifera]	140	4	11S globulin seed storage protein [Amaranthus hypochondriacus] 11S seed storage globulin [Chenopodium quinoa]
	UPI00019839EA	PREDICTED: hypothetical protein isoform 3 [Vitis vinifera]	140	4	legumin A precursor, putative [Ricinus communis] 11S legumin protein [Carya illinoinensis]
5	UPI000198433F	PREDICTED: hypothetical protein [Vitis vinifera]	134	2	Nonspecific lipid-transfer protein A, putative [Ricinus communis] lipid transfer protein 2 [Euphorbia lagascae]

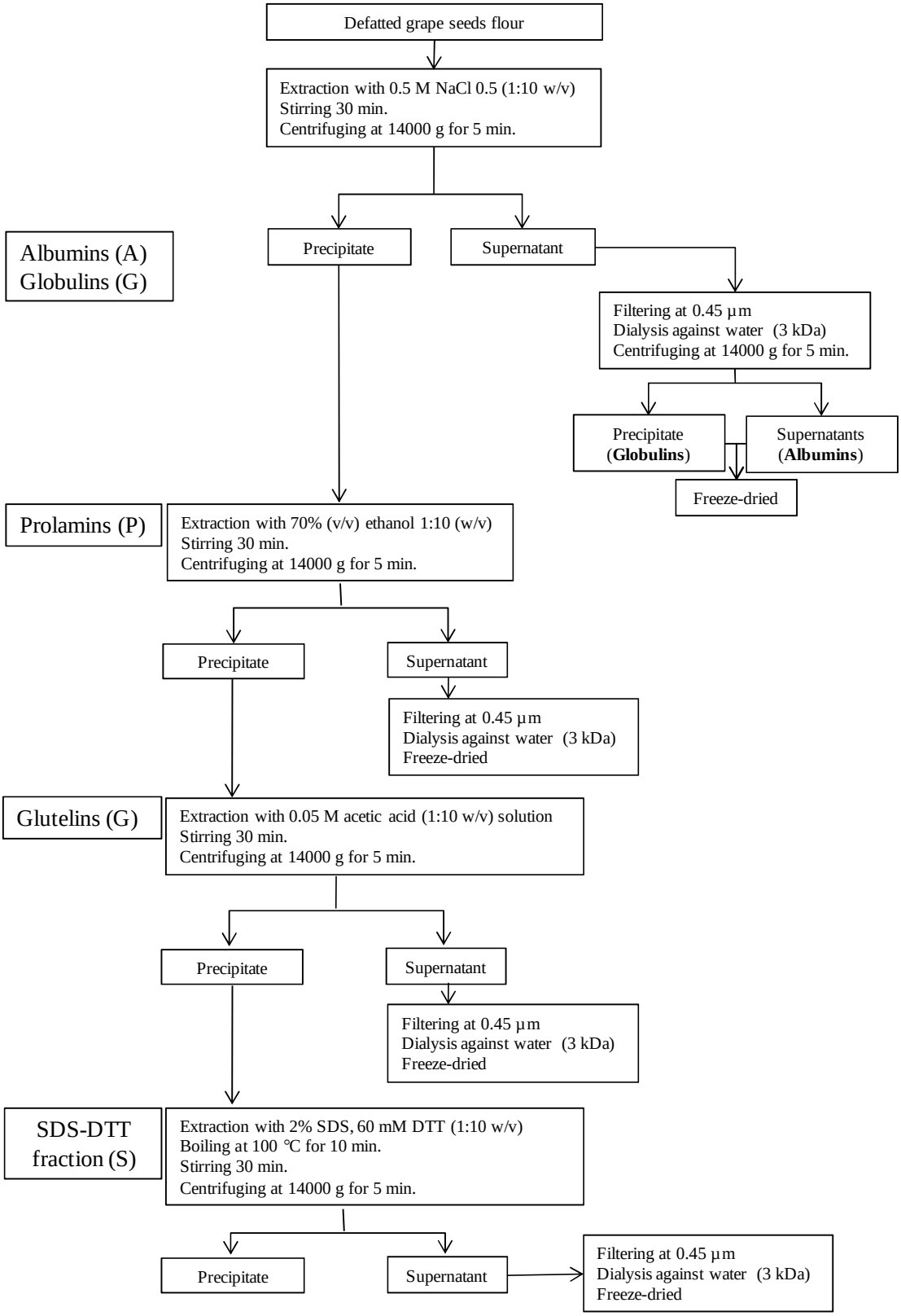

Figure 1. Scheme for protein extraction from grape seed flour (*Vitis vinifera* cv. Glera) according to the Osborne method.

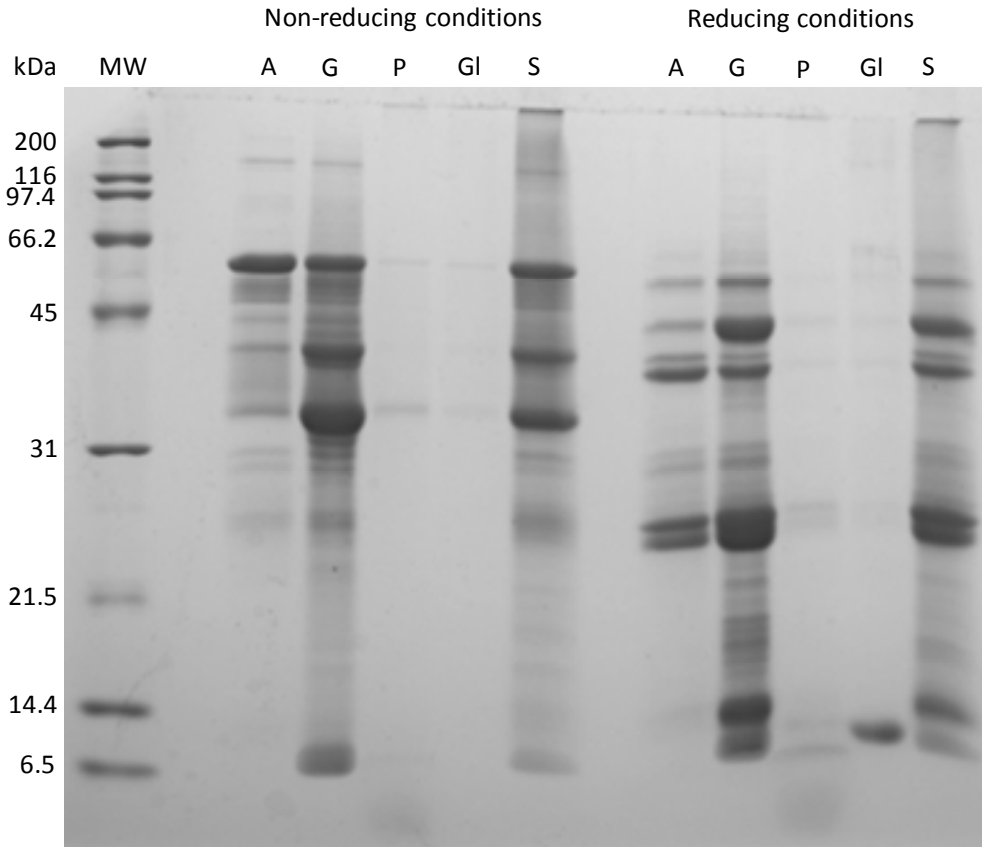

Figure 2. SDS-PAGE under non-reducing and reducing conditions of albumins (lane A), globulins (G), prolamins (P), glutelins (Gl), fraction extracted with SDS-DTT (S). MW: molecular mass markers (kDa). The gel was stained with Colloidal Coomassie Brilliant Blue G-250.

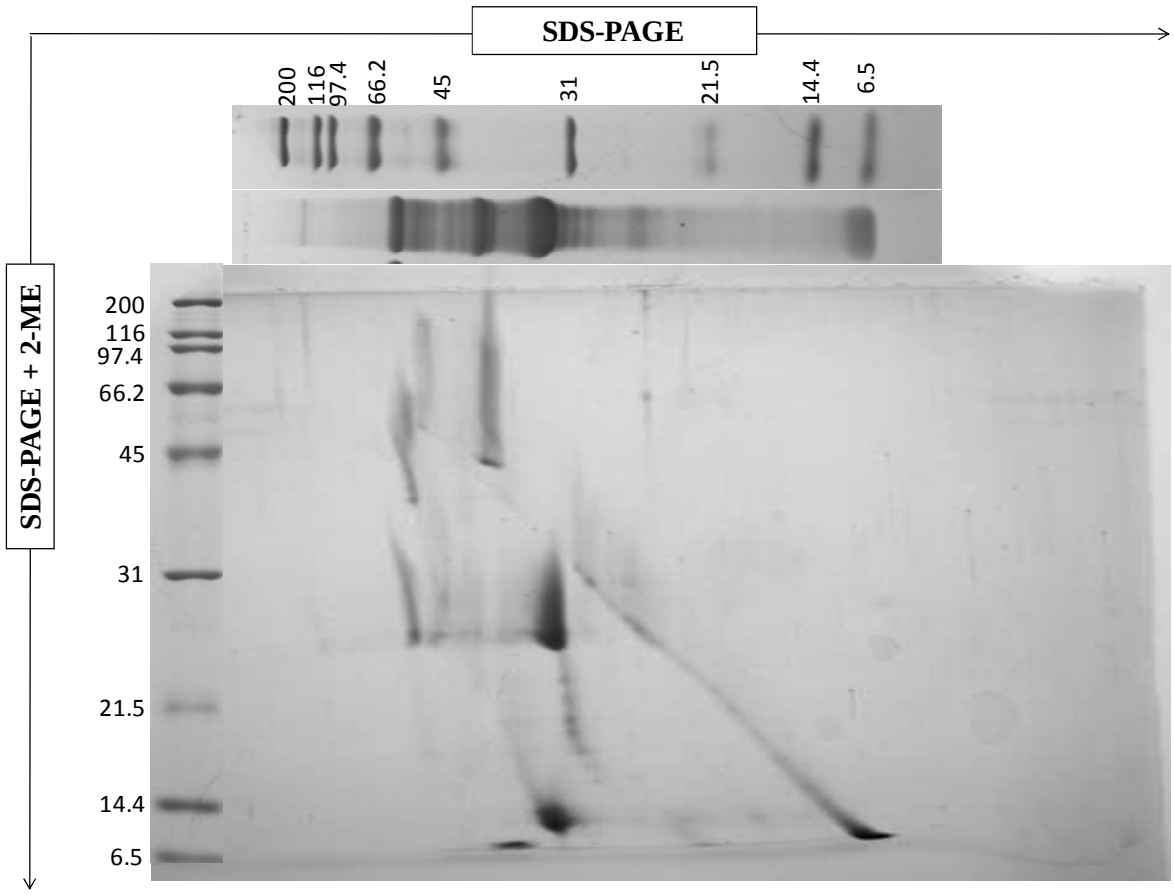

Figure 3. Trasversal (two-dimensional) electrophoresis (unreducing X reducing SDS-PAGE) of the globulin fraction. The gel was stained with Colloidal Coomassie Brilliant Blue G-250.

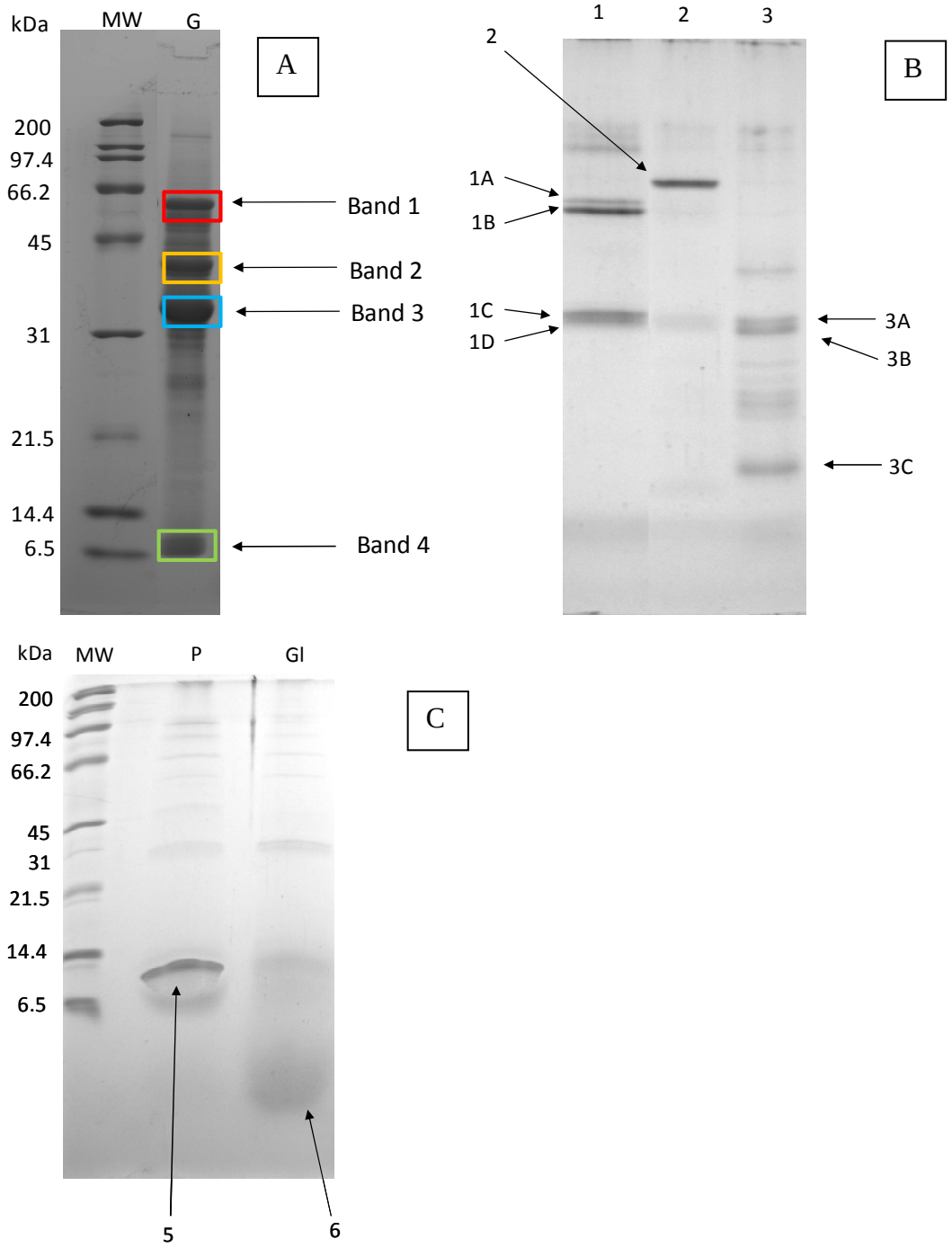

Figure 4. SDS-PAGE gels showing the bands submitted to identification by LC-MS/MS analysis. Bands 1, 2 and 3, from globulin fraction loaded in SDS-PAGE under non-reducing conditions (A), were cut and loaded again in SDS-PAGE under reducing conditions (B). Bands 5 and 6 obtained by extraction with a 70% ethanol (lane P, panel C) and 0.05 M acetic acid solutions (lane Gl, panel C) (1:10 w/v) loaded in SDS-PAGE under reducing conditions.

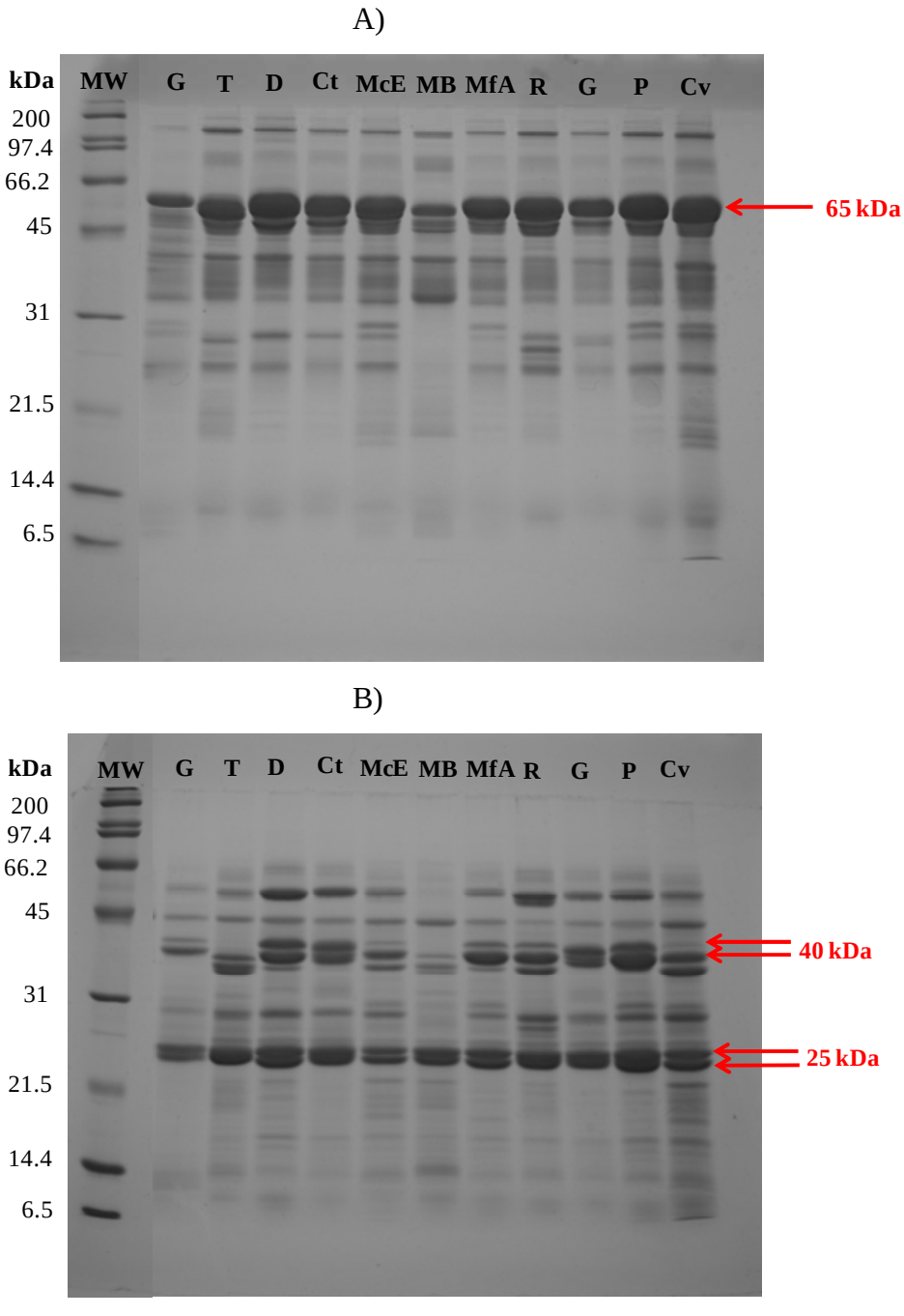

Figure 5. SDS-PAGE under non-reducing (A) and reducing conditions (B) of the albumin fraction from different varieties of *Vitis vinifera*: Glera (G), Trebbiano (T), Durella (D), Cortese (Ct), Moscato Colli Euganei (McE), Manzoni Bianco (MB), Moscato Fiori d'Arancio (MfA), Raboso (R), Garganega (G), Prosecco Serprino (P), Corvina (Cv).

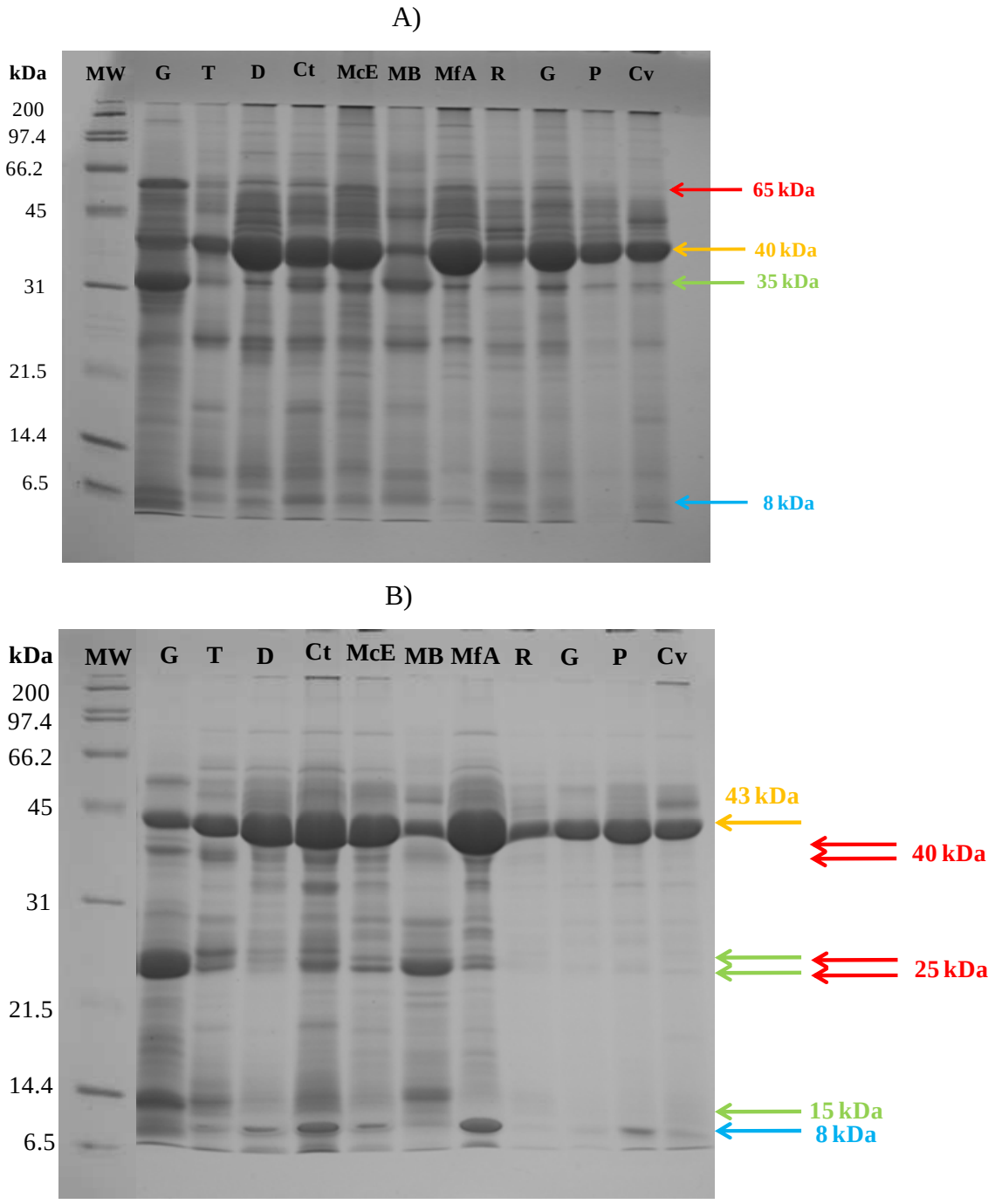

Figure 6. SDS-PAGE under non-reducing (A) and reducing conditions (B) of globulin fraction from different varieties of *Vitis vinifera*: Glera (G), Trebbiano (T), Durella (D), Cortese (Ct), Moscato Colli Euganei (McE), Manzoni Bianco (MB), Moscato Fiori d'Arancio (MfA), Raboso (R), Garganega (G), Prosecco Serprino (P), Corvina (Cv).

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CHAPTER 6

Conclusions

Excessive proteins remaining soluble in white wines is one of the major causes of wine haze. Proteins are present in wines in small amounts but they may denature during storage causing aggregates that settle in bottled wines. Brightness and clarity of wines are the most apparent and important characteristics to consumers and thus of major concern to oenologists. Any unattractive haze or amorphous sediments forming in wines can damage the quality and the value of these products, causing a severe economic problem for producers. Therefore, protein depletion in white wines is a crucial issue in winemaking as well as the process of protein haze formation.

Unstable wine proteins have been found mainly to be derived from the grape and most are pathogenesis-related, namely TLPs and chitinases. Recent publications have reported that grape chitinases seem to be more prone to precipitate and form haze than TLPs. Therefore, a better knowledge of these enzymes is required.

By means of two chromatographic techniques (AEC and HIC) and electrophoretic analyses, at least 5 different grape chitinase isoforms were identified in Manzoni Bianco grape juice. Coomassie staining of SDS-PAGE gels containing increasing amounts of glycol chitin (0, 0.01 and 0.05%) under non-reducing conditions showed a progressive shift for almost all bands. On the contrary, reduced samples showed the same migration pattern independently from the quantity of glycol chitin incorporated into the gel. The retarding effect of glycol chitin involved also the 50 kDa bands suggesting that they could be chitinases. MS analysis by means of MALDI-TOF/TOF MS allowed to confirm that all isoforms belonged to class IV chitinases (*Vitis vinifera*), although it is not yet possible to establish if these proteins are indeed different, being a chance that the selected proteins do not exactly match corresponding database entries; there is also the possibility that the isforms come from the protein degradation of the same original protein. Moreover, chitinases seem to be present in the grape juice also in the form of S-S linked dimers, whose effects on wine remain to be established. It is not yet clear whether the dimers are

naturally present in grape juices or they are artefacts that occur during the protein extraction. If the first case was true, the consequences of the presence of these isoforms of active enzymes in relation to wine quality would warrent further investigation.

Grape proteins have been thought to be a prerequisite, together with other unknown compounds/factors in wine, to form turbidity and precipitate. To investigate on an as yet unknown non-proteinaceous factor X required for visible protein haze and on the roles that different protein isoforms can play during wine haze formation, white wine protein aggregation was studied by means of an innovative instrument (Izon qNano) to detect and quantify nanoparticles formed upon heating. The role played by TLPs, chitinases, phenolics and polysaccharides, all purified from the same unfined white wine, towards aggregate formation was investigated *via* reconstitution experiments. Five purified proteins, one chitinase (CHIT C) and four *Vitis vinifera* Thaumatin-Like isoforms (VVTL1 C, D, H and I), were dissolved in the starting wine from which proteins, polysaccharides and phenolics had been previously extracted, and heat tested alone or in combination with the other macromolecules. In general, the observed data highlighted how differently the proteins tested behaved, confirming results reported in the literature where different protein classes have shown different physicochemical behaviours after their heat-induced unfolding. In particular, CHIT C was more easily unfolded by heat and, probably as a consequence of this fact, more reactive with other wine macromolecules than VVTL1 C, D, H and I. However, VVTL1 I proved to be more reactive than the other TLPs. Therefore at least one isoform of TLPs, which in recent studies were considered as having a less relevant role than chitinases on haze formation in wines, showed the potential of contributing to this phenomenon, even though to a lesser extent. The more reactive forms of TLPs could be present in larger quantities in certain wines, and this could explain why there are conflicting reports in the literature about the role of TLPs in haze formation. This theory is supported by both qNano data and by the notion that, despite being heat unstable, the wine used contained a low level of chitinases. Besides, generally speaking, the addition of phenolics resulted in an increase of the number of aggregates, while the addition of only polysaccharides to the samples halved the number of aggregates both in presence or absence of phenolics, indicating a direct effect of polysaccharides on protein aggregation.

In contrast, the addition of phenolics did not affect the size of the particles formed by the proteins which, instead, were implicated in the largest aggregates in the presence of polysaccharides. The addition of both phenolics and polysaccharides yielded smaller particles than polysaccharides alone. This lead us to believe that proteins (at least TLPs) had more affinity towards polyphenols, derived from the fact that polyphenols are multidentate ligands able to bind simultaneously at more than one point to the protein surface, thus forming a high number of aggregates. On the contrary, the large-sized polysaccharides increased the diameter of aggregates but did not affect the number of particles detected.

Association of proteins with other wine macromolecules (polyphenols *in primis*) is one of the most fundamental factors affecting the quality of drinks. This work helped to deal with the existing issues that involve biochemical associations between wine macromolecules, allowing a better understanding of the functional consequences of these interactions on wine haze.

Wine protein concentrations commonly range from 10 up to 300 mg L^{-1}, depending on the grape variety, growing conditions, winemaking and analytical methods used to quantify them. Since the research in enology needs to find a precise method to quantify juice/wine proteins that should be rapid, insensitive to other juice/wine constituents and easy to perform, in this study two colorimetric assays were investigated: the Bradford method (based on the Coomassie Brilliant Blue, CBB) and the potassium dodecyl-sulphate (KDS) protein precipitation followed by BCA assay (KDS/BCA). Some main factors that can potentially affect the quantification of proteins in wine were analysed, including the presence of ethanol, polyphenols and protein glycosylation. Moreover, the response of different proteins towards CBB and BCA reagents was studied.

The Bradford assay did not prove to be accurate for wine protein quantification as it was affected by the presence of interfering substances in the matrices and by the aminoacid composition of the proteins tested. In particular, the presence of ethanol decreased the protein response by 28% and the polyphenols concentration of 200 mg L^{-1} caused a statistically significant decrease (16%) in the slope of the protein calibration curve.

Moreover, lysozyme showed a significantly higher average response than the other

proteins, while ovalbumin the lowest. In contrast, by applying the BCA (with water and model wine as matrixes) or the KDS/BCA method (with more complex matrixes), the matrix didn't show any statistically significant effect on the slope of the protein calibration curve and there were less differences between the proteins examined.

Moreover, Bradford is not a reliable method to quantify glycosylated proteins. Probably Coomassie G-250 dye is not able to bind proteins effectively because it is hindered by the presence of glycosylated parts in the macromolecules, confirming previous reports, while BCA was less influensed by the presence of sugar moieties on the proteins.

In conclusion, the BCA method proved to be superior to the Bradford assay for protein quantification in wines and juices, showing to have the significant advantage that many potentially interfering wine compounds that create problems in the Bradford method do not affect the protein concentration (for instance, ethanol) or are eliminated by the KDS precipitation step (phenolics).

In the last part of this thesis, the storage proteins expressed in the grape seed endosperms were systematically studied for the first time, through the fractional extraction proposed by Osborne, electrophoretic analyses and mass spectrometry.

An effective dehulling of the seeds, removing part of the fibers and the polyphenolic constituents, improved the protein extraction that was thus performed on the defatted endosperms. In fact, the protein content of the defatted flour (≈ 40%) was the highest ever observed in the studies about protein extraction from grape seeds.

The seed storage proteins were fractionated by applying the sequencial extraction based on solubility criteria proposed by Osborne. Albumins and globulins were the most represented fractions in seed endosperms, representing respectively 29.6% and 30.8% of total protein. Even if the Osborne's method presented some degree of inefficiency towards the extraction of globulins, implying that the total amount of globulins would be higher than that estimated, the approach followed was a convenient step to start the characterization of the grape seed storage proteins.

Since the protein extraction was not performed on the whole ground seeds but on the manually extracted endosperms, thus avoiding possible interferences by fibers/ polyphenols, more bands were detected in SDS-PAGE.

Using a combination of electrophoretic techniques (SDS-PAGE under reducing and non-reducing conditions, trasversal electrophoresis) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses, two 11S globulin-like proteins of 65 and 35 kDa respectively were isolated from seed endosperms (*Vitis vinifera* L. cv. Glera) and identified. The first one, consisting of two subunits with MW of 40 and 25 kDa has already been discovered in recent studies while this is the first report on the identification of a 11S globulin-like protein which resolves into a major band of 35 kDa on SDS-PAGE under non-reducing conditions and two subunits of 25 and 15 kDa on SDS-PAGE under reducing conditions.

It is also the first time that a putative 7S seed globulin precursor is found in *Vitis vinifera* seeds. It migrated as a single band with a slight delay on SDS-PAGE under reducing conditions, suggesting to be a monomer stabilized by intramolecular disulphide bonds. Surprisingly no carbohydrates were detected on the grape seed proteins using the PAS test.

The obtained results provided new information about the identity of grape storage proteins, their structure and subunit composition, thus facilitating the utilisation of these proteins in pharmaceutical and nutraceutical/functional food applications for humans.

Furthermore, a comparison was made between protein profiles obtained from grape seeds belonging to different italian varieties of *Vitis vinifera*. It was observed that the doublet at 40 kDa (subunit of the 65 kDa protein) could be used as a potential molecular marker because there are differences attributable to this band in the number of isoforms, electrophoretic mobility and relative intensity of the bands.

Seed protein profiles have been shown to be a discriminating tool for the identification of *Vitis vinifera* varieties. SDS-PAGE banding patterns of grape seed proteins can serve as a “fingerprint” for the purpose of seed identification, providing evidence of origin and genetic relationships of the grape cultivars. Furthermore, SDS-PAGE of proteins from grape seed endosperms can be considered a rapid and reliable tool for varietal identification.