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TITOLO TESI

Caratterizzazione biochimica e funzionale delle proteine dei vini bianchi e studio di metodi per la prevenzione della loro instabilità

Biochemical and functional characterisation of the proteins of white wines and studies on methods to prevent their instability

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Thesis summary

The presence of a residual amount of unstable proteins in wines is a concern for winemakers because these polymers can precipitate from solution during storage causing appearance of sediments and hazes. Such precipitates are commonly the result of denaturation and subsequent aggregation of heat-unstable wine proteins. It has been demonstrated that the vast majority of the wine proteins derive from grapes and that proteins responsible for haze formation are Pathogenesis-Related (PR) proteins, in particular thaumatin-like (TL) proteins and chitinases. Moreover, these proteins are highly resistant to acidic pHs, proteolysis and fermentation conditions. However, they can became insoluble during wine storage and thus originate perceptible turbidity in the bottles. Despite the efforts made in the recent past, the white wine protein instability is still a main problem during white wine making and bentonite treatments are even now indispensable to stabilise white wines.

This thesis is focused on the study of grape and wine proteins in relation to white wine instability. This three-years study has been done by facing different problems.

Firstly, the effects of the alcoholic fermentation on the macromolecules of a white wine was evaluated, in order to make clear how this process can affect the heat-stability of different wine protein fractions, as obtained through Anion-Exchange Chromatography (AEC). In particular, through the study of the macromolecular composition of a must/wine throughout the alcoholic fermentation and by the study of the intrinsic heat-instability of fractionated proteins, the variation in both quantity and relative proportion of macromolecules and stability of particular proteins was assessed.

Besides, a method suitable for fractionation and purification of grape and wine proteins was set up by using Hydrophobic Interaction Chromatography (HIC). This method was used to purify a thaumatin-like protein. Moreover, 26 grape juice proteins were identified by matching peptide LC-MS/MS spectra with theoretical peptides from a plant protein database.

Furthermore, HIC was also used as a method to prepare grape juice and wine protein fractions differing in hydrophobicity. After partial characterisation of

these fractions by means of different chromatographic techniques, protein hydrophobicity was studied in relation to the heat-stability of the separated fractions and also to their capability in forming insoluble aggregates through reaction with seed tannins.

At the same time, the study of methods alternative to bentonite fining for protein removal of from wines has been carried out. In particular, this problem was faced trying to find proteolytic enzymes, active at wine pH, able to degrade the grape PR-proteins. To this aim, the acidic protease activity of four phytopathogenic fungal strains was tested.

During the study of one of these fungi, we noticed that a polysaccharide (scleroglucan) emitted by the fungus *Sclerotium rolfsii* during its growth, had the ability to adsorb grape and wine proteins in solution. For this reason, the functionality of scleroglucan has been studied to verify the possibility of its utilization as a new material suitable for protein removal from wine.

Riassunto

La presenza di quantità residue di proteine instabili nei vini è un motivo di apprensione per i produttori, in quanto tali polimeri possono divenire insolubili durante lo stoccaggio dei vini e precipitare, causando la comparsa di sedimenti e torbidità. Tali precipitati sono generalmente il risultato di una denaturazione e successiva aggregazione delle proteine instabili del vino. È stato dimostrato che la maggioranza delle proteine del vino derivano dall'uva e che le proteine responsabili per la formazione di torbidità sono proteine legate alla patogenesi (PR proteins), in particolare proteine taumatina-simili (TL) e chitinasi. È stato osservato che tali proteine sono resistenti a pH acidi, alla proteolisi ed alle condizioni di fermentazione, anche se questa resistenza può venire meno durante lo stoccaggio del vino. Nonostante gli sforzi fatti nel recente passato, l'instabilità proteica è ancora il principale problema di origine non microbiologica nella produzione di vini bianchi e i trattamenti con bentonite sono ancora oggi indispensabili durante il processo di produzione di tali vini.

In questa tesi si sono volute studiare le proteine dell'uva e del vino in relazione al problema dell'instabilità proteica dei vini bianchi e questo studio è stato condotto in questo contesto cercando di dare delle risposte a diversi problemi.

Sono stati valutati gli effetti della fermentazione alcolica sulla componente macromolecolare di un vino (Manzoni bianco), al fine di chiarire come la fermentazione influisse sulla stabilità al calore di frazioni proteiche ottenute mediante cromatografia a scambio anionico (AEC).

In seguito, è stato messo a punto un metodo per il frazionamento e la purificazione delle proteine di uva e vino mediante cromatografia a scambio idrofobico (HIC), e tale metodo è stato utilizzato per la purificazione all'omogeneità di una proteina taumatina-simile. Inoltre, 26 bande proteiche ottenute dal frazionamento di proteine di mosto Semillon sono state analizzate mediante LC-MS/MS ed identificate per mezzo di comparazione delle sequenze depositate in database.

Inoltre, la cromatografia ad interazione idrofobica è stata utilizzata anche come metodo preparativo finalizzato ad ottenere frazioni proteiche caratterizzate

da differenti livelli di idrofobicità. Dopo una prima caratterizzazione delle frazioni ottenuta mediante varie tecniche cromatografiche, l'idrofobicità delle frazioni proteiche ottenute è stata messa in relazione alla loro stabilità al calore ed alla loro capacità di reagire con tannini di vinaccioli formando composti insolubili.

Parallelamente, il lavoro di tesi si è incentrato sullo studio di metodi alternativi alla bentonite per la rimozione delle proteine fonte d'instabilità proteica nei vini bianchi. In particolare, si è cercato di risolvere tale problema mediante la ricerca di proteasi attive a pH acidi in grado di degradare le PR-proteins. A tal fine sono stati studiati quattro ceppi fungini per valutarne l'attitudine a produrre enzimi proteolitici in grado di degradare le proteine dell'uva.

Dallo studio di uno di questi funghi (*Sclerotium rolfsii*) si è notato che un polisaccaride da esso prodotto durante la sua crescita, lo scleroglucano, aveva la capacità di adsorbire le proteine di uva e vino. Per questo motivo è stata studiata la funzionalità di tale polimero al fine di verificare la possibilità di un suo possibile utilizzo per la rimozione delle proteine dal vino.

List of abbreviations

aa: amino acid;

AEC: Anion Exchange Chromatography;

Asx: Aspartic acid or asparagine (undefined);

B24: *Sclerotinia sclerotiorum*;

BC: *Botrytis cinerea*;

BSA: bovin serum albumin;

Cyt C: horse heart cytochrome C;

Glx: Glutamic acid or glutamine (undefined).

HIC: Hydrophobic Interaction Chromatography;

LTP: Lipid Transfer Protein;

MW: molecular weight;

MWCO: molecular weight cut off;

PAS: Periodic acid-Schiff staining;

pI: Isoelectric point;

PR-proteins: Pathogenesis-related proteins;

RI: refractive index;

RT: Retention time;

SEC: Size exclusion chromatography;

SM: *Sclerotinia minor*;

SR: *Sclerotium rolfsii*;

TL-protein: thaumatin-like protein;

UF: Ultrafiltered wine;

VvTL: *Vitis vinifera* Thaumatin Like protein.

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CHAPTER 1

Introduction

Soluble heat-unstable proteins, mainly deriving from grapes, are recoverable in white wines also after bottling. Haze formation in white wine is still a matter of concern for winemakers, and the presence of residual protein in fined wines is mainly related to the possible appearance of haze during wine storage in the bottle. Haze appearance is considered the worst fault of non-microbiological origin affecting white wines, leading consumers to refuse the product also if it is no significantly modified from a sensorial point of view (Bayly and Berg, 1967; Hsu and Heatherbell, 1987a; Waters *et al.*, 1992).

Proteins are one of the three main macromolecular compounds of must and wine together with polysaccharides and polyphenols. The proteins responsible for haze have been identified as Pathogenesis-Related (PR) proteins, in particular thaumatin-like (TL) proteins and chitinases, deriving from grape berries (Waters *et al.*, 1996, 1998). These PR-proteins are likely to protect the berry during ripening against fungal pathogens (Høj *et al.*, 2000).

As reported by several authors (Bayly and Berg, 1967; Somers and Ziemelis, 1973; Hsu and Heatherbell, 1987a; Murphey *et al.*, 1989a; Murphey *et al.*, 1989b; Dorrestein *et al.*, 1995; Santoro, 1995; Pocock *et al.*, 1998), the protein level measured in wines can be very variable due to the numerous factors (variety, climate, ripening time, harvest methods, type of winemaking, stabilising treatments, assay adopted to measure protein content, etc.) affecting it. Generally protein amounts varying from few to several hundreds milligrams per litre are detectable in white wines. However, no relationship between protein content and wines instability has been found to date (Sarmento *et al.*, 2000; Ferreira *et al.*, 2002).

Despite bentonite is effective in protein removal from wines (Blade and Boulton, 1988; Achaerandio *et al.*, 2001; Ferreira *et al.*, 2002), its utilisation is not without consequences on wine quality. In particular, bentonite is considered as responsible for simplification of the aromatic profile of the wine and for the loss of colour and of compounds useful for the wine structure (Høj *et al.*, 2000).

Moreover, the wine volume lost after bentonite fining can vary from a 3 to 10 % (Tattersall *et al.*, 2001) with high costs for wineries (Høj *et al.*, 2000).

For these reasons, the research is active on trying to improve the bentonite efficacy (Muhlack *et al.*, 2006; Nordestgaard *et al.*, 2007) but also in finding alternative methods economically convenient and with a less dramatic impact on wine quality. To these aims several techniques have been studied such as wine ultrafiltration (Hsu and Heatherbell, 1987b; Peri *et al.*, 1988; Flores *et al.*, 1990), addition of enological tannins (Weetall *et al.*, 1984; Powers *et al.*, 1988), use of haze protective factors (Waters *et al.*, 1994; Moine-Ledoux and Dubourdieu, 1999; Dupin *et al.*, 2000), protein adsorption on different matrices (Pachova *et al.*, 2004a, Vincenzi *et al.*, 2005), polysaccharide finings (Marchal *et al.*, 2002; Cabello-Pasini et. al, 2005) and use of proteolytic enzymes (Feuillat *et al.*, 1980; Bakalinsky and Boulton, 1985; Lagace and Bisson, 1990).

Protein haze formation in wines can be induced by factors as pH changes, inappropriate storage temperature and/or reaction with polyphenols (Siebert *et al.*, 1996; Sarmento *et al.*, 2000; Mesquita *et al.*, 2001). The mechanism of haze formation is probably related to the slow denaturation of heat-unstable proteins during wine storage (Tattersall *et al.*, 2001) although recently it has been suggested that the sulphate content of the wine can play a key role in the haze development process (Pocock *et al.*, 2007).

This thesis is focused on the characterization of the still unclear grape and wine protein characteristics which can be involved in haze formation, such as the haze potential and tannin reactivity of the different proteins fractions.

Moreover, the search for methods alternative to bentonite fining for wine protein stabilisation was carried out by using phytopathogenic fungi as the source of proteolytic enzymes and suitable polysaccharides to be used to remove proteins from wine.

THE ORIGIN OF THE WINE PROTEINS

The origin of the wine proteins has been extensively investigated from the fifties, although contradictory conclusions have been reported. Wine proteins have

long been considered as a mixture of grape proteins and proteins from autolyzed yeasts. This supposition was disproved by Bayly and Berg (1967) which showed that, after the fermentation of a model must, the yeasts contribution to the final protein level was not significant. Lee (1985) suggested that the main protein source on wines is the grape berry and that the final wine protein level is especially affected by the variety, the ripening grade of grapes and the climate. Several authors, by using more modern techniques, reached at the same conclusion (Hsu and Heatherbell, 1987a; Ruiz-Larrea *et al.*, 1998; Ferreira *et al.*, 2000; Dambrouck *et al.*, 2003). However, other authors suggested that some differences between grape and wine protein composition were noticeable by detecting, in the wine, proteins of yeast origin (Yokotsuka *et al.*, 1991; Monteiro *et al.*, 2001; Kwon, 2004). According to this idea, Waters and colleagues (1994) isolated two mannoproteins from white and red wines fermented with *Saccharomyces cerevisiae* strains. They affirmed that these compounds were released from yeasts during both the exponential phase of growth and wine fining on lees. Similar results have been achieved by Yokotsuka and co-workers (1997) which demonstrated that some glycoproteins recoverable from red wines were from yeasts and that they appeared during both alcoholic and malolactic fermentations. With a chromatographic approach, Lugera *et al.* (1998) observed that alcoholic fermentation and the successive stabilisation processes led to a decrease on the protein content of a Chardonnay wine. In this study, authors highlighted that no proteins release occurred from yeasts through the fermentation but only after 18 months of fining on lees.

However, yeasts can influence the wine protein composition in two ways: through protein transfer into the wine during the autolysis process and/or through the emission of extracellular proteolytic enzymes that contribute to the must protein hydrolysis (Feuillat *et al.*, 1980).

As above discussed, it is then possible to generally affirm that wine proteins come mainly from grapes, although a certain percentage of them can derive from micro-organisms, particularly yeasts (Marchal *et al.*, 1996; Lugera *et al.*, 1998; Goncalves *et al.*, 2002).

Proteins synthesis proceeds rapidly after veraison (Luis, 1983). Nevertheless, the proteins present in white wines do not correspond to a

representative fraction of the grape pulp proteins, since most of them 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.*, 1989a). The low protein levels typically found in wines are mainly due to proteolysis and denaturation of the grape proteins during fermentation, caused by protease activities and changes in pH, respectively (Bayly and Berg, 1967; Feuillat, 1980; Murphey *et al.*, 1989a). Moreover, it has been estimated that half of the grape proteins are bound to polyphenols and consequently they incur in precipitations during winemaking (Somers and Ziemelis, 1973).

CHARACTERISTICS OF THE WINE PROTEINS

The introduction of new analytical techniques gave a large impulse to wine proteins characterization. In the sixties, four protein bands were discovered by electrophoresis by the Berg group (Moretti and Berg, 1965; Berg and Bayly, 1967), showing a variable concentration depending on the type of wine and on the *Vitis Vinifera* cultivar. These researchers have been the firsts to hypothesize that only some wine protein fractions, and not their whole pattern, can be responsible for the protein instabilities in white wine. By using size exclusion chromatography, Somers and Ziemelis (1973) fractionated wine proteins from other components and concluded that the wine protein size was between 10 to 50 kDa. In 1987 Hsu *et al.*, by removing phenolic compounds from white wines before the protein assay, discovered many fractions with molecular weights (MW) in the range 11.2 - 65 kDa. Following studies (Hsu and Heatherbell, 1987b) led to the hypothesis that low MW proteins (20-30 kDa) were the most important for haze formation compared to higher MW fractions. This guess has been lately confirmed by Waters and colleagues (1991, 1992) which described three major wine protein fractions (from *V. vinifera* cv. Muscat Gordo Blanco; respectively with MW of 24, 32 and 63 kDa) and highlighted that the fraction of 24 kDa produced up to 50 % more haze than the other two fractions. Besides, the protein of 63 kDa was found to be the more termostable and this finding was deeper studied with researches for natural haze-protective factors in wines (Waters *et al.*, 1993). Further studies (Waters *et al.*, 1996) showed that the wine proteins of 24

and 32 kDa presented high homology with PR-proteins from other plants, and particularly with thaumatin and chitinases.

In addition to the studies conducted on the size of wine proteins, several investigations have been carried out to determine their isoelectric point (pI). At the wine pH, proteins are positively charged, and this fact permits their removal by bentonite (negatively charged) treatments and could play also a role in the interaction between proteins and non-protein factors leading to haze formation. Proteins with low pI represent the main part of the wine proteins (Moretti and Berg, 1965) and have been claimed as the principal responsible for haze formation (Bayly and Berg, 1967). Several authors confirmed this idea (Lee, 1985; Hsu and Heatherbell 1987a; Paetzold *et al.*, 1990), reporting that wine proteins have pI values between 4 and 7.

After wine protein fractionation based on their pI, Dawes *et al.* (1994) found that the five obtained fractions were all able to develop turbidity when heat tested. The insoluble particles formed showed different sizes and this observation led to the conclusion that, to deeply understand the mechanism of haze formation, it was necessary to consider other wine components such as phenolic compounds.

To date, wine has been reported to contain polypeptides ranging in molecular mass from 9 to 63 kDa and having isoelectric points from 3 to 9 (Hsu and Heatherbell, 1987b; Lamikanra and Inyang, 1988; Brissonet and Maujean, 1993). However, the vast majority of the wine proteins exhibit low molecular masses (20-30 kDa) and low isoelectric points (4.1-5.8), possessing a positive charge at the pH values encountered in wines (Brissonet and Maujean, 1993; Hsu and Heatherbell, 1987b; Ferreira *et al.*, 2000).

Using two-dimensional (2D) electrophoresis, it was possible to highlight a high variability of the protein profiles of grapes which is undetectable with normal (one-dimensional) SDS-PAGE techniques. In particular, it was possible to obtain two-dimensional maps of the grape berry in which the presence of about 270 protein spots was detected (Sarry *et al.*, 2004). However, wine protein profiles very often results surprisingly simple with the predominance of low MW bands (Hsu and Heatherbell, 1987b; Murphey *et al.*, 1989a; Pueyo *et al.*, 1993).

It seems that the sudden pH variation and the interaction with tannin during grape crushing causes the precipitation of a high number of proteins

resulting in a simplified electrophoretic profile. The recoverable proteins are those able to remain soluble at acidic pH, resistant to both endogenous protease action and precipitation by tannins (Sarry *et al.*, 2004). Moreover, to these proteins surviving the pre-fermentation processes, it is necessary to subtract those that are degraded or precipitate during fermentation mainly because of the yeast and ethanol actions. However, this decrease in grape protein content should be partially compensated by the emission of protein by the fermenting yeasts during and after fermentation.

The described selection process leads to the presence in wine of proteins with a high resistance to variations in the external factors and proteolysis. As a matter of fact, several authors reported that proteins responsible for haze formation in white wines (PR-proteins) are very stable against both the conditions of fermentation and proteolysis although, paradoxically, they became unstable during the wine storage (Feuillat and Ferrari, 1982; Waters *et al.*, 1992; Waters *et al.*, 1995).

PROTEIN HAZE IN WHITE WINES

In white wine winemaking, the appearance of haze during storage in the bottle is a frequent problem. Different types of hazes can occur in wines after bottling and they can be both of microbiological or chemical origin.

The most important non-microbiological haze is due to the presence in wine of heat-unstable proteins (Høj *et al.* 2000; Tattersall *et al.* 2001; Ferreira *et al.* 2002). These proteins are the grape (*Vitis vinifera*) Pathogenesis-Related (PR) proteins, namely, thaumatin-like proteins and chitinases that tend to aggregate during wine storage, resulting in formation of light-dispersing particles (Høj *et al.* 2000; Tattersall *et al.* 2001; Ferreira *et al.* 2002), which above certain dimensions can be visually detected as haze. Although white wine containing protein haze is not dangerous for consumption, it becomes unattractive, and thus, tends to be rejected by consumers, resulting in a great economical damage.

During winemaking, grape proteins undergo to the “stressful” conditions of the fermentation process. Consequently, the less resistant grape proteins are degraded or precipitated during this step, with a sort of selection of the grape PR-

proteins that are highly resistant to the fermentation conditions (Waters *et al.*, 1992). These proteins, that are very stable in the short-medium period, became insoluble during the long term storage and thus originate perceptible turbidity.

The full mechanism of protein haze formation is not fully understood despite much research has been done worldwide on this problem. Slow denaturation of wine proteins is thought to lead to protein aggregation, flocculation into hazy suspension and, finally, formation of precipitates (Bayly and Berg, 1967; Hsu and Heatherbell, 1987a; Waters *et al.*, 1991, 1992)

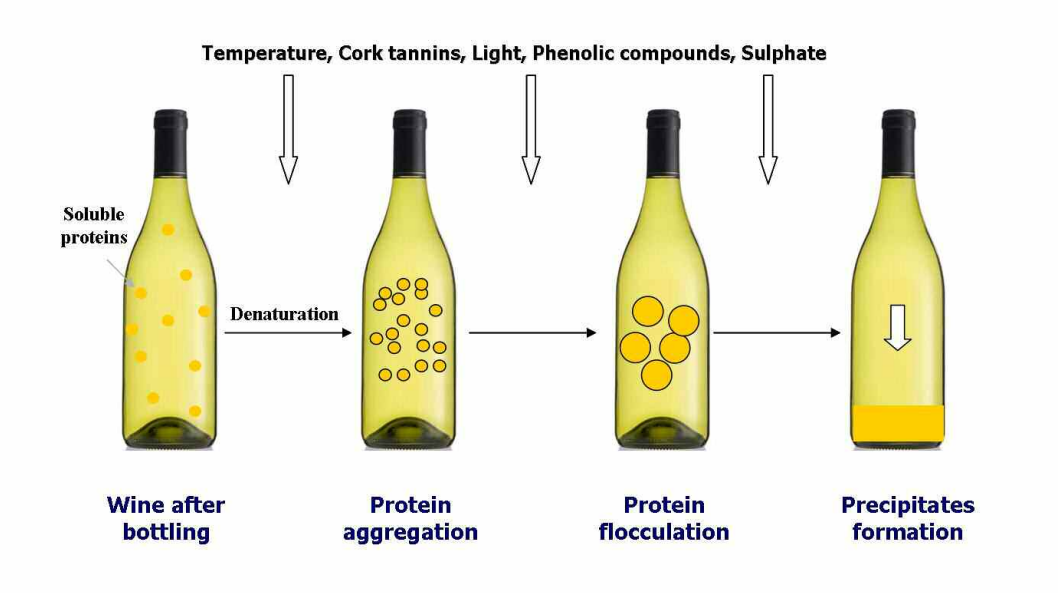

Figure 1.1. *Hypothetical haze formation mechanism in a bottle of white wine during storage.*

GRAPE PATHOGENESIS RELATED PROTEINS (PR-PROTEINS)

The concept of pathogenesis-related (PR) protein was introduced in 1980 to designate any protein coded by the host plant in response to pathological or related situations (Antoniw *et al.*, 1980). In general, PR proteins are known to be acidic, of low molecular mass, highly resistant to proteolytic degradation and to low pH values (Ferreira *et al.*, 2007). The induction of some PR proteins under pathological conditions suggests, but does not prove, a role for these proteins in plant defence (van Loon, 1990).

To date, seventeen classes of PR-proteins are known, numbered in the order in which they were discovered from PR-1 to PR-17. It is noteworthy that

among PR-protein families many proteins homologues to common food allergens can be found (Van Loon and Van Strien, 1999; Hoffmann-Sommergruber, 2002, Pastorello *et al.*, 2002).

In grapevine berries there are evidences of a strong constitutive expression of some PR-proteins, that are simply regulated by the developmental stage of the plant (Derckel *et al.*, 1996; Robinson *et al.*, 1997). The synthesis of PR-proteins occurs predominantly in the skin of the grapes. Therefore, their expression in the grape berry is regulated in a developmental and tissue specific manner (Igartuburu *et al.*, 1991; Pocock *et al.*, 1998, Monteiro *et al.*, 2001).

In all cultivars of *V. vinifera* studied so far, Thaumatin-Like (TL) proteins and chitinases have been found to be the major soluble proteins of grapes (Peng *et al.*, 1997; Tattersall *et al.*, 1997; Pocock *et al.*, 1998, 2000). In *V. vinifera* cv. Muscat Gordo Blanco, the levels of the major TL protein increased dramatically after the beginning of veraison and continued during ripening (Tattersall *et al.*, 1997; Salzman *et al.*, 1998). Therefore, it was presumed that the haze-forming potential increases during berry ripening (Murphey *et al.*, 1989a; Tattersall *et al.*, 1997; Pocock *et al.*, 2000).

Grape PR-proteins demonstrate antifungal activity *in vitro* against common fungal pathogens of grapevine (Giananakis *et al.*, 1998; Salzman *et al.*, 1998; Tattersall *et al.*, 2001; Jayasankar *et al.*, 2003; Monteiro *et al.*, 2003). Girbau and colleagues (2004) showed that grape bunches infection with powdery mildew had a significant impact in the haze potential of wine as assessed by the heat test. On the contrary, Marchal *et al.* (1998) showed that berry infection by *Botrytis cinerea* resulted in a juice with a reduced protein level, suggesting a proteolytic action of this pathogen against grape proteins. Cilindre *et al.* (2007) have recently confirmed these results by means of 2D electrophoretic analyses of *B. cinerea* infected grapes.

About the 19% of the total proteins from grape berry mesocarp belongs to the PR-protein category. Among these proteins, the most represented are TL proteins, chitinases, β-glucanases and an isoflavon reductase-like protein, presumably involved in the synthesis of phytoalessins (Sarry *et al.*, 2004).

The total quantity of PR-proteins detectable in the ripe grape berry depends on the variety, on the geographical collocation of the vineyard, on the

climate and on the agronomical practices (Ferreira *et al.*, 2002). Also the post-harvest practices, as mechanical harvest, are known to lead to a general increase in PR-proteins content of the grape juice because of the physical damages that mechanical operations causes to the plants and the bunches (Pocock *et al.*, 1998).

In any way, the majority of soluble proteins in grape juice have been identified as chitinases and TL proteins (Tattersall *et al.*, 1997). Thanks to their intrinsic resistance, these proteins endure to the fermentation and remain in the wine, where they can cause haze appearance during storage.

Chitinases (EC 3.2.1.14) constitute the second largest group of antifungal proteins after the PR 1 family (Jayaraj *et al.*, 2004; Ferreira *et al.*, 2007). These proteins have been found in a very wide range of organisms, containing or not containing chitin, such as viruses, bacteria, fungi, plants (gymnosperms and angiosperms) and even animals (insects, snails, fish, amphibians and mammals) (Goormachtig *et al.*, 1998). Chitinases catalyse the hydrolytic cleavage of β-1,4-glycoside bonds present in biopolymers of N-acetyl-d-glucosamine, mainly in chitin (Kasprzewska, 2003). In general, these enzymes catalyse chitin degradation, acting mostly as endochitinases and producing chito-oligosaccharides made of 2 to 6 N-acetyl-d-glucosamine residues (Stintzi *et al.*, 1993). The antifungal activity displayed by many chitinases was initially assumed to derive from their ability to digest chitin, leading to a weakening of the 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 the chitinolytic activity (Ferreira *et al.*, 2007).

In grape, chitinases represent about 50% of the total must proteins and are considered the main responsible, along with the thaumatin-like proteins, for protein haze formation in white wines (Waters *et al.*, 1998).

The Thaumatin-Like proteins and the Osmotin-Like proteins are basic, 24-kDa proteins belonging to the PR-5 family. These proteins share high homology with Thaumatin, a sweet-tasting (to humans) protein from the South African Ketemfe berry bush (*Thaumatococcus danielli*) (van der Wel and Loeve, 1972). It is likely that these proteins act by inducing fungal cell leakiness through a specific

interaction with the plasma membrane that results in the formation of transmembrane pores (Roberts and Selitrennikoff, 1986; Kitajima and Sato, 1999). As observed for chitinases, these proteins exhibit antifungal activity *in vitro* (Woloshuk *et al.*, 1991; Melchers *et al.*, 1993; Liu *et al.*, 1994). Furthermore, the simultaneous presence of both Osmotin and TL-protein from grapevine displays a synergistic antifungal effect (Monteiro *et al.*, 2003).

The TL proteins are, after the chitinases, the most represented grape and wine proteins (Waters *et al.*, 1998, Pocock *et al.*, 2000; Hayasaka *et al.*, 2001).

After these two main classes of grape PR-proteins, other proteins belonging to these groups are detectable in grapes: plant Lipid Transfer proteins (LTP) and β-glucanases.

LTPs (PR-14) are small, basic proteins, stabilized by four disulphide bonds, which transfer phospholipids between membranes. LTPs contain an internal, tunnel-like hydrophobic cavity that runs through the molecule (Selitrennikoff, 2001; Cheng *et al.*, 2004). 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). In grapevine, a LTP of 9 kDa having high homology with that of peach and cherry has been detected and indicated as the main grape and wine allergen (Pastorello *et al.*, 2002). In the same study, also a type 4 endochitinase and a TL protein of 24 kDa were indicated as minor allergens in grape and wine.

Plant β-1,3-glucanases are referred to as PR-2 proteins (Ferreira *et al.*, 2007). They participate in several physiological and developmental plant processes. In addition, class I β-1,3-glucanases exhibit antifungal activity both *in vitro* and *in planta*, as shown by using transgenic plants over-expressing a PR-2 protein (Mauch *et al.*, 1988; Joshi *et al.*, 1998). Class II β-1,3-glucanases exhibit *in vitro* antifungal activity only if applied in combination with chitinases or class I β-1,3-glucanases (Theis and Stahl, 2004).

ENOLOGICAL CONTROL OF WHITE WINES PROTEIN INSTABILITY

1.1.1 HISTORY OF WINE PROTEIN FINING

The presence of proteins in wines has been a matter of concern since the beginning of the nineteenth century. In 1904, Laborde suggested heating the wine at 70-80 °C for 15 minutes to eliminate proteins. The use of a cation exchanger was firstly proposed in 1932 by using caolin, although too high dosages were required to eliminate protein. In 1934 Saywell proposed bentonite as a tool for protein removal because of its net negative charge at wine pH that allowed the electrostatic interaction with the positively charged wine proteins producing their flocculation (Hsu and Heatherbell, 1987a; Lamikanra and Inyang, 1988; Ferreira *et al.*, 2002). Since then, bentonite fining was developed and this technique is still the most used treatment for protein removal from wines. However, the doses of bentonite required to stabilize white wines has increased over the last 25 years, passing from 0.2-0.4 g/L to 0.8-1 g/L (Hsu and Heatherbell, 1987a; Paetzold *et al.*, 1990).

Alternative fining treatments to bentonite have been extensively studied over the last 30 years but none of them resulted successful.

1.1.2 BENTONITE FINING

Bentonite (a montmorillonitic clay) is commonly utilised in winemaking for prevention of wine protein instability. Wine protein adsorption by bentonite is due to its cation exchange capability. Indeed, at acidic pH, grape and wine proteins are positively charged, hence they can bound to bentonite that is negatively charged at wine pH (Blade and Boulton, 1988; Høj *et al.*, 2000; Ferreira *et al.*, 2002).

One of the main problems of bentonite fining is that this clay is not specific for wine proteins adsorption, but may adsorb other molecules, including aroma compounds. From a sensorial point of view, the effects on bentonite addition to wine are still not clear. Some authors affirmed that this treatment does not lead to sensible variations of the aromatic profile of wines (Leske *et al.*, 1995; Pocock *et al.*, 2003), while other authors state that bentonite addition on musts

and wines leads to a decrease of aromatic compounds concentration (Miller *et al.*, 1985; Rankine 1989, Pollnitz *et al.*, 2003). However, it is generally assumed that bentonite fining has a detrimental effect on wine aroma and flavour (Waters *et al.*, 2005).

Several authors have investigated the adsorption mechanism of bentonite against different standard proteins in model solutions (Lee, 1985; Blade and Boulton, 1988; Achaerandio *et al.*, 2001; Gougeon *et al.*, 2002, 2003). These studies led to the statement that bentonite acts very rapidly in protein adsorption (30 s – 1 min), but no relevant evidences about bentonite specificity against standard proteins were detected.

Another problem related to bentonite in winemaking is the high quantity of waste deriving from its the use. For instance, the bentonite used from Spanish wineries is about 4000 tonnes a year, and their annual bentonite sludge production is this figure plus the weight of adsorbed proteins and other impurities. This estimate gives some idea of the size of the bentonite waste disposal (Arias-Estévez *et al.*, 2007). In order to solve the problem related to the bentonite waste treatment, the possibility of bentonite regeneration has been considered by several authors (Armstrong and Chesters, 1964; Fogler, 1992). The most efficient technique was based on the bentonite treatment with sodium hydroxide, but this application did not found large application.

Finally, also the problem of the wine losses resulting from bentonite treatments should be highlighted. As reported by Høj *et al.* (2000), some 3 to 10% of the wine can be lost as bentonite lees, resulting in great economical damage.

1.1.3 BENTONITE ALTERNATIVE TECHNIQUES FOR PROTEIN REMOVAL

During the last 30 years, several techniques alternative to the bentonite fining have been studied but, for the present, none of those resulted suitable for fully substitute bentonite in treating wines for prevention of protein hazing. Generally, these studies were focused on techniques exploiting ultrafiltration, proteolytic enzymes, flash pasteurization and different adsorbent materials.

1.1.3.1 Tangential ultrafiltration

This technique has been object of several studies focused on its effect in

protein stabilization of wines (Hsu *et al.*, 1987c; Flores *et al.*, 1990). The increment of soluble proteins retention according to the diminution of the membrane pore size, reaching a 99% of protein removal with MWCO of 10 kDa, was shown. However, Hsu *et al.* (1987c) demonstrated that 3-20 mg/L of protein are often detectable in ultrafiltered wine, which can lead to haze formation. Although protein stability is not always achievable with 10 or 30 kDa of MWCO ultrafiltration, these treatments allow reducing the required bentonite up to 95%. However, ultrafiltration is still unattractive for use because it leads to great losses in important organoleptic compounds, does not eliminate all the proteins from the wine and requires high set up and running costs (Miller *et al.*, 1985; Feuillat *et al.*, 1987, Voilley *et al.*, 1990).

1.1.3.2 Immobilized phenolic compounds

Tannins are well known to interact with proteins, resulting in mutual precipitation. A method proposed in 1984 by Weetall and colleagues suggests the possibility to stabilise wine protein by using immobilised phenolic compounds (condensed seed tannins) to bind proteins. The treatment with proanthocyanidins resulted in a stable wine. Powers and co-workers (1988) showed that by immobilising proanthocyanidins in an agarose matrix it was possible to prepare a column for continuous wine stabilisation. However, trials to regenerate the column matrix showed a reduction in protein-binding capacity after a small regeneration cycles.

1.1.3.3 Alternative adsorbents

A range of alternative adsorbents including other clays, ion exchange resins, silica gel, hydroxyapatite, amberlite and alumina have been evaluated (Gump and Huang, 1999; Sarmento *et al.*, 2000) for their ability to stabilise white wines. Some of the ion exchange resins showed favourable behaviour in packed bed applications. Besides, metal oxide materials, in particular zirconium oxide, for continuous flow applications have been proposed as alternatives to bentonite fining (Pachova *et al.*, 2002; Pachova *et al.*, 2004 a, b). However, wine protein adsorption resulted relevant only at flow rates too low to propose the use of this system in winemaking conditions.

Another application regarding a protein removal in a continuous flow application was suggested by Vincenzi *et al.* (2005). The authors utilised chitin, the natural substrate of chitinases, to bind this protein considered one of the main haze-forming component (Waters *et al.*, 1998). By using a chitin column, it was possible to reach a good protein removal but not the complete wine stabilisation because of the presence of the other heat unstable proteins in wine.

Finally, the addition of polysaccharides of seaweed origin was suggested by Cabello-Pasini *et al.* (2005). The authors tested the binding capability of negatively charged polysaccharides such as agar, carragenans and alginic acid and found that the maximum adsorption was at protein content lower than 50 mg/L although a certain effect was detected until 400 mg/L of protein. However, a non specific adsorption effect was highlighted, with a behaviour similar to that of bentonite.

1.1.3.4 Flash pastorization.

Ferenczy (1966) suggested that flash pasteurisation has negative effects on wine quality, but subsequent researches affirmed that a short time heating at 90°C of the wine do not have those negative effects from the sensorial point of view (Francis *et al.*, 1994; Pocock *et al.*, 2003). Moreover, it has been demonstrated that short term heating allow a reduction of the bentonite required between 50 and 70%. Pocock *et al.* (2003) proposed to couple the flash pasteurisation with an enzymatic treatment and found that a further reduction of the bentonite required was achievable with this method. These studies are promising but expensive in a large-scale application in terms of energy and apparatuses.

1.1.3.5 Proteolytic enzymes

The endogenous and exogenous proteolytic enzymes have been largely studied in musts and wines because the possibility to exploit their activity to reduce or eliminate unstable proteins from wine is considered the best alternative to bentonite fining (Lagace and Bisson, 1990; Waters *et al.*, 1992; Dizy and Bisson, 2000). Several authors have investigated the effects of addition of microbial proteases such as those from *Aspergillus niger* (Bakalinski and Boulton, 1985), *Saccharomyces cerevisiae* (Feuillat *et al.*, 1980; Lurton *et al.*, 1988), and

Botrytis cinerea (Marchal *et al.*, 1998, Girbau *et al.*, 2004; Marchal *et al.*, 2006; Cilindre *et al.*, 2007). However, in each study, the enzymes showed not to be able to effectively degrade grape PR-proteins because of their high resistance to proteolysis and for the unfavourable conditions for the enzyme activity existing during winemaking conditions (Heatherbell *et al.*, 1984; Waters *et al.*, 1992; Waters *et al.*, 1995; Modra and Williams, 1988).

1.1.3.6 Haze protective factors

In the nineties, technique alternative to bentonite fining has been proposed by means of polysaccharide-rich proteins having a protective effect against haze formation (Waters *et al.*, 1993; 1994; Dupin *et al.*, 2000). A main compound showing protective effect resulted to be a 420 kDa mannoprotein of which about 30% was protein (Waters *et al.*, 1994). Besides, other glycoproteins have been shown to exhibit haze protective activity such as yeast invertase (McKinnon, 1996; Moine-Ledoux and Dubourdieu, 1999) and its fragments (Ledoux *et al.*, 1992; Moine-Ledoux and Dubourdieu, 1999; Lomolino and Curioni, 2007), a wine arabinogalactan-protein, and also arabinogalactan-protein from apple (Waters *et al.*, 1994).

The exact mechanism by which mannoproteins prevent haze formation is still unclear. It has been demonstrated that mannoproteins do not aggregate on their own, although their presence in wines together with wine proteins results in a decrease in the particle size of the haze formed after heating from 30 to 5 µm, resulting in visually undetectable particles (Waters *et al.*, 1993).

1.1.3.7 Genetic methods

A possibility to overtake the problem of white wines hazing would be to modify grape genes in order to not allow PR-proteins production by the plant. While this hypothesis seems interesting, researchers generally think that this way will have little chance to solve the problem of protein instability in wines without incurring in other inconveniences such as high susceptibility of vines to fungal attacks or to stresses in general (Ferreira *et al.*, 2002; Waters *et al.*, 2005).

SCIENTIFIC OBJECTIVES OF THE THESIS

This thesis aims to improve the knowledge on grape and wine proteins by studying both their chemical nature and functionality.

Taking into account that, in general, hydrophobicity can have a great effect on protein behaviour and that this aspect has not been studied in detail for the grape and wine proteins, this work has been focused on testing the suitability of the Hydrophobic Interaction Chromatography non only for protein purification, but also for their characterisation in functional terms. In particular the aim was to clear the relationship occurring between the hydrophobicity of fractionated proteins and their haze potential and to study the reactivity of protein fractions differing in hydrophobicity with tannins.

Besides, the thesis work was focused on the effects of the fermentation process, in order to identify the critical steps which can have an effect on both the quantity and heat stability of individual grape proteins. Also this aspect, at the moment, is non completely clarified.

Finally, this thesis aimed to find alternative methods for the removal of haze-forming proteins from white wines. Starting from the idea that some phytopathogenic fungi are able to grown in the presence of the (haze-forming) grape PR proteins, one can suppose that these fungi must possess some mechanisms to prevent the well-known toxicity of these grape proteins. Therefore, the strategy adopted was that to focus on the substances that the fungi emit to eliminate or remove the PR proteins from the medium in conditions similar to those of winemaking in order to identify those that can be proposed as possible means to degrade or remove the haze-forming proteins from white wines.

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CHAPTER 2

White wine protein evolution during fermentation and post-fermentation operations: relationship with protein stability

ABSTRACT

Wine proteins play a key role in determining the quality of white wines, mainly because they are unstable, thus causing haze formation during wine storage. It has been demonstrated that the vast majority of the wine proteins derive from grapes, but the stability of the grape proteins has never been studied. Grape proteins undergo some modification during winemaking. However, it is not clear whether this modification affects their instability in wine. In this chapter, the effect of the fermentation process on both the quantity and heat stability of individual grape proteins is described.

Key words: Wine, PR-proteins, haze, proteases.

INTRODUCTION

Proteins are typically present in wines at low concentration; however, they have a considerable technological and economical importance because they greatly affect clarity and stability of white wines. The presence of haze in bottled white wines results in a serious quality defect because turbidity makes the wine undesirable for consumers and wine proteins, which have the tendency to become insoluble during wine storage (Bayly and Berg 1967, Hsu and Heatherbell 1987, Waters *et al.*, 1992), are the main cause for this defect. However, the hazing potential of a wine does not seem to correlate with its total protein concentration (Bayly and Berg 1967), suggesting a different contribution of individual protein components to the phenomenon of haze formation (Hsu and Heatherbell 1987, Waters et al. 1992). Proteins present in wine have long been considered as a mixture of grape proteins and proteins from yeasts. Moreover, yeasts may affect the wine protein composition directly by releasing cell wall components and/or

indirectly by secretion of exocellular proteases that might contribute to the hydrolysis of the proteins in the must.

Ruiz-Larrea *et al.* (1998), by comparing must and wine proteins by SDS-PAGE analysis concluded that wine proteins come exclusively from grape. Accordingly, Lugera *et al.* (1998) observed that alcoholic fermentation lowered the total protein content and that no release of yeast proteins was detectable before 18 months of wine ageing on lees. By using an immunologic approach, Ferreira *et al.* (2000) confirmed that no anti-yeast antibody-reactive proteins were present in a white wine. Conversely, Waters *et al.* (1994) purified two *Saccharomyces cerevisiae* mannoproteins both in white and red wines, and found that yeasts released these proteins already during the exponential phase of growth. Marchal and colleagues (1996) gave another evidence of this statement by purifying seven wine glycoproteins through affinity chromatography on Concanavalin A, finding that several of these glycoproteins were of yeast origin. In addition, Monteiro and colleagues (2001) showed a high degree of homology between the N-terminal sequence of several proteins purified from a Moscatel wine and some microbial and yeast proteins. These disagreeing results might depend on the particular composition of yeast mannoproteins, which are characterized by high sugar content (Waters *et al.*, 1994), which makes difficult their detection with the common protein staining methods.

MATERIALS AND METHODS

1.1.4 WINE PREPARATION

Grapes of *V. vinifera* white cv. "Manzoni bianco" harvested in 2006 (about 100 kg) were pressed at ≤ 2 atm. The must was treated with SO_2 (50 mg/L) and racked before fermentation by settling for 24 h with pectolytic enzymes (Everzym MPL, Ever) at 4°C. The fermentation took place in stainless steel tanks (100 L) at 15-18°C after addition of a selected *S. cerevisiae* strain (Anchor VIN13, Ever). At the end of fermentation (7 days), two wine rackings were carried out after 10 and 29 days, respectively. Samples were taken before fermentation (before and after settling, 11 and 12 September, respectively), during fermentation (every day), and

after each of the two rackings (28 September and 17 October).

1.1.5 ANALYTICAL METHODS

Total and volatile acidity, reducing sugars, alcohol content and pH, were determined following the O.I.V. (Office International de la Vigne et du Vin) official methods of analysis (1990).

1.1.6 PROTEIN CONTENT DETERMINATION

The protein content determination was performed according to Vincenzi *et al.* (2005b). Firstly, proteins were precipitated from 1 mL of wine with the KDS method (Zoccatelli *et al*. 2003). The pellets were dissolved into 1 mL of distilled water and quantified by using the BCA-200 protein assay kit (Pierce). The calibration curve was prepared by using serial dilution of bovine serum albumin (BSA, Sigma) in water. The measurements were performed spectrophotometrically at 562 nm (Shimadzu UV 6010).

1.1.7 TOTAL POLYSACCHARIDE CONTENT DETERMINATION

The polysaccharide content was determined colorimetrically according to Segarra and co-workers (1995). After addition of 5 volumes of absolute ethanol (Baker), samples were left at 4°C overnight before centrifugation (30 min, 14000g). The collected pellets were washed twice with ethanol (Baker) and then dissolved in bi-distilled water. 1 mL of sample was then added of 25 µL of 80% phenol (w/w, Fluka) and 2.5 mL of sulphuric acid (Merck). Samples were mixed and the reaction carried on for 30 minutes at room temperature. Absorbance values were spectrophotometrically measured at 490 nm (Shimadzu UV 6010). The calibration curve was prepared by using serial dilution of galactose (Fluka) in water.

1.1.8 TOTAL POLYPHENOLS CONTENT DETERMINATION

The phenolic content in sample was determined colometrically according with the method proposed by Singleton and Rossi (1965) optimised for small

sample volumes by Waterhouse (2002). 200 μL of water diluted sample (1:10 v/v) were added with 1 mL of water diluted (1:10 v/v) 2N Folin-Ciocalteau reagent (Sigma). 800 μL of 7.5% (w/v) Na_2CO_3 (Merck) solution were added to the sample and the incubation carried out for 30 min at 40°C. The calibration curve was prepared by using serial dilution of gallic acid (GAE, Fluka) in water. The measurements were performed spectrophotometrically at 725 nm (Shimadzu UV 6010).

1.1.9 TOTAL PROTEIN PREPARATION

Musts and wines were dialysed against distilled water in 3500 Da porosity dialysis bags (Spectrapore) and passed on solid phase extraction C-18 cartridge (1 mL resin, Supelco) to "clean" the protein extract from residual polyphenols. Afterwards, the obtained preparations were frozen, freeze-dried and dissolved in a small volume of water for long term storage at -20°C.

1.1.10 ANION EXCHANGE CHROMATOGRAPHY (AEC)

The chromatographic separations were performed by means of a HPLC (Waters 1525) equipped with a Dual λ Absorbance Detector (Waters 2487) and a Refractive index detector (Waters 2414). Collected data were processed by Waters Breeze™ Chromatography Software (Version 3.30). Total protein preparations from must and wine were solubilized in 20 mM Tris-HCl pH 8.5 (eluent A) and loaded onto a Resource™ Q column (Amersham) equilibrated with the same buffer at a flow rate of 1 mL/min. Bound proteins were eluted with a gradient of eluent B (eluent A added with 1M NaCl) as follows: from 0% to 14% of eluent B in 70 minutes, then to 50% B in 30 minutes and then to 100% B in 1 minute. This latter concentration was kept for 15 minutes. Protein peaks were collected, dialyzed and freeze-dried.

1.1.11 REVERSE PHASE (RP)-HPLC

The protein composition of wine fractions was determined by HPLC, according to the method proposed by Peng *et al.* (1997).

100 µL of sample was loaded at 1 mL/min onto a semi-preparative C18 column (4.6 x 250 mm, Vydac 218 MS 54, Hesperia, CA) fitted with a C18 guard column (Vydac 218 MS 54, 4.6 x 5 mm, Hesperia, CA) equilibrated in a mixture of 83% (v/v) solvent B [0.1% trifluoroacetic acid (TFA) in 92% Acetonitrile] and 17% solvent A [80% Acetonitrile, 0.1% (v/v) TFA] and held at 35°C. Proteins were eluted by a gradient of solvent A from 17% to 49% in the first 7 minutes, 49% to 57% from 7 to 15 minutes, 57% to 65% from 15 to 16 minutes, 65% to 81% from 16 to 30 minutes and than held at 81% for 5 minutes before re-equilibrating the column in the starting conditions for 6 more minutes. Peaks were detected at 220 nm.

1.1.12 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970). Samples to be analysed were dissolved in a 0.5 M Tris-HCl pH 6.8 buffer containing 15% (v/v) glycerol (Sigma) and 1.5 % (w/v) SDS (Bio-Rad) (loading buffer) and heated at 100°C for 5 minutes before loading. For SDS-PAGE in reducing conditions, 3% (v/v) of 2-mercaptoethanol (Sigma) was also added to the loading buffer. Electrophoresis was performed in a Mini-Protean III apparatus (Bio-Rad) with T = 14% (acrylamide-N, N' metylen-bisacrylamide 29:1; Fluka) gels. The molecular weight standard proteins were Myosin (200,000 Da), β-galactosidase (116,250 Da), Phosphorylase b (97,400), Bovine Serum Albumin (66,200 Da), Ovalbumin (45,000 Da), 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). After electrophoresis, gels were stained for 18 h with Coomassie brilliant blue R-250 (Sigma) and then destained with 7 % acetic acid for 24 h (Koenig *et al.*, 1970). The PAS (Periodic Acid-Schiff) method was used to stain glycoproteins as suggested by Segrest and Jackson (1972).

1.1.13 HEAT TEST

Solutions in ultrafiltered (3.5 kDa MWCO) wine of both the total protein

fraction (300 mg/L) and each HPLC fraction (150 mg/L) were heated at 80°C for 6 h and placed at 4°C for 16 h. Hazing was then assessed by calculating the difference (before and after heating) in the absorbance at 540 nm (Pocock and Rankine, 1973; Waters *et al.*, 1991).

RESULTS

The must obtained from white grapes of *V. vinifera* cv. "Manzoni bianco" was initially treated with pectolytic enzymes to allow static settling. The turbidity measured before yeast inoculum was 181 NTU, which is considered to be in the range for an optimal alcoholic fermentation (Singleton *et al.*, 1975). Must and wine samples were collected before (M, must; M+P, must after pectolytic enzyme treatment overnight), during (F1-F7, from day 1 to 7 of fermentation) and after (R1 and R2, first and second racking, respectively) the alcoholic fermentation.

The analysis of the reducing sugars showed a regular trend of fermentation. In addition, total acidity and pH did not show any considerable variation during the same period. At the time of the second racking, the volatile acidity was 0.23 g/L, showing no acetic bacteria development. This data is significant because acetic bacteria possess the ability to produce extracellular proteases able to degrade wine proteins (Bossi *et al.*, 2006).

Sampling	pH	Total acidity (g/L tartaric acid)	Reducing sugars (% w/v)	Ethanol (%)
M (day 0)	3.32	7.0	22.9	-
M + P (day 1)	3.26	7.0	24.1	-
F1 (day 2)	3.22	7.3	18.2	-
F2 (day 3)	3.21	7.6	14.7	-
F3 (day 4)	3.20	7.8	9.6	-
F4 (day 5)	3.23	7.5	3.2	-
F6 (day 7)	3.25	7.2	< 1	13
R1 (day 17)	3.21	7.1	-	13.4
R2 (day 36)	3.22	7.2	-	13.3

Table 0.1. *Main wine analytical parameters during fermentation of the Manzoni Bianco wine.*

The total polysaccharides content showed a significant decrease (46%)

after the settling process (fig. 2.1), probably due to a pectin degradation leading to fragments that are more difficult to be precipitated by ethanol during the analytical test (Pérez-Magarino *et al.*, 2001).

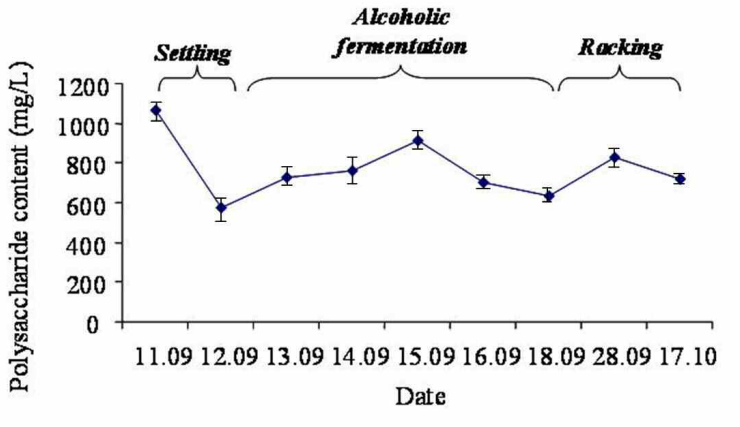

Figure 0.1. *Total polysaccharides content (as mg/L of galactose) during the vinification.*

The polysaccharide content slowly increased during the first days of fermentation, followed by a fluctuation between 631 and 916 mg/L. The first increase might be due to a polysaccharide release by yeast cells. Actually, during fermentation in synthetic solutions, yeasts demonstrated their ability to produce extracellular polysaccharides, although in lower quantity than in the autolysis phase (Llaubères *et al.*, 1987). The quantity of polysaccharides released by yeasts was shown to depend on both the strain and the conditions of fermentation, varying from 50 to 250 g/L for commercial yeast strains at 20°C (Llaubères *et al.*, 1987). The molecular structure of such exocellular polysaccharides is very similar to that of cell wall mannoproteins released during autolysis (Villetaz *et al.*, 1980).

However, the greatest fraction of total polysaccharides arises from grape, being mainly constituted of pectin, cellulose and hemicellulose (Ribèreau-Gayon, 2003). The effect of these kinds of polysaccharides on wine instability is not clear. In a study on protein instability in beer, the collected haze particles contained a substantial portion (as much as 80%) of carbohydrates, although the authors concluded that these carbohydrates are entrained or co-precipitated with proteins or polyphenols and that are not involved in the haze formation mechanism (Siebert, 1996). In wine, Mesquita and colleagues (2001) found that, after addition of wine polysaccharides, the protein instability of the wine increased, particularly

under moderately high temperatures (40-50°C). In this case, however, the purified polysaccharides had a significant absorption at 280 nm, leading the authors to suggest that the hazing effect was due to the presence of contaminant polyphenols. On the contrary, yeast polysaccharides, particularly mannoproteins, have been showed to have a protective effect against haze formation in wines (Moine Ledoux and Dubourdieu, 1999; Dupin *et al.*, 2000).

The electrophoretic analysis of glycoproteins (obtained by ethanol precipitation) by PAS staining SDS-PAGE gels confirmed the augment of soluble glycosilated compounds during fermentation, showing a smear of increasing intensity through the fermentation time (not shown). When proteins were precipitated by SDS-KCl (Vincenzi *et al.*, 2005b), the glycoprotein pattern resulted poorer (fig. 2.2), but still showed an increase in glycosilated proteins, in particular of those having an apparent high MW (white arrow in fig. 2.2). These data confirmed the observations of Llaubères and colleagues (1987) that showed, by mean of gel permeation chromatography, that the exocellular polysaccharides released by yeasts during fermentation in a synthetic medium were composed up to 80% of mannoproteins, with MWs between 100 and 200 kDa. By SDS-KCl precipitation also a band of about 45 kDa became visible (indicated by black arrow in fig. 2.2) in the samples obtained at first and second racking.

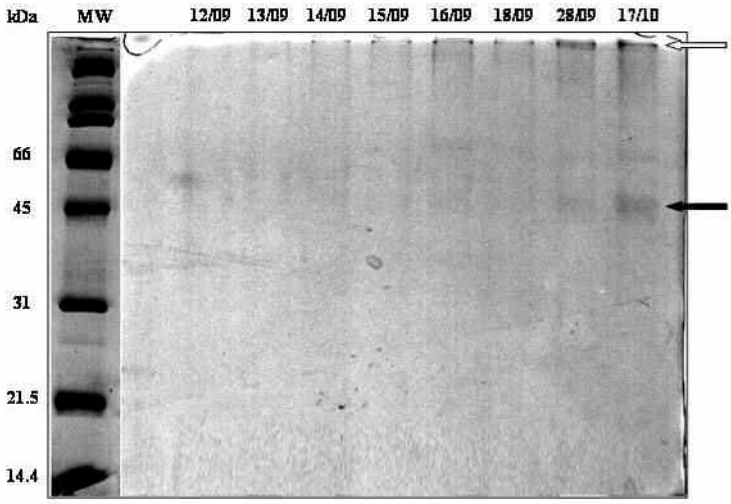

Figure 0.2. *Glycocompounds (PAS method) staining after the SDS-PAGE separation of the proteins precipitated (KDS method) from samples collected at the different times of vinification.*

The total polyphenols content was measured in all the samples with the

Folin-Ciocalteau reagent, because polyphenols, and in particular tannins, have the capacity to bind proteins and polysaccharides, leading to the formation of haze and sediments (Siebert, 1996). The pectinolytic enzyme treatment caused only a low reduction on total polyphenols, confirming the results of Pérez-Magarino and co-workers (2001) (fig. 2.3).

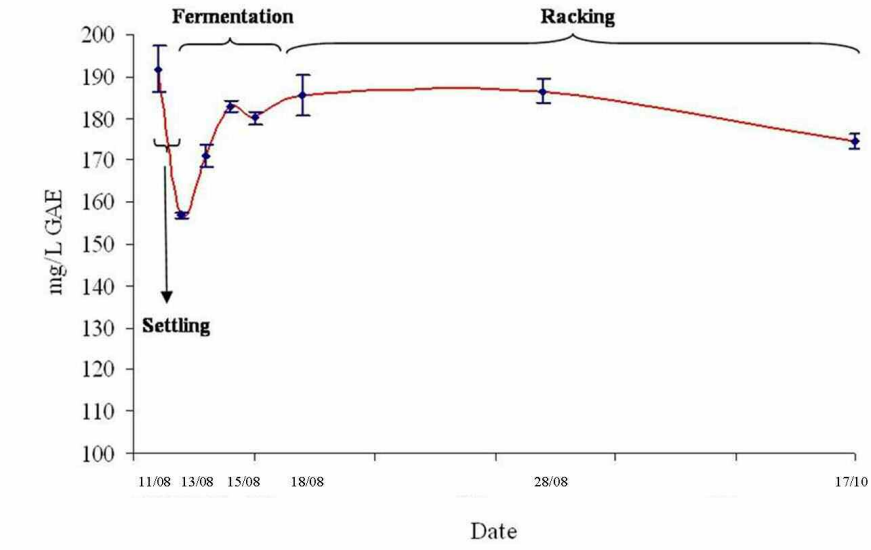

Figure 0.3. *Total polyphenol content [as mg/L of gallic acid equivalent (GAE)] of the samples collected at the different times of vinification.*

However, during the first two days there was a decrease of about 18% in the polyphenol content, probably due to their adsorption by the yeast cell walls (Caridi, 2006). Only at third day, the polyphenols started to become soluble again, possibly for the increased ethanol concentration. After the end of the fermentation process, the polyphenol content started to decrease, probably due to precipitation or complexation with other wine components. Moreover, the occurrence of some polyphenol modifications (i.e. oxidation, etc.) affecting the reactivity with the Folin-Ciocalteau reagent could not be excluded. However, the decrease resulted slow, with a polyphenol content after one month from the end of fermentation that was still over 90% of the initial concentration in must.

During alcoholic fermentation, the protein content (measured after SDS-KCl precipitation) raised by almost 30%, passing from 270 mg/L in must to 350 mg/L in wine at the end of the fermentation process. This behaviour has already

been shown in other experiments (Gasparini, 2004; Dizy and Polo, 1996) and could derive from protein release from yeasts. During the post-fermentation period the protein content slowly decreased until reaching, at the second racking, a value similar to that measured in the grape juice (fig. 2.4).

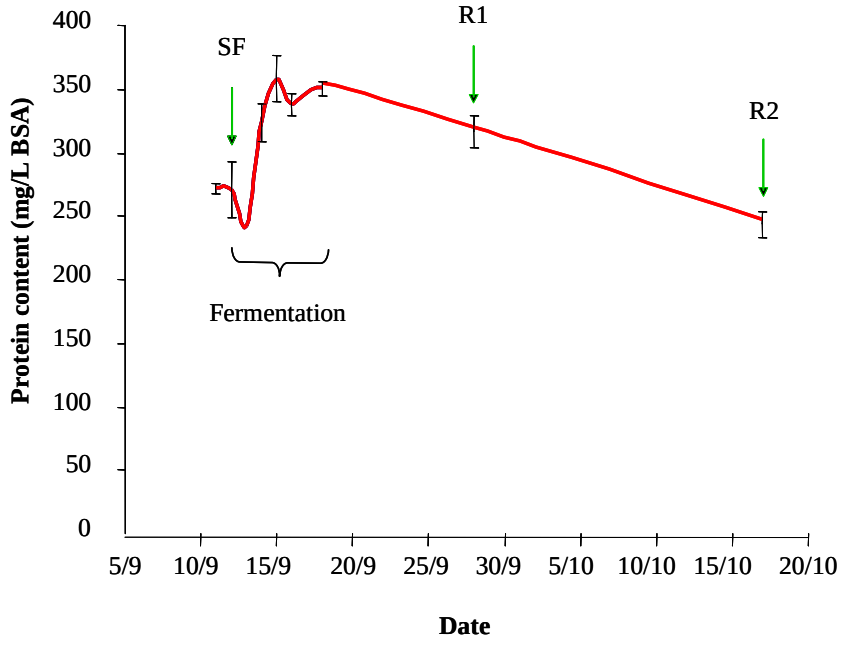

Figure 0.4. *Total protein content (as mg/L of Bovine Serum Albumin, BSA) of the grape juice during and after fermentation. The dates of sample collection are indicated. SF: start of fermentation; R1 and R2: first and second racking, respectively.*

The must electrophoretic analysis in reducing conditions showed the presence of 4 bands with apparent MWs of ≈ 60, 32, 24 and 14 kDa. This profile is surprisingly simple, but it has been reported that this is due both to a precipitation of many proteins during berry crushing and to the large number of polypeptides with different isoelectric point values but similar molecular masses (Monteiro *et al.*, 2001). The analysis of all samples in non-reducing conditions did not show any modification in the protein profile during fermentation (not shown). A better separation of grape and wine proteins by SDS-PAGE in non-reducing than in reducing conditions was highlighted (Gasparini, 2004; Vincenzi, 2005a; see fig. 4.5), probably due to the presence of proteins having different patterns of disulfide bonding. In the unreduced state, these proteins could partially maintain a more compact structure and therefore increase their apparent electrophoretic mobility with respect to the fully denaturated polypeptides. As a matter of fact, by

adopting non-reducing conditions more bands became visible in the must by SDS-PAGE (fig 2.5) compared to what can be detected after reduction of the protein samples (Vincenzi, 2005a; Vincenzi and Curioni, 2005).

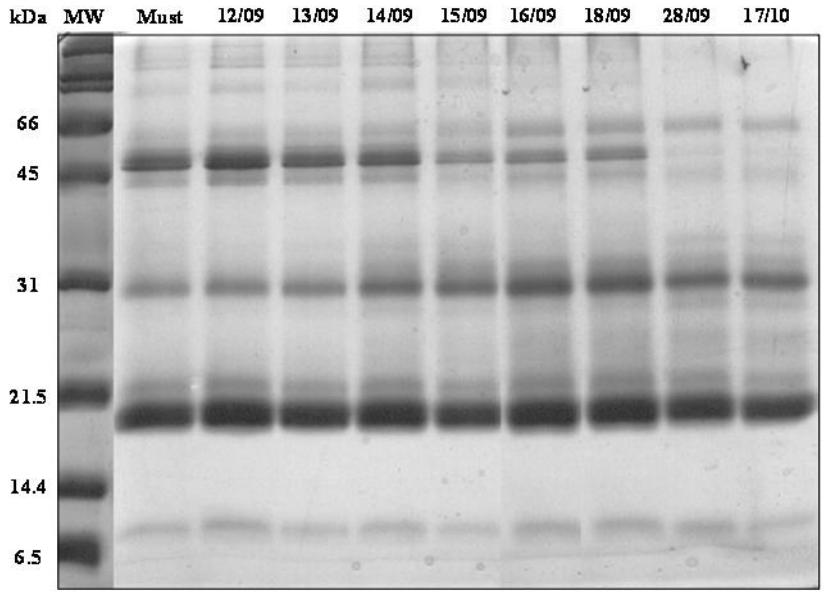

Figure 0.5. *SDS-PAGE (T=14%, C= 3.3 %) in non-reducing conditions of the proteins of the samples collected during and after fermentation. Each lane contains proteins from 100 µL of sample.*

During fermentation, no additional bands appeared, whereas an increase of the intensity of several bands, particularly that at 31 kDa, was evident. Instead, the decrease in the total protein content after the end of fermentation (fig. 2.4) seemed to be due to a degradation of the ≈ 50 kDa protein, probably a grape invertase. Therefore, these data show that no proteins release was observed, although yeasts could contribute to the variations of the total polysaccharide content (fig. 2.1). This is consistent with the results of Charpentier and Feuillat (1993) that observed proteins release from yeasts only during autolysis, which occurs several months after the end of the alcoholic fermentation. In addition, Lugera and co-workers (1998) showed a release of proteins after 18 months of contact with lees in Chardonnay wine, whereas a decrease of total protein content during the alcoholic fermentation was observed.

The protein content increase observed during fermentation can be due to a release and solubilisation of proteins from the berry particles still present in the

must. This release, probably enhanced by the yeast action and by the increase of ethanol concentration, can explain the observation that during fermentation only a change in the bands intensity is detectable in the must, without the appearance of new bands. However, the presence in must of heavily glycosilated yeast proteins not stained by Coomassie cannot be excluded.

Good separation of grape and wine proteins was achieved with anion exchange chromatography (AEC), as already reported by other authors (Waters *et al.*, 1992; Dorrestein *et al.*, 1995; Pastorello *et al.*, 2002). In order to eliminate the polyphenols from the dialyzed samples, a passage trough a cartridge was performed. This operation might result in a certain quantitative protein loss, but does not affect protein composition of the sample, as previously reported (Waters *et al.*, 1992).

In must, six peaks were detected at 280 nm by AEC (fig. 2.6). The same peaks were detected in all must/wine samples collected during vinification (fig. 2.7) and two new small peaks were detected only in the wine sample collected one month after the end of the alcoholic fermentation (fig. 2.8). However, the relative proportion between the different peaks changed during the time, as measured by the quantification of the peak areas (fig. 2.9).

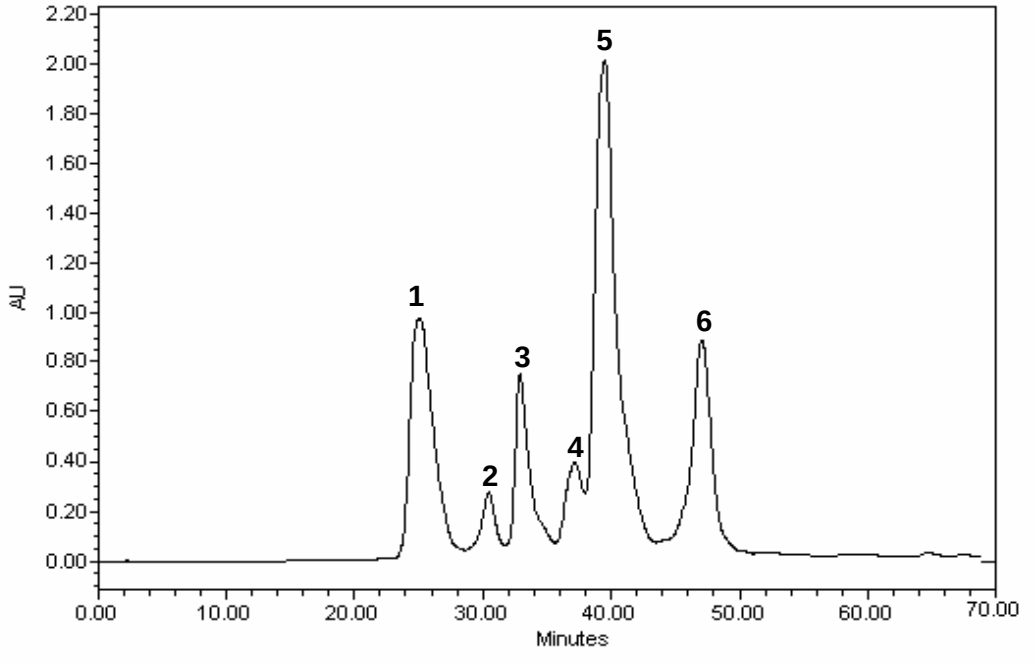

Figure 0.6. *Anion-Exchange Chromatography of the proteins obtained starting from 100*

mL of must (treated with pectolytic enzymes) before fermentation (sampling date: 12 September). Absorbance was measured at 280 nm.

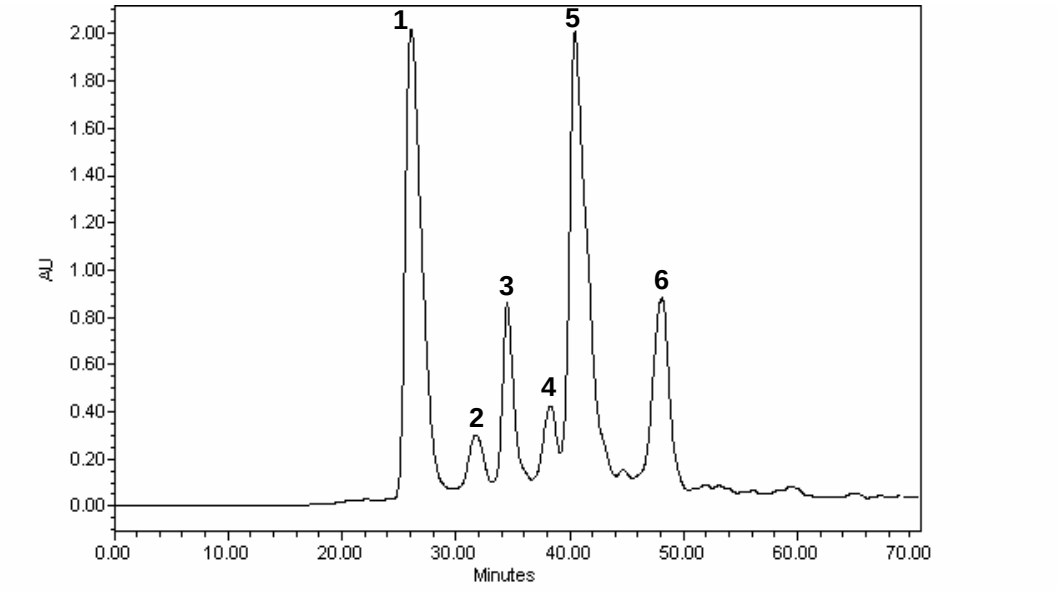

Figure 0.7. *Anion-Exchange Chromatography of the proteins obtained starting from 100 mL of wine after the end of alcoholic fermentation (sampling date: 18 September). Absorbance was measured at 280 nm.*

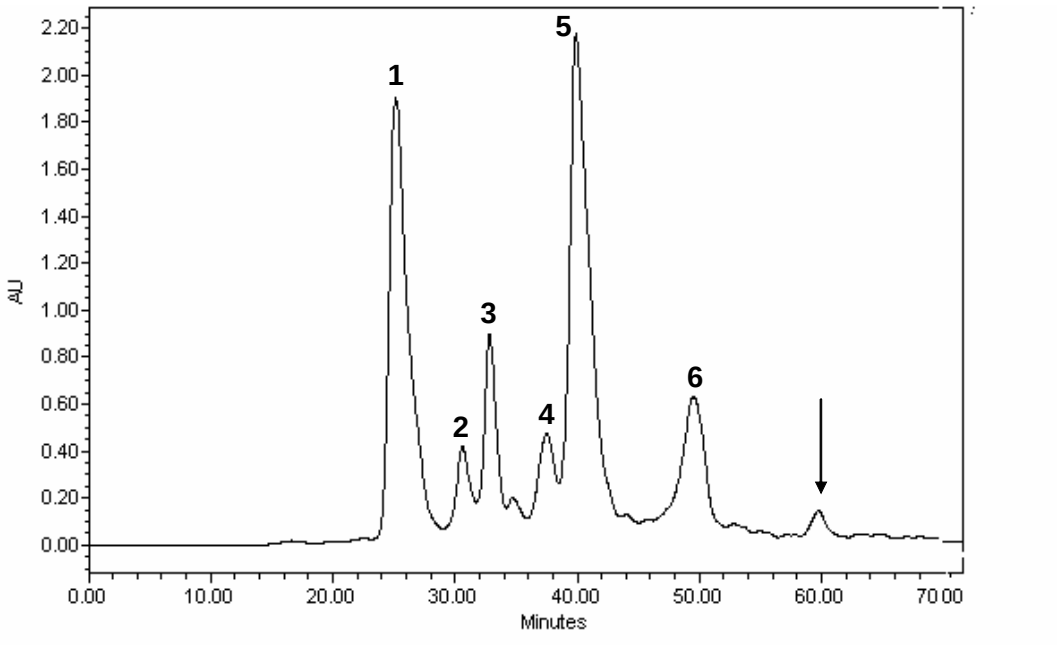

Figure 0.8. *Anion-Exchange Chromatography of the proteins obtained starting from 100 mL of wine after 1 month from the end of alcoholic fermentation (sampling date: 17 October). Absorbance was measured at 280 nm. The arrow indicates a peak formed after fermentation.*

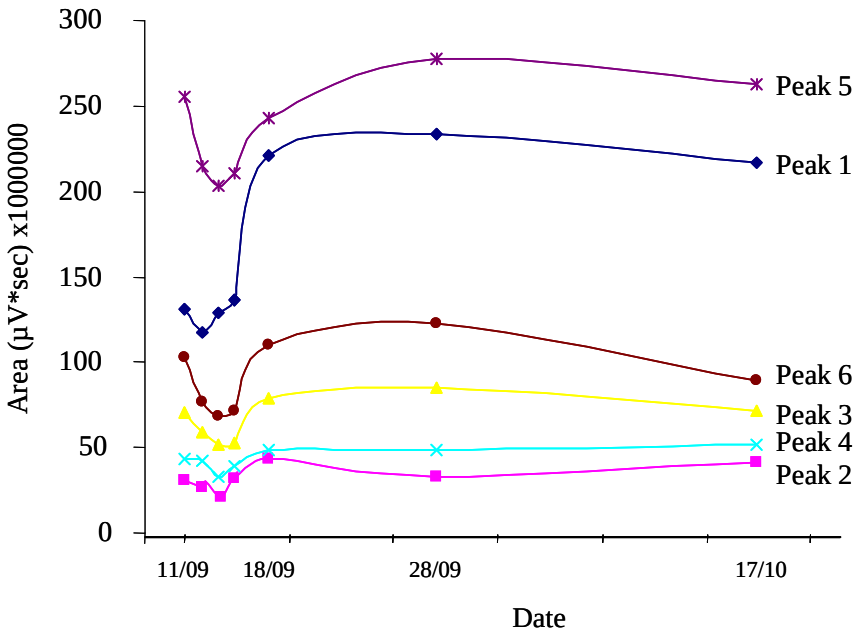

Figure 0.9. *Variation of the Anion-Exchange Chromatography peak areas during the time of the experiment.*

During the time of the experiment, the peak areas showed a trend similar to that of the total protein content, with a drop in the first three days followed by an increase until the end of fermentation and a slight decrease afterward. The increase of 30% observed in the total protein content (fig. 2.4) seems to be due in particular to the increase of AEC peak 1 (+ 72%) (fig. 2.9). The analysis of this peak by Reverse-Phase (RP) HPLC showed the presence of 98% of thaumatin-like proteins with a little contamination of chitinases, as assessed on the basis of the retention time (not shown). The AEC peak 5 contributed from 35 to 42% to the total peak area, confirming our previous data on Manzoni bianco wine (Gasparini, 2004; Vincenzi, 2005). The chromatographic analysis by RP-HPLC showed this peak to be mainly constituted of chitinases (not shown), in accordance with the findings obtained previously by chitinase activity detection on SDS-PAGE gels (Gasparini, 2004; Vincenzi and Curioni, 2005). These results confirmed that the PR-proteins are the main proteins in grape and wine (Waters *et al.*, 1992).

The six peaks obtained from AEC separation of the must proteins were collected and analysed by SDS-PAGE (fig. 2.10).

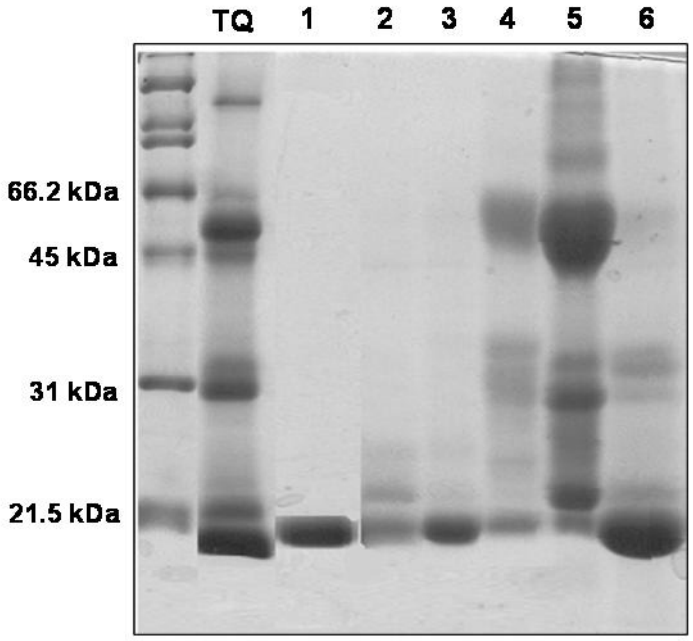

Figure 0.10. *SDS-PAGE (T=14%, C=3.3%) in non-reducing conditions of the peak fractions collected from Anionic Exchange Chromatography of the wine proteins at the end of fermentation (sampling date: 18 September). Each lane contains 15 µg of protein. TQ: total proteins of the wine before fractionation.*

With the exception of peaks 1 and 3, which both showed an only band, all the AEC fractions contained two or more protein bands. In particular, a band at ≈ 20 kDa was present in every peak, confirming that grape contains a large number of polypeptides with different pI values (affecting the elution from the AEC column) but similar apparent molecular masses in SDS-PAGE (Monteiro *et al.*, 2001).

Considering that individual wine proteins can differently contribute to the phenomenon of haze formation (Hsu and Heatherbell 1987, Waters *et al.* 1992), these changes in the relative concentrations of the AEC peaks during fermentation might be related to variations in the hazing potential of the mixture of the grape proteins passing from must to wine.

To evaluate the haze stability throughout the fermentation process, the total proteins from must/wine samples were heat tested (Pocock and Rankine, 1973), with a slightly modified method (Waters *et al.*, 1991). It has been demonstrated that the environmental conditions, and in particular the pH and the

ethanol concentration, have a great influence on wine protein stability (Siebert, 1999). Possible interferences due to the different composition of samples (sugars, ethanol, etc.) collected from the start to the end of fermentation were eliminated by using the proteins precipitated from each sample dissolved in an ultrafiltered wine (3 kDa MWCO) at the same concentration of 300 mg/L. The protein solutions were then heat tested and the turbidity was measured by mean of the absorbance at 540 nm (fig. 2.11).

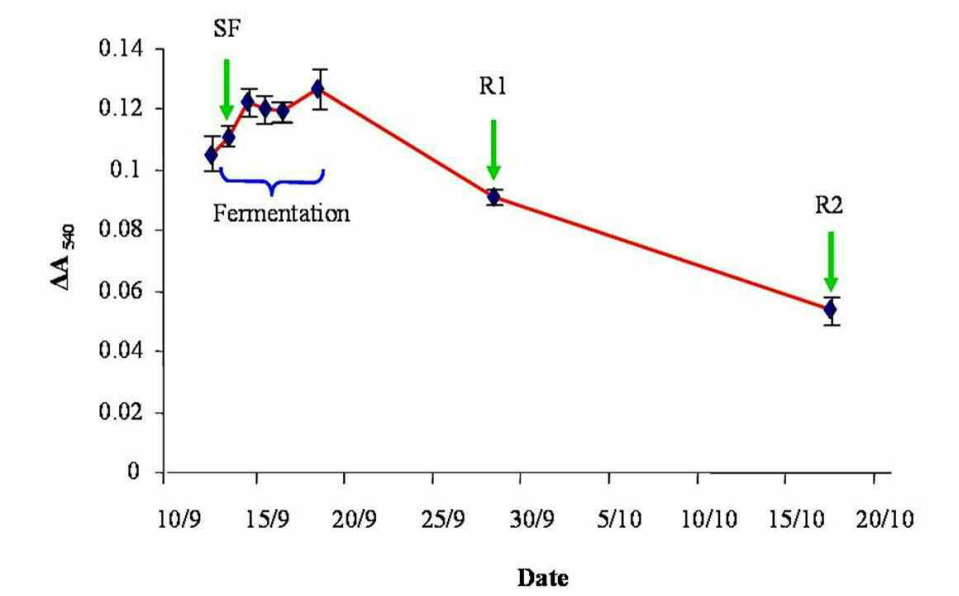

Figure 0.11. *Haze formed after the heat test (in de-proteinised wine) by the total proteins of the samples collected during the experiment. SF: start of fermentation; R1 and R2: first and second racking, respectively.*

The results showed that the total protein instability slowly increased throughout the alcoholic fermentation. This behaviour could indicate that the quantitative variation of the different protein fractions observed by AEC, such as the increase of thaumatin-like proteins in peak 1, caused an increase in haze formation (fig. 2.11). To confirm this observation, the instability of the precipitated proteins of the individual AEC peaks, dissolved at 150 mg protein/L in ultrafiltered wine, was tested. The total proteins precipitated from the wine at the end of alcoholic fermentation and dissolved at 150 mg/L in an ultrafiltered (3000 Da MWCO) Manzoni bianco wine were also used as a control (fig. 2.12).

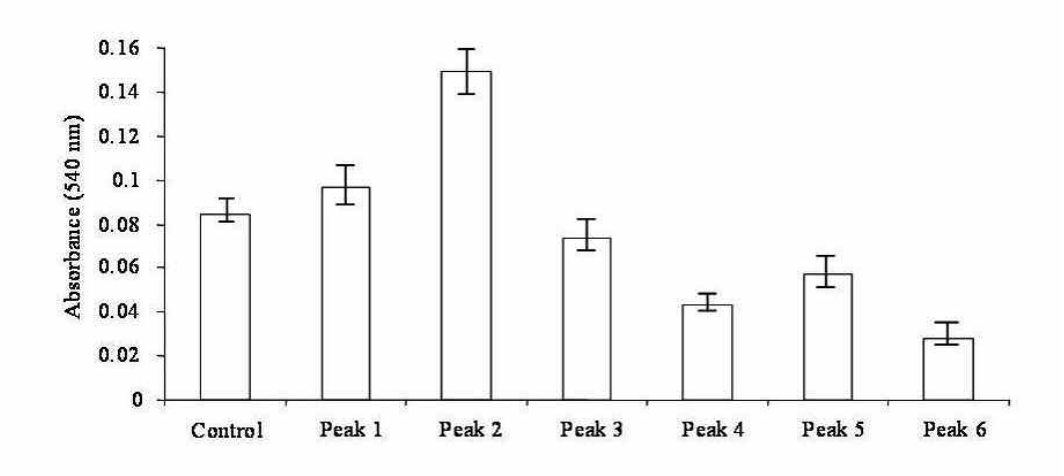

Figure 0.12. *Heat test results of the proteins of the fractions collected from Anion-Exchange Chromatography of the sample taken at the end of fermentation (sampling date: 18 September) (see fig. 2.7). Proteins from each fraction were dissolved at a 150 mg/L in ultrafiltered (3000 MWCO) Manzoni bianco wine. Control: 150 mg/L of total wine protein in ultrafiltered Manzoni bianco wine.*

Only proteins contained in peak 1 and 2 showed an intrinsic instability higher than that of the total wine proteins. However, peak 2 accounted only for a minimal part of the total wine proteins (2-5.1 % of the total area of the chromatograms, fig. 2.9), thus contributing only little to the total turbidity formed. On the contrary, peak 1 contained 21-30.5% of the total proteins (based on the chromatogram area), being the second most abundant peak in the samples (fig. 2.9). Moreover, the area of peak 1 was that showing the higher augment (+ 72 %, fig. 2.9) throughout the fermentation, with this increment likely responsible for the total proteins heat-instability increase detected in this phase (fig. 2.11). Indeed, taking into account both the actual concentration and the intrinsic instability of each peak, peak 1 was that giving the higher contribution (more than 40%) to the total wine protein instability (fig. 2.13). From the notion that peak 1 was mainly composed of a thaumatin-like protein, this finding is consistent with results recoverable in literature (Waters *et al.*, 1998) and confirmed in chapter 4 of this thesis (see fig. 4.10), in which this class of protein is indicated as the main responsible for haze formation in white wines.

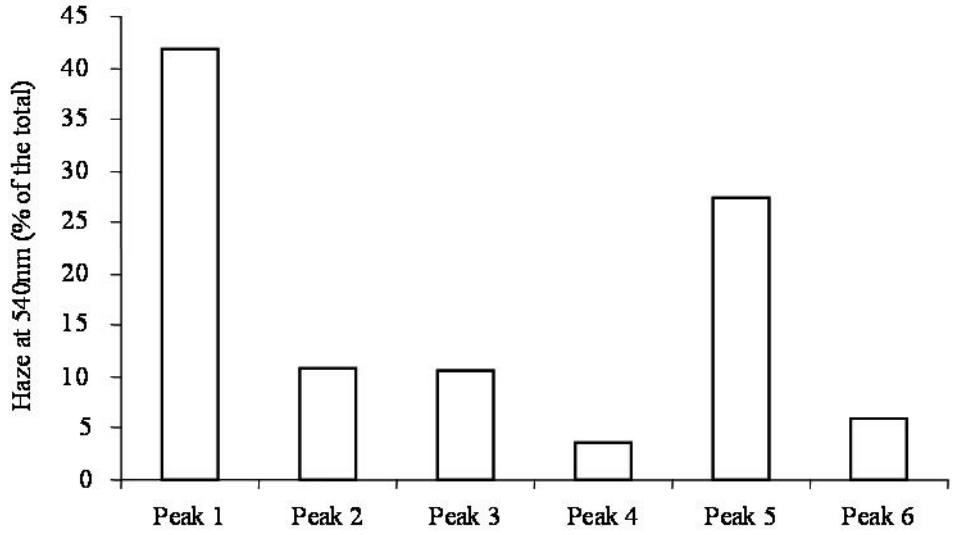

Figure 0.13. *Percent contribute of each peak to the total turbidity developed (by the heat test) in wine at the end of fermentation (sampling date: 18 September).*

After the end of fermentation, the instability of the total proteins tended to decrease and the heat-induced haze at second racking was even lower than that of the starting must. However, this variation could not be ascribed to a modification in the relative protein concentrations, because the ratios between the different protein peaks remained quite constant during the one-month storage. This stability increase could be instead attributed to the mannoproteins released by yeasts during the storage (on total lees), which actually seem to appear in the wine (fig. 2.2).

1.1.14 DISCUSSION

During winemaking, some modifications of the must proteins profile are detectable. Several authors highlighted the release of protoplasmatic proteases from yeasts during autolysis (Feuillat *et al.*, 1980; Lurton, 1988). However, these enzymes do not significantly influence the protein pattern, although it is not clear whether these enzymes are inhibited by wine polyphenols or by the low pH of the must. Lurton (1988) showed that the *S. cerevisiae* endoprotease A was still active at pH 3.0, leading to the release of peptides into the wine. In optimal conditions (pH 4.5-5.0, 35-40°C) the cell disorganization of yeasts starts in several hours,

while the same process in wine takes up to 2-3 months (Flanzy, 1998).

However, *S. cerevisiae* resulted able to hydrolyse haemoglobin and casein *in vivo* by secreting extracellular proteases in the fermenting medium (Rosi *et al.*, 1987; Feuillat *et al.*, 1980). Moreover, *S. cerevisiae* possess some periplasmic peptidases, involved in the active transport system, that are able to partially degrade the proteins of the medium (Cartwright *et al.*, 1989). Actually, wine yeasts are able to grow in a medium containing only must proteins as the nitrogen source (Conterno *et al.*, 1994).

Only few studies have analysed the fate of must proteins during vinification, and all postulated a decrease of the total protein content during this process (Lugera *et al.*, 1998; Moreno-Arribas *et al.*, 1996). These data are in disagreement with our results and with the observation that grape proteins are protease-resistant and stable at acidic pH (Modra and Williams, 1988; Waters *et al.*, 1992; Waters *et al.*, 1995). Beside, it is important to consider that the cited works aimed to clear protein changes occurring during the second fermentation in sparkling wines production and after a bentonite treatment (Lugera *et al.*, 1998; Moreno-Arribas *et al.*, 1996). Moreover, the variation of the nitrogen compounds was monitored for long times (up to 18 months), that are compatible with the degradation and precipitation even of the more resistant wine proteins. In particular, Manteau *et al.* (2003) stated that grape proteins tend to disappear during the Champagne wine production. These authors suggested that this phenomenon was mainly due to protein precipitation, adsorption on cell walls of the yeasts and denaturation. In this case, the reducing environment (150 mV) of the bottle during the second fermentation might determine a partial reduction of disulfide bonds, increasing protein susceptibility to the action of proteases secreted by yeasts (Manteau *et al.*, 2003).

In a normal white wine vinification, however, the yeast cells remain in contact with the wine for a time too short to allow autolysis. The few works that have studied the modifications of grape proteins in the early stages of vinification showed that during the first alcoholic fermentation little quantitative variations occur resulting, in most cases, in an increase of the protein content (Bayly and Berg, 1967; Dizy and Polo, 1996). This increase was considered to be due to a protein release from the yeast cells. By means of chromatographic and

electrophoretic techniques, Bayly and Berg (1967) showed both quantitative and qualitative modifications in wine proteins during fermentation, with the appearance of new electrophoretic bands.

The results here obtained indicate that the soluble proteins of the berry of a white grape variety vary during and after the alcoholic fermentation in both quantity and relative proportion. According to what stated in another work (Vincenzi *et al.*, 2006), the protein fraction of AEC peak 1, containing a single 20 kDa band (probably a thaumatin-like protein), showed the lowest heat-stability when individually heat-tested in de-proteinised wine. The same protein fraction is that with the largest quantitative increase during fermentation, constituting a large proportion of the total wine proteins. Taking into account that bands with the same SDS-PAGE mobility, corresponding to that of the TL protein family, could be detected also in other AEC fractions, the precise nature of the protein of peak 1 warrants further investigation.

Moreover, from the results here reported, it is confirmed that the release of compounds of polysaccharidic nature by the fermenting yeast (probably mannoproteins) results in an increased heat stability of the total wine proteins, despite the increase in the relative proportion of their most unstable component.

Acknowledgements

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CHAPTER 3

Fractionation of grape juice and wine proteins by hydrophobic interaction chromatography

ABSTRACT

Thaumatin-like (TL) proteins and chitinases are the predominant proteins in ripe grapes. TL proteins and chitinases inhibit fungal growth *in vitro* and likely play a role in grape pathogen defence.

In this work, a method to separate grape juice and wine proteins by hydrophobic interaction chromatography (HIC) using Phenyl Sepharose® High Performance resin is proposed. The purification was performed in two steps: protein precipitation with ammonium sulphate followed by HIC fractionation. HIC fractions of ammonium sulphate precipitated juice and wine proteins were further fractionated by reverse phase HPLC and SDS-PAGE to assess their nature and purity. Grape juice proteins were also identified by matching peptide LC-MS/MS spectra with theoretical peptides from a plant protein database. Identifications of twenty-six LC-MS/MS samples included several TL-proteins and chitinases, vacuolar invertase, and a lipid transfer protein.

Keywords: grape, protein, wine, HIC, LC-MS/MS, thaumatin-like protein, chitinases.

INTRODUCTION

Because of the very low amount of proteins recoverable in grape and wines, researchers need to find easy and high-yield protein purification procedures. A wide variety of protein purification techniques are available today, however, different types of chromatography have become dominant due to their high resolving power. In gel filtration chromatography, ion-exchange chromatography, affinity chromatography and hydrophobic interaction chromatography (HIC), protein separation is dependent on their biological and

physico-chemical properties: molecular size, net charge, biospecific characteristics and hydrophobicity, respectively (Kennedy, 1990; Garcia, 1993). The result of a protein purification procedure is evidently dependent on the choice of separation equipment and techniques. The order in which the different techniques are combined is also of great importance. The development of techniques and methods for the separation and purification of proteins has been essential for many of the recent advances in biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contaminants, but also the concentration of the desired protein and their transfer to an environment where it is stable and in a form ready for the intended application (Queiroz *et al.*, 2001).

Techniques like ion exchange chromatography and gel filtration have been widely used in fractionations of grape and wine macromolecules (Bayly and Berg, 1967; Somers and Ziemelis, 1973; Waters *et al.*, 1992, 1993; Dawes *et al.*, 1994; Dorrestein *et al.*, 1995; Canals *et al.*, 1998; Monteiro *et al.*, 1999, 2001; Pastorello *et al.*, 2002). Other chromatographic methods, such as HIC, have been only recently applied in wine studies by Brissonet and Maujean (1993), which used HIC for the characterization of foaming proteins of champagne wine.

HIC takes advantage of the hydrophobicity of proteins promoting its separation based on hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the surface of proteins. The adsorption increases with high salt concentration in the mobile phase and the elution is achieved by decreasing the salt concentration of the eluent (Melander and Horváth, 1977; Fausnaugh and Regnier, 1986; Roe, 1989). Therefore, the term ‘salt-promoted adsorption’ could be used for this type of chromatography (Porath, 1986).

Different types of elution conditions can be used for purification of complex mixtures of proteins that would be difficult to separate using other chromatographic techniques. In fact, HIC has been successfully used for separation purposes as it displays binding characteristics complementary to other protein chromatographic techniques (Janson and Rydén, 1993). Van Oss *et al.* (1986) proposed that the van der Waals forces are the major contributing factor to the hydrophobic interactions (‘interfacial forces’) despite the complex mechanism involved. HIC that today is now an established and powerful bioseparation

technique in laboratory-scale, as well as in industrial-scale purification of proteins (Wu and Karger, 1996; Sofer, 1997; Grund, 1998).

In this chapter, the study of both Semillon grape juice and wine proteins by means of HIC is illustrated. The method of protein fractionation involved protein salting out followed by direct fractionation through a HIC matrix. Fractions collected were characterised by means of HPLC, SDS-PAGE, and LC-MS/MS techniques.

MATERIALS AND METHODS

1.1.15 MATERIALS

Grape and wine proteins were purified from Semillon variety harvested on 2006 in Adelaide Hills region (South Australia).

1.1.16 AMMONIUM SULPHATE PROTEIN PRECIPITATION

Grape proteins from Semillon juice were concentrated by ammonium sulphate precipitation. The salt was added at 80% of saturation to the grape juice, previously buffered to pH 5.0 (with KOH). After 16 hours at 4ºC, the pellet collected by centrifugation (30 min, 14000g, 4ºC) was treated in two ways: i) dissolved in 30 mM citrate buffer (pH 3.50) and desalted through passage on a column equipped with 20 mL of Bio-Gel® PD-10 resin (Bio-Rad) and freeze-dried before being dissolved in 50 mM citrate buffer (pH 3.5); ii) directly dissolved in the eluant A of HIC fractionation (see below).

1.1.17 RESIDUAL AMMONIA DETERMINATION

The ammonia content on purified fractions was determined by means of the UV test for the determination of Urea and Ammonia in foodstuffs (r-biopharm; Roche).

1.1.18 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970). The samples were dissolved in a Tris-HCl pH 6.8 buffer containing 15% (v/v) of glycerol (Sigma) and 1.5 % (w/v) SDS (Bio-Rad) and heated at 100°C for 5 minutes before to be loaded for the analysis performed by using a Mini-Protean III apparatus (Bio-Rad). Each analysis was conducted in non-reducing conditions. The molecular weight standards were the Broad Range (Bio-Rad). Ready Tris-HCl Gels [Bio-Rad, 4-20%, 15-well, 15 µl, 8.6 x 6.8 cm (W x L)] were used. Gels were alternatively stained with Bio-Safe™ Coomassie stain (Bio-Rad) or Silver stain procedure for high sensibility protein detection, according to Blum *et al.* (1987).

1.1.19 GRAPE AND WINE PROTEIN CHROMATOGRAPHY

The chromatographic separations were performed by means of:

- An ÄKTA Prime FPLC (Amersham Biosciences, Sweden) equipped with an UV detector (λ Absorbance Detector). Data collected were elaborated by the PrimeView software.
- A RP-HPLC Agilent 1200 Series (Agilent Technologies, Germany) equipped with autosampler, fraction collector and Diode Array Detector.

Every solution utilised and sample loaded had previously been filtered with cellulose acetate filters (Millipore) with pore size of 0.20 µm and degassed.

1.1.20 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Grape and wine proteins were fractionated with an ÄKTA Prime FPLC system (Amersham Bioscience, Sweden). Five columns (1 mL resin each) contained in the HiTrap Hydrophobic Interaction Chromatography (HIC) kit (Pharmacia) were used. The matrixes of the kit were: Phenyl Sepharose® High Performance, Phenyl Sepharose 6 Fast Flow (low substitution), Phenyl Sepharose 6 Fast Flow (high substitution), Butyl Sepharose 4 Fast Flow and Octyl Sepharose

4 Fast Flow.

The Phenyl Sepharose® High Performance resin was also utilized to pack a chromatography glass column (55 mL) having a diameter of 1.6 cm. Eluent A was 50 mM Sodium Phosphate + 1.25 M Ammonium sulfate, pH 5.0 and eluent B was eluent A without ammonium sulfate. Samples were loaded at 3 mL/min onto the resin previously equilibrated in 100% of buffer A. The loading lasted for 200 mL before the beginning of a linear gradient to 0% A (from 200 to 800 mL of elution volume). After the end of the gradient, 100% buffer B was applied for 400 mL to re-equilibrate the column in the starting conditions.

1.1.21 RP-HPLC PROTEIN ANALYSES

1.1.21.1 Analysis and quantification of protein by PR-HPLC

The protein composition of the grape and wine fractions was determined by reversed phase HPLC, according to the method proposed by Peng *et al.* (1997).

Samples (100 µL) were loaded at 1 mL/min onto a semi preparative C8 column (4.6 x 250 mm, Vydac 208 TP 54, Hesperia, CA) fitted with a C8 guard column kit (Vydac 208 GK 54, 4.6 x 5 mm, Hesperia, CA) equilibrated in a mixture of 83% (v/v) solvent B [0.1% trifluoroacetic acid (TFA) in 92% Acetonitrile] and 17% solvent A [80% Acetonitrile, 0.1% (v/v) TFA] and held at 35°C. Proteins were eluted by a gradient of solvent A from 17% solvent A to 49% solvent A in the first 7 minutes, 49% to 57% from 7 to 15 minutes, 57% to 65% from 15 to 16 minutes, 65% to 81% from 16 to 30 minutes and than held at 81% for 5 minutes before to re-equilibrate the column in the starting conditions for 6 more minutes. Elution was followed by absorbance at 210, 220, 260, 280 and 320 nm. From the 210 nm spectrum, their identity was assigned by comparison of their retention times to those of purified grape PR proteins (Waters *et al.*, 1996) and quantified by comparison to the peak area of two standard proteins: horse heart cytochrome C (cyt C) (Sigma) or bovin serum albumin (BSA) (Sigma).

1.1.21.2 HPLC protein analyses by Size Exclusion Chromatography

Protein fractions were analysed by Size Exclusion Chromatography using a RP-HPLC equipped with a Phenomenex BioSep SEC S2000 (7.8 x 300 mm) column with guard column at 1 mL/min in 50 mM KH_2PO_4 buffer at ambient

temperature. 20 µL of sample were injected. The absorbance was detected at 210 nm.

1.1.22 SAMPLES DESALTING

Samples were desalted by using an ÄKTA Prime FPLC apparatus equipped with a 20 mL column containing Bio-Gel® P-10DG gel resin (Bio-rad). The separation was performed in isocratic conditions (50 mM Citric acid-NaOH buffer, pH 3.50). The flow rate was 2 mL/min and the maximum loaded volume was 2 mL.

1.1.23 PROTEIN IDENTIFICATION THROUGH LC-MS/MS ANALYSES

Grape proteins were excised after electrophoretic separation and sent to Australian Proteome Analyses Facility Ltd. Bands were reduced (25mM Dithiorethiol/50mM NH_4CO_3 at 56°C) and alkylated (55mM Iodoacetamide/50mM NH_4CO_3 at room temperature in the dark) followed by a 16 hour tryptic digestion at 37°C. Samples were extracted with 0.1%TFA/2%CH_3CN and sonicated in water bath for 15 minutes. The instrument used for nanoLC was Agilent 1100 Series (Agilent Technologies, Germany). Samples were injected (40µL each) onto a peptide trap (Michrome peptide Captrap) for pre-concentration and desalted with 0.1% TFA, 2% Acetonitrile at 10µL/min. The peptide trap was then switched into line with the analytical column containing C18 RP silica (SGE ProteCol C18, 300A, 3µm, 150µm x 10 cm). Peptides were eluted from the column using a linear solvent gradient from H_2O:CH_3CN (95:5; + 0.1% formic acid) to H_2O:CH_3CN (70:30, + 0.1% formic acid) at 600nL/min over a 60 min period. The LC eluent was subject to positive ion nanoflow electrospray analysis on an Applied Biosystems QSTAR XL mass spectrometer (ABI, CA, USA). The QSTAR was operated in an information dependant acquisition mode (IDA). In IDA mode a TOFMS survey scan was acquired (m/z 370-1600, 1.0s), with the three largest multiply charged ions (counts >50) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s (m/z 100-1600). Results were a peak list in Mascot Generic Format using MASCOT.dll script (Applied Biosystems) and mascot search result PDF file with NCBInr

database with Viridiplantae taxonomy.

A second batch of analyses was performed by excising protein bands from a SDS-PAGE gel. Peptides were prepared in 10 µL of 1% formic acid, loaded by auto sampler onto a 5 cm x 100µm ID C18 column. The gradient was 0-50%B in 35 min after 12 minutes from the loading. Eluant A was 5% Acetonitrile, 0.1% formic acid. Eluant B was 90% Acetonitrile, 0.1% formic acid. LCQ Deca Ion trap mass spectrometer, set to do MS 400-1500amu, MS/MS of 3 most intense ions, dynamic exclusion enabled. The raw files were converted to mzxml format with the program readw.exe. Files in mzxml were submitted to X!tandem database via locally installed GPM-XE version. Parent ion mass window 4 da, fragment ion tolerance 0.4 Da, Cys + 57 for iodoacetamide, met differentially modified with oxidation. Reverse database searching enabled concurrently. PlantProtein database downloaded from TAIR as fasta file, converted to .pro file for GPM use.

1.1.24 AMINO ACIDS ANALYSIS

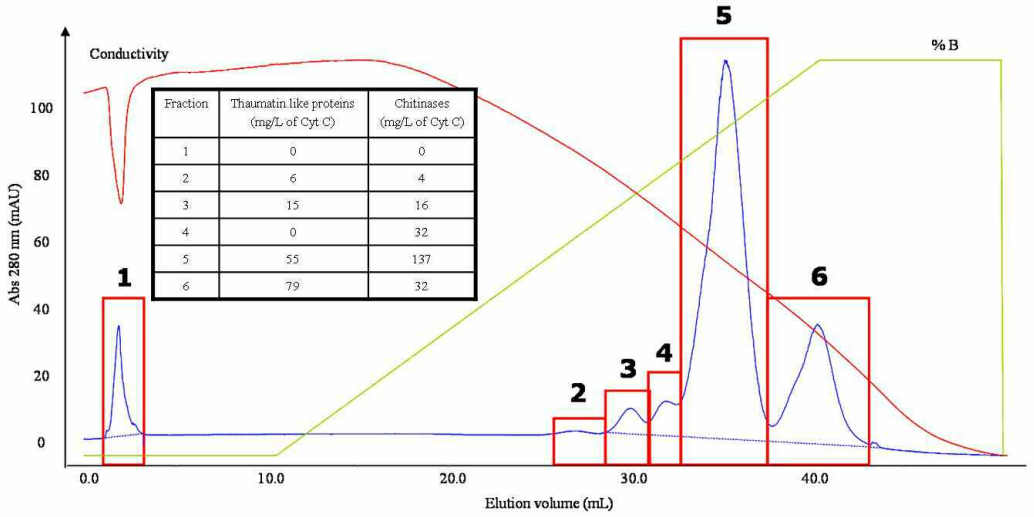
Amino acids analysis was performed by the Australian Proteome Analyses Facility Ltd. The sample underwent 24 h to a gas phase hydrolysis with 6N HCl at 110°C. Amino acids were analysed using the Waters AccQTag chemistry. Cysteine was separately analysed by using performic acid oxidation followed by 24 h acid hydrolysis with 6N HCl at 110°C. Samples were analysed in duplicate and results were expressed as an average.

RESULTS AND DISCUSSION

1.1.25 GRAPE PROTEINS FRACTIONATION: RESIN SELECTION

In order to verify the ability of hydrophobic interaction chromatography to fractionate grape proteins, a HiTrap HIC kit (Pharmacia) was utilised. An initial screening was performed using different resins, conditions of pH, buffers and gradients, to find the most suitable matrix for grape protein purification (data not shown).

The Phenyl Sepharose® High Performance resin was chosen for its good protein separation ability with an easy fractionation of 5 well defined peaks in a short gradient length (fig. 3.1).



Fraction	Thaumatin like proteins (mg/L of Cyt C)	Chitinases (mg/L of Cyt C)
1	0	0
2	6	4
3	15	16
4	0	32
5	55	137
6	79	32

Figure 0.1. *Semillon juice protein fractionation achieved using an ÄKTA Prime FPLC (Amersham Biosciences, Sweden) equipped with a column containing 1 mL of hydrophobic interaction resin (Phenyl Sepharose® High Performance). In the table: protein composition and concentration of fractions collected (expressed in equivalent of Cytochrome C) determined by RP-HPLC (Waters et al., 1996; Peng et al., 1997; Pocock et al., 2000).*

To determine the protein composition of the peaks, the 6 fractions obtained were analysed by HPLC. Results showed the absence of proteins on fraction 1 (flow through) that showed a peak due only to the conductivity fall corresponding to the sample exit from the column. The two main classes of grape

proteins (PR-protein) were detected in fractions 2 to 6. Particularly, TL-proteins and chitinases were both present at a similar concentration in the lower peaks (2 and 3). The other fractions appeared more interesting because of the presence of chitinases without TL-proteins on fraction 4, the predominance of chitinases on fraction 5 and of TL-proteins on fractions 6. These preliminary results showed the possibility to use hydrophobic interaction chromatography to fractionate grape proteins in general and PR-proteins in particular by exploiting their different hydrophobic characteristics.

1.1.26 GRAPE PROTEINS FRACTIONATION: LARGE SCALE EXPERIMENTS AND VVTL PROTEIN PURIFICATION

A chromatographic column (Ø = 1.6 cm) was packed with 55 mL of Phenyl Sepharose® High Performance resin (GE-Healthcare). Passing from a small to a bigger scale, the separation efficiency was noticeably improved.

Two methods for the sample preparation were applied to an unfined Semillon grape juice:

i. Addition of ammonium sulphate to 80% of saturation (567 g/L at 25°C), collection of the pellet by centrifugation (15000g, 4°C, 30 min), dissolution of it in 50 mM Na-phosphate pH 5.0 buffer (eluent A) until the required salt concentration (1.25 M, starting conditions for HIC fractionations) was reached.

ii. Addition of ammonium sulphate at 80% of saturation (567 g/L at 25°C), collection of the pellet by centrifugation (15000g, 4°C, 30 min). The pellets were washed with citrate buffer and concentrated by ultrafiltration (3000 MWCO) until reaching a protein content of ≈ 20 mg/mL. Grape protein stock so prepared were diluted 1:1 with 100 mM Na-phosphate pH 5.0 + 2.5 M ammonium sulphate before the HIC fractionation.

No differences in protein fractionation were seen with both methods (data not shown). Therefore, the first method was chosen because of its simplicity and shortness. For this reason, the obtained pellet from an unfined Semillon juice was treated as previously described and loaded directly into the HIC column (fig 3.2).

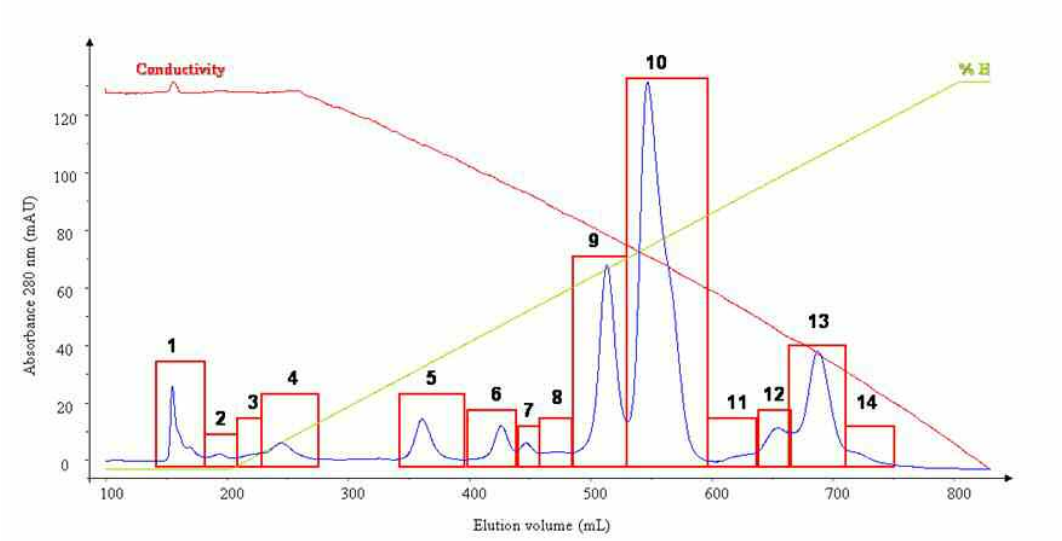


Figure 0.2. *Semillon grape protein fractionation by hydrophobic interaction chromatography with a column (Ø = 1.6 cm) containing 55 mL of Phenyl Sepharose® High Performance resin. Total protein content loaded: ≈ 170 mg. Red rectangles indicate the fractionation scheme.*

The resulting chromatogram showed a good protein separation, with the presence of a larger number of peaks compared to the fractionation obtained with the 1 mL column (fig. 3.1). Two fractionations were performed with ≈ 170 mg of protein loaded on each. The profiles were almost identical. Consequently, the 14 fractions collected were pooled prior to analysis by RP-HPLC to determine both their protein composition and concentration (tab. 3.1).

As a comparison, the juice before HIC fractionation was also analysed and included in the table. The profile of the whole juice assessed by HPLC (fig. 3.3) showed the high number of peaks that were fractionated with HIC, as summarised in table 3.1.

According to Peng *et al.* (1997), peaks from RP-HPLC chromatograms were assigned to different protein classes. In particular, it was assumed that peaks with a retention time between 8.7 and 12 minutes belonged to the TL-protein classes, while peaks eluted from 18.5 and 24.5 were considered to be chitinases. Consequently, the three main peaks observed in fig. 3.3 were likely TL-proteins (9.3 and 10.9 min) and chitinases (19.6 min).

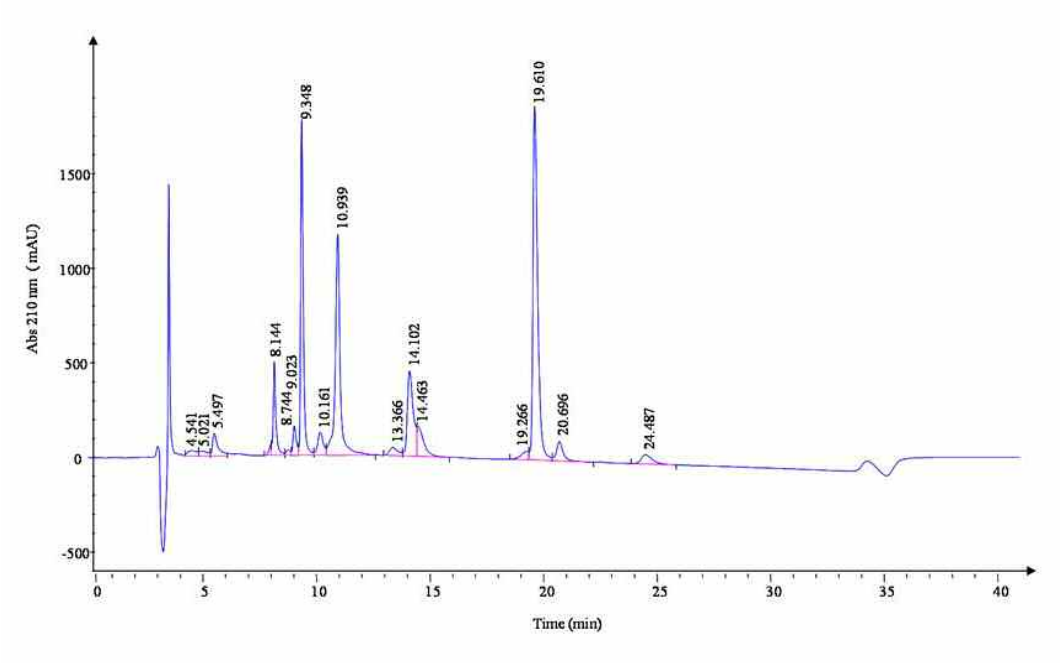


Figure 0.3. *RP-HPLC chromatogram of the unfractionated Semillon juice.*

Fraction	VvTL peaks number	Area %	Chitinases peaks number	Area %	Number of other peaks	Area %	Protein concentration (mg/L BSA)	Total Protein content (mg)
Juice	5	41.7	4	37.3	6	21	1874.1	337.5
1	4	60	1	40	\	\	11.8	1.1
2	2	61	1	39	\	\	18.6	1.1
3	2	70	1	30	\	\	35.1	2.7
4	2	67	1	27	1	6	62.9	6.2
5	1	0.5	1	28.5	1	**71**	129.4	13.6
6	1	0.8	2	**97.5**	1	1.7	110.0	7.5
7	\	\	4	**88**	3	12	115.2	5.2
8	2	2.2	4	**58**	3	39.8	207.8	4.2
9	2	12.2	4	34.4	2	**52.5**	783.9	58.8
10	4	38	3	**59.5**	5	12.5	484.2	98.3
11	4	**87**	1	10.3	2	2.7	74.2	3.0
12	4	**88**	1	4.6	2	7.4	225.6	12.9
13	4	**92**	1	2	1	6	293.8	35.3
14	2	**82**	3	11	1	7	28.0	2.8

Table 0.1. *Protein class, concentration and area percentage of fractions collected after Semillon juice HIC fractionation. In total ≈ 340 mg of protein from Semillon juice were loaded while 252.7 mg were recovered. The chromatograms from which these data were acquired are shown in figure 3.8. Bold fonts indicate the main protein class of each fraction.*

The data of table 3.1 indicated that grape proteins could be divided in three

classes based on their hydrophobicity. Particularly, in the first four fractions proteins were eluted in absence of a gradient. Consequently, this elution occurred in a non-specific way since the proteins did not interact with the resin. HPLC identification indicated that they mainly belong to the thaumatin-like and chitinases classes. However, their concentration represented only a minimal part of the total amount of proteins loaded. Two hypotheses have been formulated to explain this unspecific elution: i) column overloading; ii) a role of polyphenols still present on loaded sample. The second hypothesis seemed the most probable because of some evidence that will be discussed in chapter 4.

Once the gradient started, the chitinases and TL-protein content of the peaks changed during the fractionation, in a rate that was possible to summarize as follows: low thaumatin-like proteins content until fraction 10; a prevalence of chitinases from fraction 6 to 10; a thaumatin-like proteins preponderance on the last four fractions. Hence, in these experimental conditions, chitinases behaved in a less hydrophobic way than thaumatin-like proteins, confirming the preliminary results achieved with the 1 mL kit column. Several fractions were enriched in a particular class of protein in terms of peak area percentage in RP-HPLC. For instance, the chitinase peak area of fractions 6 and 7 was ≈ 90% of the total, while fractions 11, 12, 13 and 14 were mainly consisting of thaumatin like proteins, with a minimum peak area of 82 %.

The two highest HIC fractions (9 and 10) contained a large number of peaks belonging to different protein classes (fig. 3.4). In particular, peak 9 showed four main peaks, recognised as two TL-proteins, one chitinases and an unknown peak. In fraction 10 there were two main peaks recognizable as a chitinases and a TL-protein.

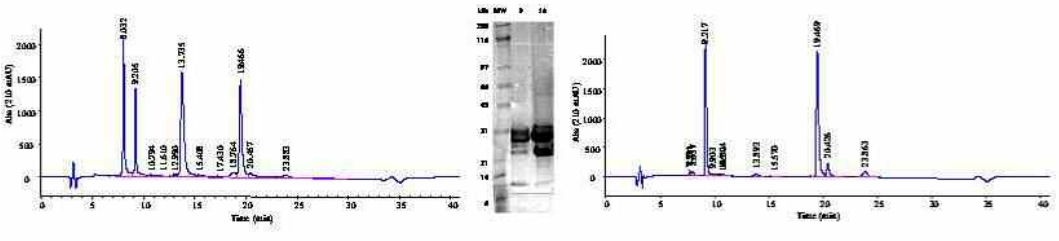


Figure 0.4. *RP-HPLC chromatogram of HIC fraction 9 (left) and 10 (right). In the middle: SDS-PAGE profile of the two fractions.*

It resulted evident the discrepancy in MW and in RT of bands supposed to be TL-proteins, with the lower molecular weight of the TL-proteins of fractions 10 in comparison to that of following fractions (not shown). These differences were attributable to the presence of different TL-proteins isoforms (Peng *et al.*, 1997).

Preliminary SDS-PAGE showed that fractions 12, 13 and 14 contained a protein of about 65 kDa in addition to the TL protein (not shown). This additional higher MW protein was not visible on the RP-HPLC chromatograms (eg fraction 13, figure 3.5).

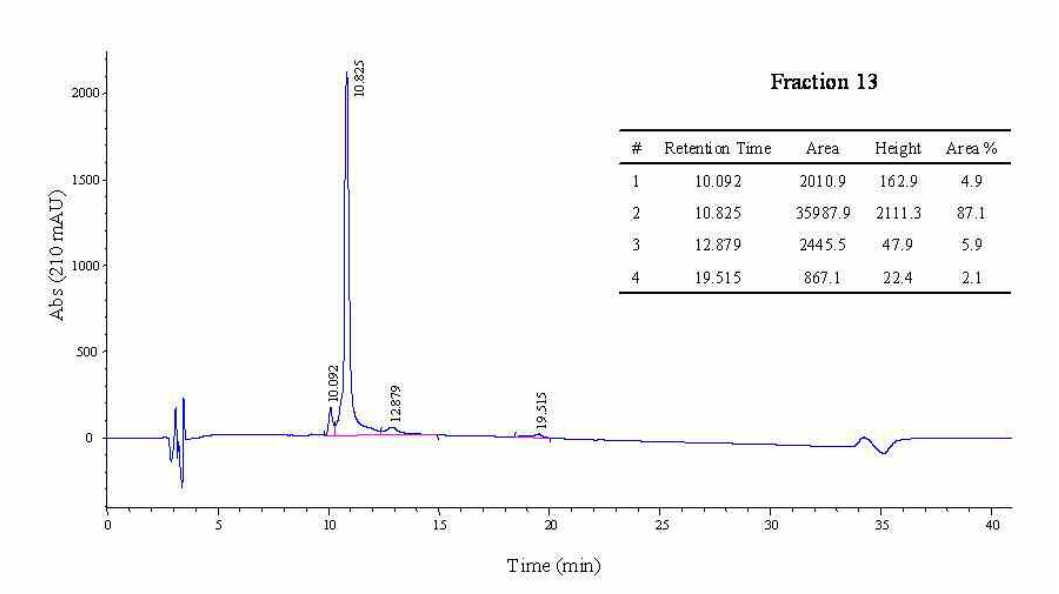


Figure 0.5. *HPLC profile of faction 13 obtained by hydrophobic interaction chromatography. In the table: retention tine, area (absolute value and percentage) and height of each peak detected.*

The chromatogram showed the presence of 1 main peak that was assumed to be a TL-protein with an estimate purity of 87.1%. Two lateral peaks were detected at 10.0 and 12.8 minutes of retention time that were respectively recognised as a minor thaumatin like protein (the first) and an unknown protein the second, probably an invertases as following shown (figures 3.6 and 3.7).

In any case, these data indicated the possibility to nearly purify a TL-protein from grape juice in a one-step preparative process based on the different hydrophobicity characteristics of grape proteins.

To confirm HPLC results, a further SDS–PAGE of fraction 13 was

performed after a desalting step (fig. 3.6). The sample desalting was made in order to eliminate all the ammonia salt from fraction 13 because this fraction was subsequently utilised as a substrate for a microbial growth (data not shown).

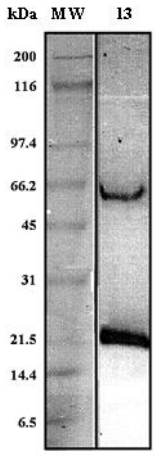


Figure 0.6. *SDS-PAGE (T = 4-20%) on non-reducing conditions of the fraction 13 obtained from the HIC chromatographic separation.*

Unexpectedly, fraction 13, which looked very pure by RP-HPLC, showed two bands instead of one. The band intensity (measured with ImageJ software) of the fraction at about 22 kDa (presumably a VvTL protein) resulted to be the 74.4% while the 65 kDa band (presumably grape invertase) was at the 25.6% of the total band intensity. Because these data disagreed with the RP-HPLC chromatograms, it was necessary to further investigate this fraction's purity. Some authors (Kwon, 2004) reported difficulties on the SDS-PAGE Coomassie staining for VvTL proteins. It appeared possible that a non-proportional staining occurred during the SDS-PAGE analyses. This hypothesis seemed reasonable also because of the high quantity of protein loaded on each lane (50 µg). As a result of the poor staining of the VvTL band, the amount of the presumed invertase in the fraction would be exaggerated.

To certainly identify the nature of these proteins, the two bands were cut from the gel to be analysed by ESI-MS/MS (fig. 3.7). As expected, from Mascot Search results (Matrix science database) the higher band was identified ($p < 0.05$) as a vacuolar invertase 1, GIN1 [*Vitis vinifera*=grape berries, Sultana, berries, Peptide, 642 aa] (gi|1839578) (Davies and Robinson, 1996) while the lower band was classified as a thaumatin-like protein from V*itis vinifera* (gi|33329390)

(Manteau *et al.*, 2002, unpublished).

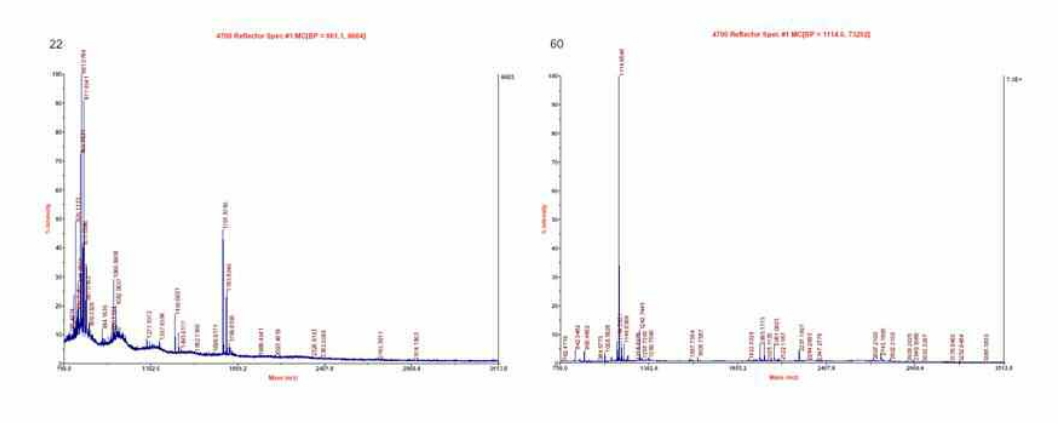


Figure 0.7. *Spectra of the lower SDS-PAGE band (left) identified as a Vitis vinifera thaumatin-like protein and of the higher band (right) identified as a vacuolar invertase 1 of Vitis vinifera.*

A further HPLC analysis was attempted to determine the content of invertase of this fraction. From the peak area obtained by using a size exclusion chromatography column it was possible to quantify the area percentage of the two proteins (fig. 3.8).

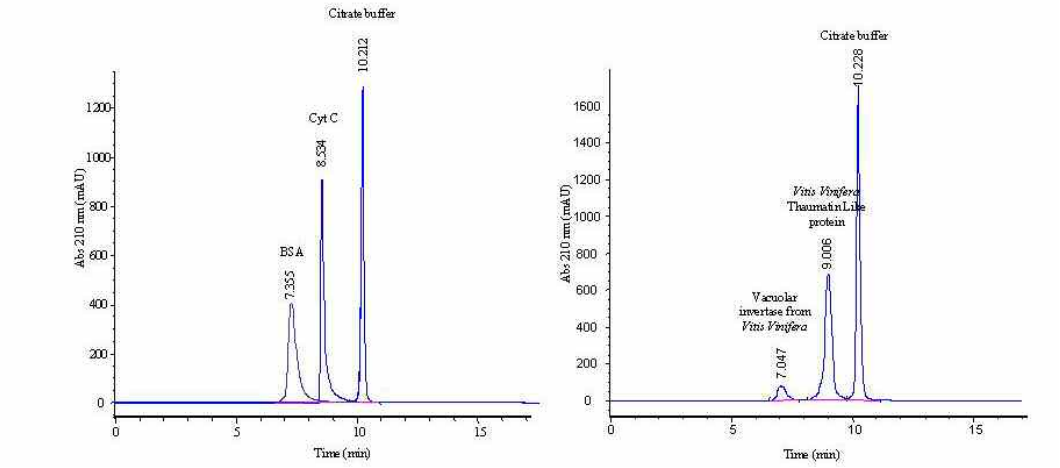


Figure 0.8. *SEC-HPLC chromatograms of fraction 13 separated with Phenomenex BioSep SEC S2000 (7.8 x 300 mm) with guard column at 1 mL/min on 50 mM KH_2PO_4 buffer, room temperature. Left: BSA (20 µL injected at 3 mg/mL concentration on 30 mM citrate buffer pH 3.50) and Cytochrome C (20 µL injected at 2.5 mg/mL concentration on 30 mM citrate buffer pH 3.50) utilized as standards. Right: fraction 13 HIC separation on 30 mM citrate buffer pH 3.50.*

From the peak area it was clear that this fraction was mostly composed of

VvTL (91%). This result confirmed that the Coomassie staining in the SDS-PAGE results overestimated the invertase content and it was in agreement with the previous RP-HPLC data.

1.1.26.1 VvTL protein quantification

To quantify the protein content of fraction 13 (63 mL in total), three different methods were used (tables 3.2 and 3.3):

i. By means of the ratio between fraction absorbance (spectrophotometrically determined at 280 nm) and the VvTL molar extinction coefficient (ε =29230 AU/M);
ii. Through a RP-HPLC analyses and the quantification based on a BSA calibration curve;
iii. By determination of the amino acid content.

ABS 280 nm	RP-HPLC	Amino acids
590 mg/L	621.23 mg/L	484.76 mg/L
35.99 mg/sample	37.89 mg/sample	30.63 mg/sample

Table 0.2. *Comparison of the protein concentration and content of fraction 13 (63 mL) measured with three methods.*

Results obtained were similar and, as stated by other authors (Fountoulakis *et al.*, 1992; Fountoulakis and Lahm, 1998) the amino acids quantification method was assumed as the most accurate, also if a quantification lower than the reality is not to be excluded for causes below discussed.

The table below (tab. 3.3) consider only 17 amino acids because under the conditions of the acidic hydrolysis performed, asparagine and glutamine are completely hydrolyzed to aspartic acid (Asx) and glutamic acid (Glx), respectively. Tryptophan is completely destroyed and cysteine can not be directly determined from the acid-hydrolyzed samples, and this is the reason for its separated determination. Tyrosine is partially destroyed by traces of impurities present in the hydrolysis agent (Fountoulakis and Lahm, 1998). Serine and threonine are partially hydrolyzed as well and usually losses of about 10 and 5%, respectively, occur (Ozols, 1990).

Amino Acid	Amino Acid ($-H_2O$) (μg/mL)[a]	Amino Acid (μg/mL)[b]	Mole (%)	Expected aa composition of TL-protein (% Mole)[c]	Expected aa composition of Invertase (% Mole)[c]
Histidine	5.06	5.70	0.87	0.40	3.00
Serine	26.61	32.35	7.18	7.60	6.70
Arginine	20.55	22.90	3.09	2.20	4.00
Glycine	29.81	39.20	12.27	9.80	7.90
Asx[e]	68.50	79.20	13.98	12.90	12.50
Glx[f]	32.56	37.10	5.92	5.40	7.00
Threonine	50.24	59.15	11.67	10.20	6.70
Alanine	26.62	33.35	8.79	8.90	6.70
Proline	26.00	30.80	6.29	5.80	6.20
Lysine	16.72	19.05	3.06	2.70	2.50
Tyrosine	29.25	32.45	4.21	3.60	4.50
Methionine	7.22	8.20	1.29	1.30	2.60
Valine	24.02	28.40	5.69	4.40	7.60
Isoleucine	13.12	15.20	2.72	2.70	4.80
Leucine	28.33	32.85	5.88	6.20	9.70
Phenylalanine	44.53	49.95	7.10	7.10	3.90
Cysteine[d]	33.65	41.9	n.d.	7.60	0.80
Total	484.76	567.75	100.00	98.80	97.10

[a] Calculation based on amino acid residue mass in protein (molecular weight minus H_2O).
[b] Calculation based on free amino acid molecular weight.
[c] Amino acid composition from the computation of the complete protein sequence obtained from the ProtParam tool of the ExPASy Proteomics Server
[d] Cysteine was separately determined
[e] Results deriving from the addition of asparagine and aspartic acid.
[f] Results deriving from the addition of glutamine and glutamic acid.

Table 0.3. *Amino acids composition of HIC fraction 13. 17 amino acids have been quantified in HIC fraction 13, while the expected amino acids composition of invertase and TL-protein is expressed as a molar percentage on 20 amino acids.*

Waters and colleagues (1992) purified a heat unstable protein with MW of 24 kDa that was following identified as a thaumatin-like protein (Waters *et al.*, 1996). The authors showed that this protein contained large amounts of aspartic acid, glycine, alanine, serine, threonine, phenylalanine and tyrosine. These results strongly agree with those of the table above. However, some differences from the comparison between the amino acids composition of HIC fraction 13 and the expected amino acid composition of the thaumatin-like protein were highlighted. In particular, fraction 13 showed a higher amount of histidine, arginine, proline and tyrosine than the expected. These differences seem attributable to the invertases contamination that, as visible from its expected amino acids content, could shift the molar percentage of some amino acid. Besides, the serine percentage resulted slightly lower than the expected. As previously discussed, the

amino acid quantification by acid hydrolysis can result in an underestimation of the level of this amino acid because of its fragility (Simpson et al., 1976; Ozols, 1990). In addition, several amino acids (alanine, proline, lysine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine and cysteine) were detected in amounts very similar to the expected. According to Waters *et al.* (1992), the amount of three amino acids (Asx, Glx and Threonine) resulted a little higher in HIC fraction 13 than the predicted, and this occurrence cannot be explained by the invertase contamination. It seems possible that these disagreeing results are due to some post-translational modification that is not taken into account in the amino acids computation of table 3.3. Looking at the glycine content of the sample, it resulted sensibly higher than the expected and not influenced by the invertase. It is possible that a chitinase contamination led to this content. In fact, the chitinase impurity (2.1%) present in this fraction (see fig. 3.5) could explain this occurrence because of the high glycine content of this protein (13%, not shown).

In general, the results formerly discussed are in accord with the observation that the most represented amino acids in grape juice are aspartic acid, threonine and phenylalanine (Yokotsuka and Singleton, 1997). Besides, a relevant amount of proline was detected. Several authors reported that polypeptides containing proline are able to form haze when combined with phenolic compounds (Asano *et al.*, 1982; Siebert *et al.*, 1996). This observation agrees with the assumption that PR-proteins in general and VvTL in particular are the main haze-forming proteins in white whines (Høj *et al.* 2000; Tattersall *et al.* 2001; Ferreira *et al.* 2002).

The whole fraction 13 (63 mL) was concentrated by using a stirred ultrafiltration cell system (Amicon) equipped with membranes with porosity of 3000 MWCO. The concentrated sample was washed with 30 mM citrate buffer (pH 3.50) in order to eliminate all the residual ammonia salt. The absence of ammonia was detected using an ammonia kit (not shown). The protein content of the concentrated sample, assessed spectrophotometrically, was 8.7 mg/mL with a total protein content of 30.26 mg (tab. 3.4).

Stage of purification	Protein concentration (mg/L)	Total protein content (mg)	Yield (%) on total protein	% Purity (by HPLC)
TL-protein (10.9 min RT) in HIC fraction 13	255.9	35.30	100	87.1%
Desalting – APAF quantification	484.76	30.63	86.77	90%
Sample concentration (3000 MWCO)	8700	30.26	85.72	91%

Table 0.4. *Fraction 13 (VvTL) protein recovery during the purification steps.*

The method previously described led to the partial purification of a thaumatin like protein in only one chromatographic step. Preliminary results show that by coupling the HIC fractionation with a cationic exchange chromatography, it is possible to solve the invertase impurity on fraction 13 (data not shown). Besides, these results showed the chance to improve the capability to purify other grape proteins with the reaching of the purification of 5 proteins that represented more than the 50% of total grape protein content (Van Sluyter *et al.*, 2007).

1.1.27 SEMILLON GRAPE PROTEINS IDENTIFICATION

The fractions collected from HIC fractionation were further analysed in order to establish their protein composition. Therefore, 14 fractions plus a whole juice sample were analysed by RP-HPLC (fig 3.9; tab. 3.1).

In total 13 peaks with different RT were detected by HPLC in the whole juice and after HIC fractionation. Therefore, it was possible to create a table summarizing, for each HPLC retention time, the HIC fractions in which the same peaks have been detected (tab. 3.5).

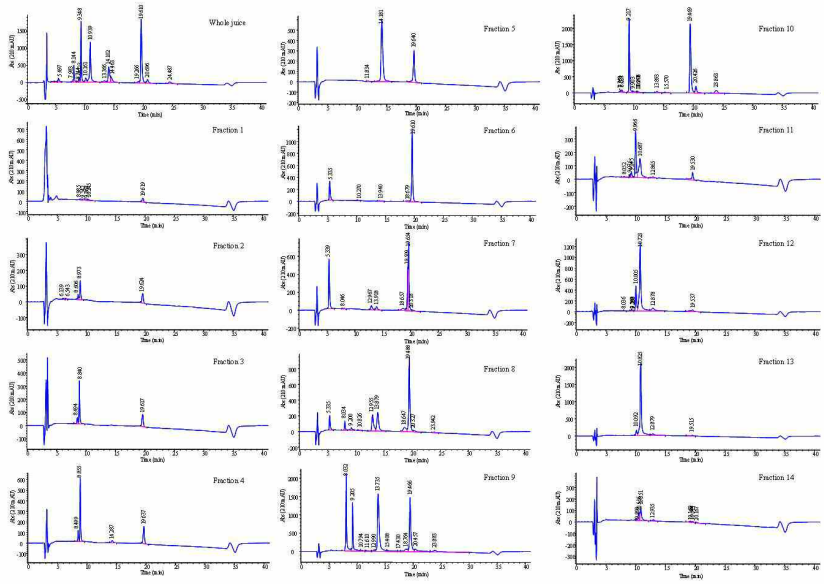


Figure 0.9. *RP-HPLC chromatograms of Semillon juice fractionated by HIC chromatography.*

RT (minutes) protein in whole juice	HIC fractions in which it was present
5.5	6-**7**-8-9
8.1 (8.0)	8-**9**-10-11
9.0 (8.9)	1-**2**-**3**-**4**
9.3	8-9-**10**-11
10.1	**12**-13
10.9	11-12-**13**-14
13.3 (12.8)	7-**8**-9-10-11-12-13-14
13.9	6-7-8-**9**-10
14.1	4-**5**
18.6	7-8-9-10
19.5 (19.6)	In every fraction
20.4	7-8-9-**10**
24.5 (23.9)	8-9-**10**

Table 0.5. *Peaks retention time of the whole Semillon juice and fraction in which they are detectable after HIC chromatography (between brackets there are the equivalent RTs obtained with a slightly different HPLC set up). Bold fonts indicate the main protein class of each fraction.*

The table summarised how grape proteins were fractionated by HIC. The peak distribution was, for all the RT considered, in adjacent HIC fractions, indirectly confirming the appropriateness of Phenyl Sepharose® HP resin for grape protein fractionation.

In detail, it seemed that the peak with 9.0 min of RT was eluted in a

similar way in the first 4 fractions in which no elution buffer was present. Consequently, this protein was not bound by the resin.

Once the elution gradient started, the first protein eluted had RT of 14.1 and came out in presence of low concentration of the elution buffer (fractions 4 and 5) suggesting a very low hydrophobicity degree for this protein.

A HPLC protein peak with RT 5.5 min was detectable in fractions 6 to 9, with a prevalence on fraction 7. The elution of this protein started with high salt concentration, showing its low-medium hydrophobicity.

A similar hydrophobicity was observed for protein with 18.6 min of RT, with its major concentration in HIC fraction 8. In the same fraction (8) the peak with RT 13.3 min was mainly recoverable, also if its presence was detected from fraction 6 to the end of the separation. As afterward demonstrated, this protein seemed to belong to the invertases classes, and the causes of this spreading will be later discussed.

Fraction 9 and 10 were those with the larger number of protein classes. In particular, protein with RT of 8.1 and 13.9 were mostly eluted on fraction 9 while protein with RT of 9.3, 19.5, 20.4 and 24.5 were mainly eluted on fraction 10.

As previously discussed, a TL-protein was mainly contained in the last part of the separation (from fraction 11). In those peaks, besides the main TL peak (RT 10.9 min) only two other peaks have been detected: a peak at 10.1 min mainly contained on fraction 12 and a peak at 19.5 min (presumably chitinases) that was observed in every HIC fraction.

Two protein peaks did not show a fractionation behaviour in agreement with the hydrophobicity. Peaks at 13.3 and 19.5 min of RT were detected respectively in fractions 7 to 14 and in all the fractions considered, respectively, showing a not strictly hydrophobic dependant elution. This occurrence need to be further investigated.

1.1.27.1 ESI-MS/MS Protein identification

Okuda and colleagues (2006) affirmed that more than 310 protein or polypeptide fractions were recoverable in Japanese Chardonnay wines belonging to the thaumatin and osmotin-like proteins, invertase, lipid transfer proteins and their hydrolysis products.

In order to identify the origin of Semillon grape proteins, 5 HIC fractions (4, 5, 9, 11 and 13) were chosen because of their protein content representative of all the HPLC retention time detectable in the whole juice. Three consecutive HPLC fractionation for each of these fractions were made and peaks, collected with a fraction collector, were pooled. In total, 12 different peaks were obtained and, after concentration and equilibration with the loading buffer, a SDS-PAGE in non-reducing conditions (fig. 3.10) was performed. Proteins have been revealed by MS-compatible stains (Bio-Safe™ Coomassie stain or Silver stain procedure).

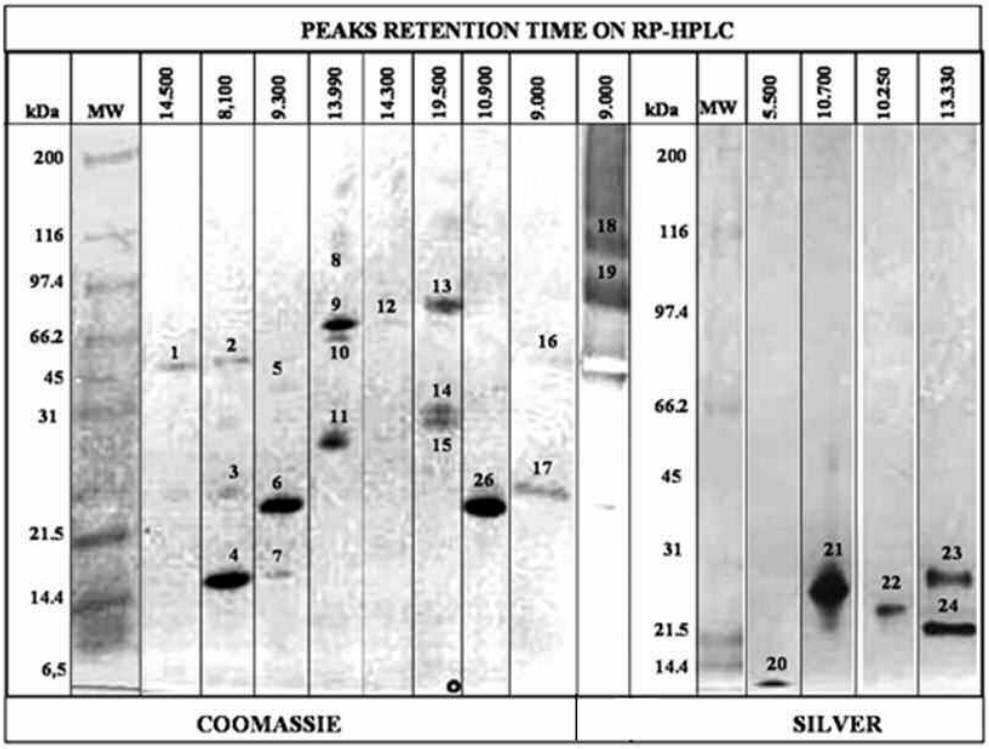


Figure 0.10. *SDS-PAGE of peaks collected by RP-HPLC. Bands were cut and analysed by ESI-MS/MS. SDS-PAGE analyses was performed according to Laemmli (1970) by using Ready Gel Tris-HCl Gel [Bio-Rad, 4–20%, 15-well, 15 µl, 8.6 x 6.8 cm (W x L)], in absence of reducing agents. RP-HPLC peaks characterized by a high protein concentration were stained with Bio-Safe® Bio-Rad Coomassie. RP-HPLC peaks with low protein content were stained with silver procedure. 26 samples in total. The sample 25 (RT 24.500) was not loaded on the gel but analysed in liquid form. Sample 26 corresponded to the VvTL protein (gi|33329390) (already identified by APAF during the VvTL protein purification of fraction 13). Sample 9.000 was first stained with Coomassie, than two bands were excised before the lane was re-stained with silver that allowed the appearance of other two bands (18 and 19).*

Unexpectedly, the SDS-PAGE showed the appearance, in some lanes, of more than one band that derived from the HPLC peak collection. Hence, a HPLC peak did not correspond to a single protein, and consequently band were analysed via LC-MS/MS analysis to understand their nature.

Results of 26 samples are summarised in table 3.6.

Spot name	HIC fraction	RP-HPLC RT	Apparent SDS-PAGE MW	Accession number	1st X! Tandem significant ID	pI	MW	2nd X! Tandem significant ID	pI	MW
1	5	14.555	50	unknown	no matches	/	/	no matches	/	/
2	9	8.100	53	gi|33414046|	class IV chitinase [Galega orientalis]	7.42	29.4	PR-4 type protein [Vitis vinifera]	5.5	15.2
3	9	8.100	24	gi|33414046|	class IV chitinase [Galega orientalis]	7.42	29.4	PR-4 type protein [Vitis vinifera]	5.5	15.2
4	9	8.100	15	gi|3511147|	PR-4 type protein [Vitis vinifera]	5.5	15.2	/	/	/
5	9	9.300	40	gi|7406716|	putative thaumatin-like protein [Vitis vinifera]	4.94	24	/	/	/
6	9	9.300	22	gi|7406716|	putative thaumatin-like protein [Vitis vinifera]	4.94	24	VVTL1 [Vitis vinifera]	5.09	24
7	9	9.300	16	gi|7406716|	putative thaumatin-like protein [Vitis vinifera]	4.94	24	PR-4 type protein [Vitis vinifera]	5.5	15.2
8	9	13.990	100	gi|33414046|	class IV chitinase [Galega orientalis]	7.42	29.4	/	/	/
9	9	13.990	70	gi|33414046|	class IV chitinase [Galega orientalis]	7.42	29.4	/	/	/
10	9	13.990	65	gi|33414046|	class IV chitinase [Galega orientalis]	7.42	29.4	/	/	/
11	9	13.990	27	gi|116329|	Endochitinase A precursor (Seed chitinase A) [Zea mais]	8.3	29.1	/	/	/
12	11	14.300	70	gi|116329|	Endochitinase A precursor (Seed chitinase A) [Zea mais]	8.3	29.1	/	/	/
13	11	19.560	80	gi|33329392|	class IV chitinase [Vitis vinifera]	5.38	27.5	/	/	/
14	11	19.560	31	gi|33329392|	class IV chitinase [Vitis vinifera]	5.38	27.5	/	/	/
15	11	19.560	29	gi|33329392|	class IV chitinase [Vitis vinifera]	5.38	27.5	/	/	/
16	4	9.000	55	gi|30679715|	early-responsive to dehydration protein-related / ERD protein-related [Arabidopsis thaliana]	8.68	87.6	class IV chitinase [Vitis vinifera]	5.38	27.5
17	4	9.000	24	gi|7406671|	putative ripening-related protein [Vitis vinifera]	4.83	22.9	/	/	/
18	4	9.000	110	unknown	no matches			no matches	/	/
19	4	9.000	90	unknown	no matches			no matches	/	/
20	9	5.570	10	gi|28194084|	lipid transfer protein isoform 1 [Vitis vinifera]	9	11.6		/	/
21	11	10.000	25	gi|33329390|	thaumatin-like protein [Vitis vinifera]	4.67	23.9	/	/	/
22	13	10.250	23	gi|33329390|	thaumatin-like protein [Vitis vinifera]	4.67	23.9	/	/	/
23	13	13.330	26	gi|1839578|	vacuolar invertase 1, GIN1 [Vitis vinifera=grape berries, Sultana, berries, Peptide, 642 aa]	4.6	71.5	/	/	/

24	13	13.330	22	gi|33329390|	thaumatin-like protein [Vitis vinifera]	4.67	23.9	/	/	/
25	9	24.500	Not loaded	unknown	no matches			no matches	/	/
26	13	10.970	23	gi|33329390|	thaumatin-like protein [Vitis vinifera]	4.67	23.9	osmotin-like protein [Vitis vinifera]	4.56	23.9

Table 0.6. *Summarizing table of the ESI-MS/MS protein identification in comparison to the HIC fraction from which they were purified and with the retention time with which they came out from the HPLC separation.*

In table 3.6, an ID is associate to each band. From the alignment of the raw data with X!Tandem database, it seemed that only 14 samples on 26 are from *Vitis vinifera*. The number of sample identified as *Vitis vinifera* protein rises to 17 considering the 2nd match recoverable in X!Tandem database.

About the 19% of the total proteins from grape berry mesocarp belong to the PR-protein category (Sarry *et al.*, 2004). From the identification of SDS-PAGE protein spots by ESI-MS/MS it was possible to identify mainly PR-proteins such as PR-4 type proteins, putative thaumatin-like proteins, class IV chitinase, putative ripening-related protein, lipid transfer protein isoform 1, thaumatin-like protein and a vacuolar invertase 1, GIN1. Besides, other protein of no grape origin have been detected, mainly belonging to the *Arabidopsis thaliana*, *Galega orientalis* and *Zea mays* species and presenting high homology with grape PR-proteins, mainly chitinases. It is worth mentioning the absence of microbial proteins, indicating the healthiness of the grape used. The non-total *Vitis vinifera* origin of the protein analysed is related to the database quality utilised for the alignment tests. In fact, preliminary results confirmed this hypothesis with the identification of other proteins of grape origin, mainly hypothetical protein from V*itis vinifera* (data not shown) that were not included in the database used for the first protein recognition (The French-Italian Public Consortium for Grapevine Genome Characterization, 2007).

Putative thaumatin-like protein [*Vitis vinifera*] and Thaumatin-like protein [*Vitis vinifera*]: the thaumatin-like proteins are, after the chitinases, the most represented grape and wine protein (Waters *et al.*, 1998, Pocock *et al.*, 2000; Hayasaka *et al.*, 2001). This statement is confirmed in the results above shown, in which 10 spots were recognised as chitinases while 7 as TL-proteins. Particularly,

three spots (5, 6 and 7) were identified as putative thaumatin-like proteins while four (21, 22, 24 and 26) as thaumatin-like proteins. These two thaumatin classes differ in both HPLC retention time (respectively 9.3 min and 10.0 to 13.3 min) and hydrophobicity characteristics (higher for the thaumatin-like proteins). It is to be noted that the three putative thaumatin-like protein bands derived all from the same SDS-PAGE lane. Particularly, the HPLC peak (9.3 min) showed the appearance of three bands in SDS-PAGE respectively at 40, 22 and 16 kDa of apparent MWs. The theoretical MW of this protein is 24 kDa, so the two other bands are likely modification of it, which nature needs to be further investigated.

Spots 21, 22, 24 and 26 were all identified as the same thaumatin-like protein. This occurrence could be explained in two ways: i) the presence of different TL-proteins isoforms in our samples recognised as the same protein in the database; ii) a partial modification of the same thaumatin-like protein that resulted in a changing of properties as its MW or hydrophobicity. The second hypothesis seems supported by data obtained by Pocock and colleagues (2000), which showed the presence of a main VvTL protein and a minor TL protein that behaved, in HPLC, similarly respectively to spot 26 and 24. Accordingly, our experimental data suggested the second hypothesis as the most probable. In particular, we retain that the thaumatin-like protein could be incurred in some modifications, likely proteolysis. A certain content of endogenous proteolytic activity is detectable in musts (Cantagrel *et al.*, 1982; Ribéreau-Gayon et al., 2003), but this activity is not sufficient to degrade the highly resistant PR-proteins (Waters *et al.*, 1992). However, these enzymes could be responsible for a partial modification of some peptides, resulting in a slightly different behaviour of the protein during the fractionation processes. In fact, the four thaumatin-like protein bands identified presented similar, but not identical, hydrophobicity. Besides, in HPLC this protein showed up at four RTs, while by SDS-PAGE showed apparent MWs from 22 to 25 kDa.

The same discussion might explain also the appearance of three bands in the same SDS-PAGE lane from only one HPLC peak (spot 5, 6 and 7) formerly discussed.

PR-4 type protein [*Vitis vinifera*]: this class of protein was mostly eluted in HIC fraction 9 and showed a HPLC RT of 8.1 min. Generally, the PR-4 class of

protein is mainly composed by chitin binding proteins (Theis and Stahl, 2004). These proteins present antifungal activity that is mainly due to their ability to bind fungal cell wall chitin (Bormann *et al.*, 1999). The presence of this class of protein in grapevine seems consequently due to some antifungal mechanism of the plant, also if Tattersall and colleagues (1998, unpublished) attributed to this class a ripening-related role. PR-4 proteins behaved, in SDS-PAGE, similarly to the putative thaumatin-like proteins, with the appearance of 3 bands at different MWs on the same lane, recognised as the same protein (spots 2, 3 and 4). Actually, only one of those spots resulted a PR-4 protein, while the other two (those with MWs not compatible with the theoretical MW of 15.2 kDa) resulted more similar to a class IV chitinases. This occurrence might be explained by the organization of the PR-4 family of proteins, which is similar to that of the plant chitinase family (Friedrich *et al.*, 1991). Moreover, Van Damme and colleagues (1999) showed the existence of hevein-like chitin-binding protein isolated from mature elderberry fruits (*Sambucus nigra*). The authors demonstrated that this protein was synthesized as a chimeric precursor consisting of an N-terminal chitin-binding domain and an unrelated C-terminal domain. Sequence comparisons indicated that the N-terminal domain had high sequence similarity with the N-terminal domain of class I PR-4 proteins, whereas the C terminus was most closely related to that of class V chitinases. This finding contributes to better explain the recognition of spots 2 and 3 as chitinases instead of PR-4 proteins, also if the reasons for their apparent MW in SDS-PAGE warrants further investigations.

C**lass IV chitinase [*Vitis vinifera*]:** among the 26 samples analysed, 10 were identified as chitinases as first X!Tandem match plus one identified as second database match. These results confirmed the predominance of chitinases in grape proteins highlighted by other authors (Waters *et al.*, 1998, Pocock *et al.*, 2000; Hayasaka *et al.*, 2001). By observing the HIC distribution of chitinases spots, it can be observed that most of these proteins were eluted in the middle of the HIC gradient (HIC fraction 9 and 11). Particularly, spots from 8 to 15 were all recognised as proteins with chitinase function. However, these eight bands did not behave similarly in SDS-PAGE in which a great MW variability in a range from ≈ 97 to ≈ 28 kDa was detectable. This SDS-PAGE mobility might be partially explained by the absence of reducing agents in the SDS-PAGE loading buffer,

which could be related to major differences in the hydrodynamic volumes of the protein deriving from structures stabilized by S-S bonds, although a different binding of the unreduced protein to the detergent SDS seems also possible (Vincenzi and Curioni, 2005). However, these notions seem not sufficient to explain these great differences in MWs, thus additional investigations are required.

Putative ripening-related protein [*Vitis vinifera*]: one spot (17) was identified as a putative ripening-related protein. This protein presented a very low hydrophobicity because it was eluted in HIC fraction 4, so before the gradient started. Besides, its HPLC RT (9.0 min) was similar to those of the putative thaumatin like proteins. This occurrence seemed explained by the slight difference in pI between this protein (4.83) and the thaumatin one (4.94), resulting in a slightly different RT in HPLC. Besides, the apparent MW in SDS-PAGE of this protein resulted higher than the hypothetical (≈ 25 instead of 22.9 kDa).

Lipid transfer protein isoform 1 [*Vitis vinifera*]: in literature there are two closely-related types of nsLTPs, types 1 and 2, which differ in protein sequence, molecular weight, and biological properties (Cheng *et al.*, 2004). Many nsLTP1 proteins, also of grapevine origin, have been characterized as allergens in humans (Pastorello *et al.*, 2002). The LTP here identified belong to the isoform 1 and showed very low MW in SDS-PAGE and early RT in HPLC, in which it was well separated from the other proteins. HIC fractionation also showed a good LTP separation ability as it was mainly eluted in fraction 7.

Vacuolar invertase 1, GIN1 [*Vitis vinifera*=grape berries, Sultana, berries, Peptide, 642 aa]: some authors (Davies and Robinson, 1996; Sarry *et al.*, 2004) highlighted the preferential expression of the GIN1 isogene with respect to the GIN2 in the pericarp, confirming the results here described. Band 23, recognised as a vacuolar invertase, did not show a MW of about 70 kDa as expected, but its apparent MW was of 28 kDa. Okuda and co-workers (2006), by using 2D-PAGE have noticed the presence of invertase fragments on Chardonnay wine with MWs of 39, 38 and 29 kDa, highlighting for the first time the presence of hydrolysed invertases in wine. However, in this study, proteins were derived from grapes. Consequently, the observed invertase hydrolysis could not have been due to the fermentation process as suggested by Okuda, but might be due to some

endogenous proteolytic activity in musts as discussed for the thaumatin-like proteins. The fragmentation of invertases could also explain the spreading of this protein throughout 8 HIC fractions. As observed in figures 3.5 and 3.6 in the studied Semillon juice, a protein with an apparent MW of ≈ 65 kDa was identified as a vacuolar invertase 1, GIN1 [*Vitis vinifera*=grape berries, Sultana, berries, Peptide, 642 aa] (gi|1839578), leading to the hypothesis that the hydrolyses occurred after the HIC fractionation step.

Unidentified spots: sample 1 isolated from HIC fraction 5 did not show any match on X!Tandem. The same behaviour was observed for samples 18, 19, 25, even though preliminary results obtained in another database (Blastp) suggested some correspondences with *Vitis vinifera* proteins. Particularly, it seemed that spots 1, 18 and 19 were Hypothetical proteins, while the sample 25 seemed more likely to be a β 1-3 glucanases (data not shown).

In order to graphically summarise the results discussed above, associations between both protein RP-HPLC retention time and hydrophobicity with the protein identities were performed (figures 3.11 and 3.12).

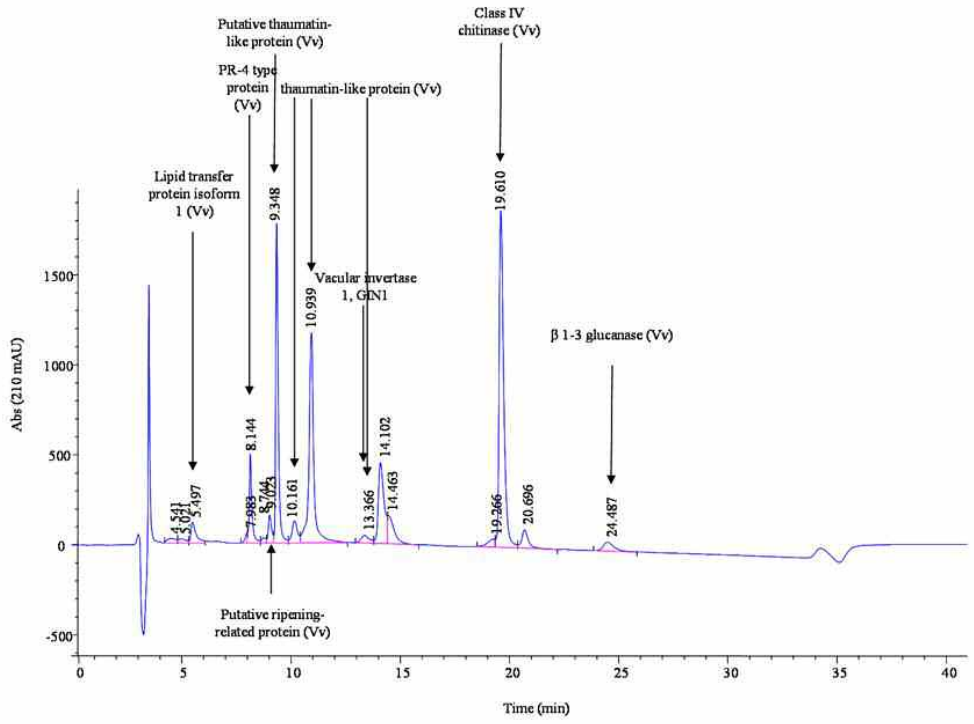


Figure 0.11. *RP-HPLC chromatogram pf whole Semillon juice with protein ID after X!Tandem database search.*

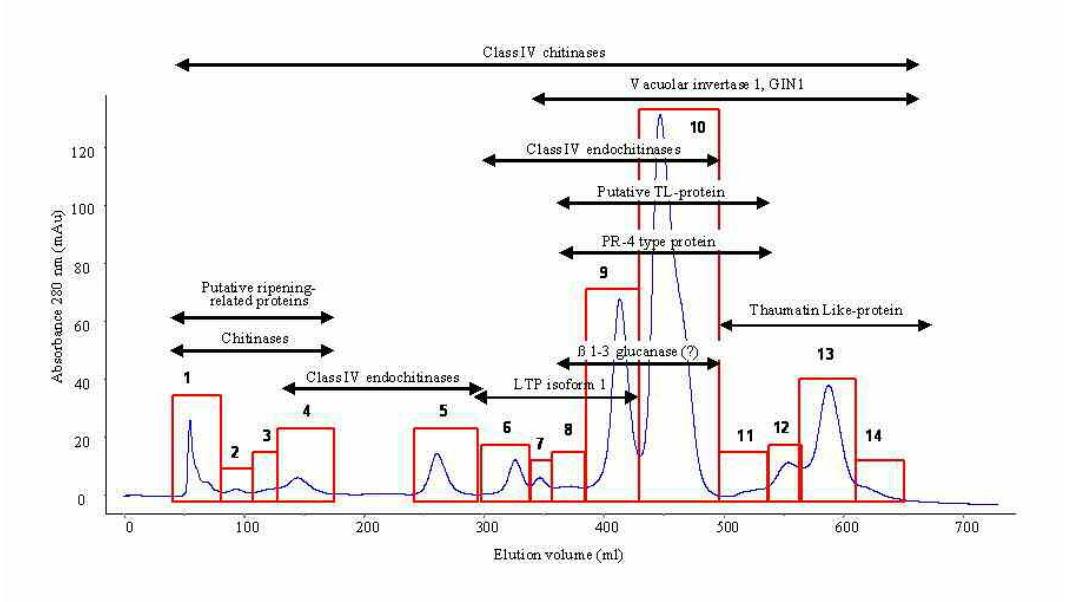


Figure 0.12. *Nature of proteins fractionated during HIC chromatography.*

From the general schemes above represented, it was possible to make some remarks:

- Putative ripening-related proteins and chitinases seem the grape proteins less influenced from the hydrophobicity. Chitinases seemed not to be affected by the resin because the elution occurred all along the gradient. The elution appeared to be not specific, also if the main chitinases concentration was detectable from fraction 5 to 10, while in the last 4 fractions chitinases were detectable only in traces. This phenomenon could be ascribed to the presence of several classes of chitinases (Waters *et al.*, 1998) that can assume a different behaviour during the gradient and/or to a fragmentation of this protein that led to its modified hydrophobicity.
- After the chitinases, the second protein more spread along the HIC fractionation was the vacuolar invertase that started to be detectable from fraction 7 until the end of the gradient. It appears that this protein was initially precipitated by the resin, while with the reaching of an ammonium sulphate concentration of about 0.8 M in the buffer, a re-solubilization of the invertase started and continued until the end of the gradient. The causes of this event

need to be investigated. We hypothesize that an invertase glycosilation resulting in an interaction with the resin could be a possible cause. Besides, an invertase fragmentation due to proteolysis as suggested by Okuda *et al.* (2006) could also explain this phenomenon. It is noteworthy that, at RP-HPLC retention time of 13.3 min were associated both invertases and thaumatin-like proteins. This fact could further explain the difficulty in obtaining a TL-protein without invertase contamination discussed in section 3.4.

- Some proteins were eluted in a small chromatogram zone, such as the Lipid Transfer Protein isoform 1, detected in the middle of the fractionation and mainly in fraction 7. This result was unexpected because the LTP is meant to be a very hydrophobic protein. In fact, LTP is known to have a hydrophobic pocket that endows them with the capacity to bind hydrophobic molecules (Blein *et al.*, 2002).
- A thaumatin-like protein (gi|33329390) resulted the most hydrophobic protein in Semillon grape juice. This fact differentiates this protein class from all the other, bringing to its high purification grade already after the HIC fractionation.

Results previously discussed highlighted that HIC chromatography can be used for grape protein fractionation and that it is a tool for partial or total protein purification. By coupling this technique with RP-HPLC analyses, SDS-PAGE and ESI-MS/MS it was possible to identify a large number of grape proteins and to obtain preliminary results on their hydrophobic characteristics.

1.1.28 SEMILLON WINE PROTEIN FRACTIONATION

To validate the data collected on Semillon grape proteins in the previous part of this work, the HIC method proposed was tested for its protein fractionation ability of Semillon wine.

To this aim, proteins from several litres of an unfined Semillon wine were

precipitated with ammonium sulphate (80% saturation) and collected by centrifugation. The protein recovery was unexpectedly low, with a total amount of protein precipitated of 145.4 mg/L (58.15 mg in total). After equilibration with the loading conditions, precipitated wine proteins were injected into the HIC column. The resulting chromatograms (fig. 3.13) showed a high similarity with that of the Semillon juice.

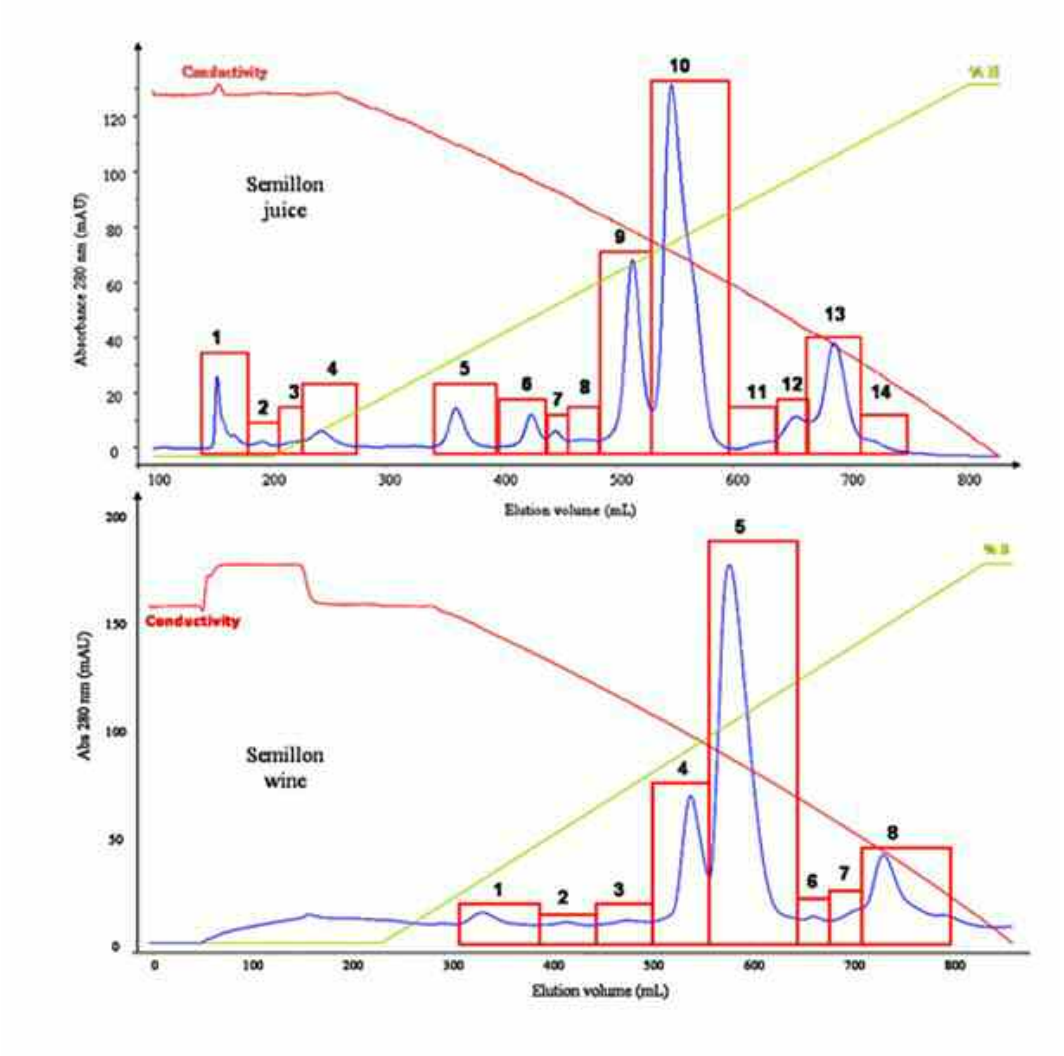


Figure 0.13. *Semillon juice and wine chromatograms of protein fractionation by hydrophobic interaction chromatography.*

Three consecutive and almost identical fractionations were performed. Consequently, fractions collected were pooled prior to be analysed by RP-HPLC (fig. 3.14; tab. 3.7) to determine both their protein composition and concentration. Differently to the juice fractionation, only 8 fractions were obtained. As reported

by several authors (Murphey *et al.*, 1989; Dizy and Bisson, 2000; Fukui *et al.*, 2003), a protein simplification can occur during the fermentation. The main differences were noticed in the first half of the gradient, in which the less hydrophobic proteins were eluted. As shown in the juice chromatogram, only small amounts of protein were detected in the first part of the fractionation. This behaviour could indicate that, during the fermentation, the less hydrophobic proteins were more affected than the most hydrophobic (mainly Chitinases and TL-proteins), which seemed not to be subjected to significant modifications during this process (Waters *et al.*, 1992).

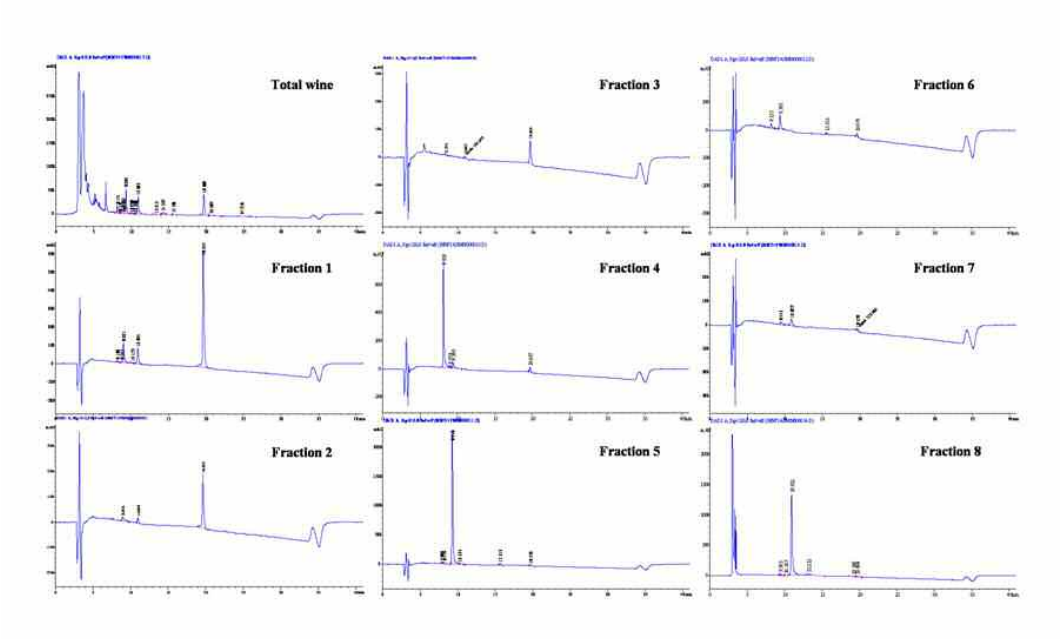


Figure 0.14. *RP-HPLC chromatograms of fractions collected through HIC Semillon wine protein fractionation.*

Fraction	VvTL peaks number	Area %	Chitinases peaks number	Area %	Number of other peaks	Area %	Protein concentration (mg/L BSA)	Total Protein content (mg)
Wine	6	52.1	3	30.9	5	17.0	145.4	58.15
1	3	24.5	1	**74**	2	1.5	78.5	9.8
2	2	20.2	1	**79.8**	1	11	24.9	2.1
3	1	8.5	1	**85.2**	1	6.2	7.8	0.9
4	2	11.7	1	5.7	1	**82.6**	53.9	6.7
5	1	**93**	1	0.9	3	4.3	171.1	13.7
6	1	**56.1**	1	16.1	2	27.7	7.0	0.4
7	2	**86**	1	14	/	/	4.8	0.7
8	3	**93.4**	2	2.8	1	3.8	132.6	14.6

Table 0.7. *Protein class, concentration and area percentage of fractions collected after Semillon wine HIC fractionation. In total 58.15 mg of protein were loaded while 48.9 mg of protein were recovered. Bold fonts indicate the main protein class of each fraction.*

On first inspection, the results showed a different protein fractionation in comparison to the Semillon juice. In wine, chitinases were mainly eluted in the first three fractions whilst in juice (table 3.1), the main chitinases concentration was observed on fractions 6, 7 and 8. Looking at the conductivity in which these fractions were eluted, however, they were comparable to fraction 1 to 3 of the wine HIC fractionation. For wine, the larger thaumatin-like protein content was observed from fraction 5 to 8. Particularly, fraction 5 (corresponding to fraction 10 of juice fractionation) had a TL-protein eluted in HPLC at 9.2 min of retention time. This retention time was different to that of the TL-protein eluted in fraction 8 (RT 10.9 min) possibly due to different VvTL classes of the two fractions. In fact, from the comparison with fig. 3.11, these proteins were likely to be a putative thaumatin-like protein and a thaumatin-like protein, respectively. These assignments agreed with those observed on juice fractionation in which a TL-protein with 9.3 min of RT was observed in fraction 10, while a TL-protein with 10.9 min RT was detected in fraction 13.

These data suggested that fermentation did not affect the main grape TL-protein classes, although a certain effect was detected for chitinases. In fact, passing from juice to wine, the number of chitinases peaks decreased, suggesting a possible role of fermentation conditions or yeast proteases on this phenomenon (Murphey *et al.*, 1987; Dizy and Bisson, 2000; Fukui *et al.*, 2003). Data showed that, in wine peaks 4 and 5, TL-proteins were the main represented classes. On the contrary, in the same peaks chitinases resulted poorly present, while in the equivalent juice peaks (fraction 9 and 10) were largely represented.

In general, results suggest that no modification in PR-protein hydrophobicity occurred during the winemaking, simply the number of PR proteins in total and thus the complexity of the PR protein profile was reduced.

In order to confirm the results achieved during the thaumatin-like protein purification from Semillon juice, a TL-protein purification was attempted also for

the wine. As above mentioned, HIC fraction 8 contained a TL-protein almost identical of that purified in juice HIC fraction 13 (see paragraph 3.4.2). Proteins of fraction 8 were precipitated through the addition of ammonium sulphate (99% of saturation) and the pellet dissolved in citrate buffer. The fraction so prepared was desalted by means of a 20 mL column containing Bio-Gel® P-10DG gel. The absence of ammonia was assessed by means of an ammonia kit (data not shown). The desalting was performed because the purified TL-protein was subsequently utilised as a substrate for a microbial growth (data not shown).

The protein content of the eluate after the desalting step was assessed spectrophotometrically (tab. 3.8).

Stage of purification	Protein concentration (mg/L)	Total protein content (mg)	Yield (%) on total protein	% Purity (by HPLC)
TL-protein (10.9 min RT) in whole wine	31.1	12.44	100	23%
TL-protein (10.9 min RT) in HIC fraction 8	123.5	12.14	97.9	91%
Desalting	187.0	6.17	42.26	93.9%

Table 0.8. *Fraction 8 (VvTL) protein recovery during the purification steps.*

The desalted sample reached a protein concentration of 187 mg/L, so the total protein collected was 6.17 mg/33 mL of final sample volume, with an estimate purity of 93.9%. In wine TL-protein purification, the yield was lower than the expected. As visible from the table, this loss was mainly due to the desalting step. In fact, in order to get rid of all the ammonia salt from the sample, the tails of the protein peaks from the desalting column were discarded, with a consequential great loss of protein (not shown).

The residual impurity of this fraction was due to 2 peaks, respectively with 9.300 and 13.200 min of retention time.

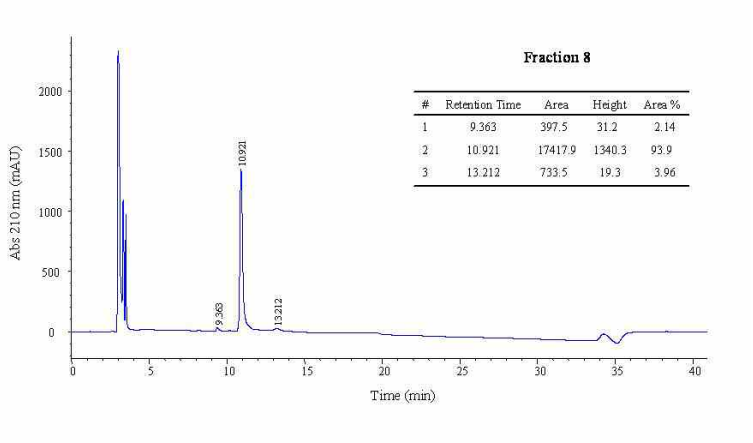


Figure 0.15 *HPLC profile of faction 8 obtained by Hydrophobic Interaction Chromatography. In the table: retention tine, area (absolute value and percentage) and height of each peak detected.*

By comparison of the RT of these two peaks with data of table 3.6, the nature of the impurity was investigated. In particular, it seemed that the peak at 9.3 min RT was a putative thaumatin-like protein, while the peak at 13.2 min RT was recognisable both as a thaumatin like protein (see spot 24, tab. 3.6) and a grape vacuolar invertase (see spot 23, tab. 3.6). It is well known that the TL-proteins are more resistant than non PR-proteins to the fermentation conditions (Tattersall *et al.*, 1997). Indeed, invertase hydrolyses products have been detected in wine by others (Okuda *et al.*, 2006). Hence, it seemed more likely that the peak at 13.2 min was a thaumatin-like protein than a vacuolar invertase. Consequently, the purity percentage of 93.9% seemed too low, with a real purity that would be more likely of 95-96%. Thus, the impurity of this fraction seemed only formed by a 2.14% of putative thaumatin-like protein.

The results above discussed confirmed the suitability of HIC chromatography in purification of protein also deriving from wine, with the reaching of a high purity percentage after a single chromatographic step.

1.1.29 CONCLUSIONS

In summary, it was possible to affirm that, with Hydrophobic Interaction Chromatography it is possible to fractionate high quantities of grape and wine

protein combining a good preparative fractionation with the reaching of high protein purity for several fractions. In particular, a *Vitis vinifera* thaumatin-like protein purification in a single step with high recovery percentages from both Semillon juice and wine was achieved.

Moreover, the chromatographic system used had shown the potentiality of purify more than one protein (especially in wine in which the profile is simplified) and, for this reason, further studies should utilize this knowledge to purify other grape and wine protein classes. Preliminary results showed the possibility to reach these objectives with a purification in a two-step chromatography of 5 grape proteins (Van Sluyter *et al.*, 2007).

It seems possible to affirm that the application of HIC chromatography in wine studies can contribute to improve the knowledge on grape and wine protein. Moreover, by coupling this technique with other chromatographic methods, a more efficient protein purification in terms of both quantity and quality can be exploited.

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CHAPTER 4

Fractionation of wine proteins based on hydrophobicity and characterization of their heat instability and reactivity with tannins

ABSTRACT

Fractionation of Manzoni bianco wine proteins was performed by using both Size Exclusion and Hydrophobic Interaction Chromatography. The obtained fractions were analysed by SDS-PAGE and HPLC. HIC fractionation resulted appropriate for both preparative and analytical aims. Besides, the relation between wine protein hydrophobicity and their aptitude to develop heat-induced haze was assessed. A certain relationship between the level of hydrophobicity and the turbidity formed was found. Identification of the proteins present in the most hazing fractions revealed the presence of thaumatin-like proteins, indicating a major role of these proteins in haze formation.

The second part of this chapter is focused on the reactivity with tannins of wine protein fractions differing in hydrophobicity. Moreover, the effects of protein denaturation by heating and sulphate addition were investigated. The turbidity developed by adding seed tannins in model wine containing increasing amounts of total wine proteins was affected only by the tannin dosage, with a maximum at 250 mg/L, which was followed by a plateau effect. Wine proteins fractionated according to their hydrophobicity were tested for tannin reactivity. Most of the protein fractions developed turbidity immediately after tannin addition and this turbidity increased according to the elution order from the HIC column, confirming that the level of protein hydrophobicity affects the reactivity with tannins. Besides, the effect of sulphate addition (0.5 g/L) was tested on this system. The samples in which sulphate was added with tannins after protein heating showed the lowest haze in 5 of the 6 fractions tested, suggesting a possible role of sulphate on this occurrence. This phenomenon did not occur only for the most hydrophobic fraction, which showed the highest turbidity level. Further analyses showed this fraction as the richest in thaumatin-like proteins, which were the most hydrophobic wine proteins.

The turbidity development for each fraction was followed during 144 hours. The highest long-term turbidity was always detected with un-heated proteins and in the absence of sulphate. It is noteworthy that a HIC fraction containing ≈ 90 % of chitinase showed an opposite behaviour, with a turbidity formation when un-heated lower than that observed when heated. However, this fraction showed a linear turbidity increase, leading to hypothesize that also chitinases might play a role in the mechanism of wine proteins-tannin haze formation.

Key words: wine, PR-proteins, haze, tannin, chromatography, HIC, chitinase, thaumatin-like protein.

INTRODUCTION

Wine proteins are generally considered to be detrimental to white wine quality due to their role in formation of hazes. During winemaking, part of the soluble grape proteins is precipitated *via* interaction with tannins (Powers *et al.*, 1988) which are, by definition, protein-binding and precipitating agents (Schofield *et al.*, 2001).

Grape and wine proteins have been extensively investigated by means of several chromatographic techniques. By using Ion-exchange chromatography on DEAE-cellulose, Bayly and Berg (1967) discovered four different wine protein bands by electrophoresis. Somers and Ziemelis (1973) studied the wine proteins by using Size Exclusion Chromatography (SEC) and concluded that the wine protein molecular weight ranges from 10 to 50 kDa.

Sodium Dodecyl Sulphate - (SDS) (Waters *et al.*, 1991; Dorrestein *et al.*, 1994), Lithium Dodecyl Sulphate (LDS) - Polyacrylamide Gel Electrophoresis (PAGE) (Hsu and Heatherbell, 1987a, 1987b, 1987c) and Isoelectric Focusing (IEF) (Pueyo *et al.*, 1995; Santoro *et al.*, 1994) have been used for fractionation and characterization of the different wine proteins with good results although these techniques resulted suitable only for analytical aims. Fast Protein Liquid Chromatography (FPLC) (Dawes *et al.*, 1995; Dorrestein *et al.*, 1994; Canals *et al.*, 1998) was more useful for grape and wine protein studies because of its

preparative characteristics. There are several evidences that hydrophobic bonding may be the major mode of interaction between condensed tannins and proteins. Oh and colleagues (1980) studied the interaction in tannin-protein complexes, concluding that the dominant mode was the hydrophobic bonding rather than hydrogen bonding as previously supposed. Siebert and colleagues (1996) confirmed this statement and defined that hydrogen bonding is not as important as hydrophobic bonding in the interaction between proteins and polyphenols. However, Hagerman and co-workers (1998) suggested that different types of tannin have modes of interaction with proteins that seemed dependent on the tannin polarity. In particular, they suggested that the interaction of a nonpolar tannin with a standard protein (BSA) resulted in precipitates due to the formation of a hydrophobic coat around the protein, whereas a more polar tannin formed precipitated by means of hydrogen-bonded cross-links between protein molecules.

Brissonet and Maujean (1993) proposed the use of Hydrophobic Interaction Chromatography for the characterization of foaming proteins of Champagne wine, but other applications of this technique to the wine proteins has never been reported.

In this chapter, wine proteins have been fractionated according to their hydrophobicity and fractions collected have been analysed to clear the relationship between protein hydrophobicity and haze potential. Moreover, the relation between protein hydrophobicity and reactivity with grape seed tannins has been studied.

MATERIALS AND METHODS

1.1.30 MATERIALS

The wine utilised in this work (Manzoni bianco, vintage 2006,) was kindly supplied by the winery of the "Scuola Enologica G.B. Cerletti" of Conegliano (Italy). This wine had an average protein content of ≈ 300 mg/L as assessed by KDS-BCA method (Vincenzi *et al.*, 2005).

The tannins used in the experiments were grape seed tannins Premium®Vinacciolo SG (Vason Group)

The model wine used was prepared with 5 g/L tartaric acid, 12 % ethanol, pH 3.20

1.1.31 PROTEIN EXTRACTION FROM WINE

1.1.31.1 Concentration by ultrafiltration

After wine sterile filtration with cellulose acetate filters (pore size of 0.20 µm, Millipore), Manzoni bianco wine proteins were , concentrated by means of a stirred cell ultrafiltration system (Amicon) equipped with 3000 Da (MWCO) membranes. The retentates (on average 20 mL from 1 litre of wine) were dialysed against 5 litres of distilled water on tubes with porosity of 3500 Da (Spectrapore) before being washed with citrate buffer and stored at -20°C.

1.1.31.2 Protein precipitation with potassium dodecyl sulphate (kds)

In order to be analysed by SDS-PAGE or to be quantified by bicinchoninic acid (BCA) method (Smith *et al.* 1985), proteins were precipitated by using the KDS method according to the procedure proposed by Zoccatelli *et al*. (2003). 10 µL of SDS (10% in water, Bio-Rad) were added to 1 mL of protein sample and heated for 5 min at 100°C. 250 µL of 1M KCl (Carlo Erba) were then added to the samples and, after at least two hours of incubation, the formed pellets were collected by centrifugation (15 min, 4°C). Further washes with 1 mL of 1M KCl were required to completely eliminate polyphenols from the sample. Every quantification was the average of at least three replicates.

1.1.32 GRAPE AND WINE PROTEIN CONTENT DETERMINATION

The protein content was determined according to Vincenzi and co-workers (2005). Firstly, proteins were precipitated from 1 mL of wine with the KDS method according to Zoccatelli *et al.* 2003 (see 4.3.2.2). After centrifugation (15 min, 4°C), pellets were dissolved into 1 mL of distilled water and quantified by using the BCA-200 protein assay kit (Pierce, Rockford, IL). The calibration curve was prepared by using serial dilutions of bovine serum albumin (BSA, Sigma) in water. Absorbance was measured at 562 nm (Shimadzu UV 6010).

1.1.33 TOTAL POLYSACCHARIDE CONTENT DETERMINATION

The polysaccharide content was determined colorimetrically according to Segarra and co-workers (1995). After addition of 5 volumes of absolute ethanol (Baker), samples were left at 4°C overnight before being centrifuged (30 min, 14000g). Collected pellets were washed twice with ethanol (Baker) before being dissolved in bi-distilled water. 1 mL of the resulting solution was added of 25 µL of 80% phenol (w/w, Fluka) and 2.5 mL of sulphuric acid (Merck). Samples were mixed and the reaction carried on for 30 minutes at room temperature. Absorbance values were determined at 490 nm (Shimadzu UV 6010). The calibration curve was prepared by using serial dilution of galactose (Fluka) in water.

1.1.34 HEAT TEST

According to Pocock and Rankine (1973), a heat test was performed to determine grape and wine protein stability. After heating (80°C for 6 hours), samples were chilled (16 hours at 4°C) and, after equilibration at room temperature, turbidity values were measured nephelometrically (Hach 2100P turbidimeter) or spectrophotometrically (Shimadzu UV 6010) at 540 nm (Waters *et al.*, 1991). Net turbidity values lower than 2 NTU (Net Turbidity Unit) or 0.02 AU (Absorbance Unit) indicated sample stability.

1.1.35 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970). Samples were dissolved in a Tris-HCl buffer pH 6.8 containing 15% (v/v) glycerol (Sigma) and 1.5 % (w/v) SDS (Bio-Rad) and heated at 100°C for 5 minutes before loading. Electrophoretic analyses were performed with a Mini-Protean III apparatus (Bio-Rad). For analyses in reducing conditions, 3% (v/v) of β-mercaptoethanol (Sigma) was added to the loading buffer. The molecular weight standard proteins were: Myosin (200,000 Da), β-galactosidase (116,250 Da), Phosphorylase b (97,400), Serum Albumin (66,200 Da), Ovalbumin (45,000

Da), 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).

Gels were generally prepared with T = 14% (acrylamide-N, N' metilen-bisacrylamide 29:1; Fluka) unless otherwise stated and alternatively stained with:

- Coomassie brilliant blue R-250 (Sigma) (18h of staining followed by 24 h of destaining with 7 % acetic acid) (Koenig *et al.*, 1970);
- The silver stain procedure according to Blum *et al.* (1987) for high sensitivity protein detection;
- The PAS (Periodic Acid-Schiff) stain procedure to stain glycoproteins as suggested by Segrest and Jackson (1972).

Gel pictures were acquired by means of ScanJet 3400C (HP) scanner and processed with the Adobe® Photoshop® 6.0 software.

1.1.36 ZYMOGRAPHY FOR CHITINASE ACTIVITY DETECTION

Chitinolytic activity 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% w/v). Glycol-chitin was prepared as reported by Molano *et al.* (1979). After protein separation, the gel was incubated overnight at room temperature in a 50 mM sodium acetate buffer pH 5.5 with 1% (w/v) Triton X-100 (Sigma). Afterwards, gel were incubated for 10 minutes with 0.5 M Tris-HCl buffer pH 8.9 containing 0.01 % (w/v) Calcofluor white MR2 , followed by a wash in bi-distilled water. Gel images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY) and photograph processed using the Adobe® Photoshop® 6.0 software.

1.1.37 WINE PROTEIN SEPARATION BY CHROMATOGRAPHY

The chromatographic separations were performed by means of four instruments:

- An ÄKTA purifier FPLC (GE-Healthcare) equipped with an UV detector (λ Absorbance Detector). Collected data were processed by the Unicorn 5.11 software.

- A HPLC (Waters 1525) equipped with a Dual λ Absorbance Detector (Waters 2487) and a Refractive index detector (Waters 2414). Collected data were analysed by the Breeze software.

Every solution utilised and sample loaded were previously filtered with cellulose acetate filters (Millipore) with pore size of 0.20 µm (MFS) and degassed.

1.1.38 SIZE EXCLUSION CHROMATOGRAPHY

An FPLC system (ÄKTA purifier, GE-Healthcare) equipped with a HiLoad 26/60 Superdex 75 prep grade (Amersham Biosciences) column was used. Fractionations were performed isocratically with 30 mM citric acid buffer pH 3.50 at a flow rate of 1.5 mL/min. Fractions were collected by a Frac-920 (GE-Healthcare) collector and concentrated through centrifugation with Vivaspin 20 devices (20 mL tubes, VivaScience).

1.1.39 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Wine proteins were fractionated with an ÄKTA purifier FPLC system (GE-Healthcare) equipped with a HIC BioSuite^{TM} Phenyl 10 µm HIC 7.5 x 75 mm column (Waters). Eluent A was 50 mM Sodium Phosphate containing 1.25 M Ammonium Sulfate, pH 5.0 and eluent B was eluent A without ammonium sulfate. The flow rate was 1 mL/min and the gradient was as follows: 0-15 mL, 100% A; 15-45 mL, 0% A (linear) and then 45-60 mL 0% A. Samples were loaded after equilibration to the starting conditions (100% A).

1.1.40 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1.1.40.1 Reverse Phase (RP)-HPLC

The protein composition of wine fractions was determined by HPLC, according to the method proposed by Peng *et al.* (1997).

100 µL of sample was loaded at 1 mL/min onto a semi-preparative C18 column (4.6 x 250 mm, Vydac 218 MS 54, Hesperia, CA) fitted with a C18 guard column (Vydac 218 MS 54, 4.6 x 5 mm, Hesperia, CA) equilibrated in a mixture of 83% (v/v) solvent B [0.1% trifluoroacetic acid (TFA) in 92% Acetonitrile] and

17% solvent A [80% Acetonitrile, 0.1% (v/v) TFA] and held at 35°C. Proteins were eluted by a gradient of solvent A from 17% to 49% in the first 7 minutes, 49% to 57% from 7 to 15 minutes, 57% to 65% from 15 to 16 minutes, 65% to 81% from 16 to 30 minutes and than held at 81% for 5 minutes before re-equilibrating the column in the starting conditions for 6 more minutes. Peaks were detected at 220 nm.

1.1.40.2 Size Exclusion (SE) - HPLC

Total and fractionated wine proteins were analysed through a Size Exclusion Chromatography column (Protein Pak 125, Waters) installed on a HPLC (Waters 1525) system equipped with a Dual λ Absorbance Detector and a Refractive Index detector. Samples were injected in a 20 µL loop. The flow rate was 0.6 mL/min in isocratic mode with tartrate buffer (5 g/L tartaric acid, pH 3.50). Absorbance was detected at 280 nm.

RESULTS AND DISCUSSION

1.1.41 FRACTIONATION OF WINE PROTEINS BY SIZE EXCLUSION CHROMATOGRAPHY

In order to fractionate proteins from an unfined Manzoni Bianco wine with a protein content of ≈ 300 mg/L, a Size Exclusion Chromatography (SEC) column was used. Initially, 5 litres of wine were concentrated (500 times) by means of a stirred ultrafiltration cell (Amicon, 3000 MWCO). The cell retentate was washed several times with 30 mM citrate buffer (pH 3.50). The sample so treated was loaded into a HiLoad 26/60 Superdex 75 prep grade chromatography column (fig. 4.1).

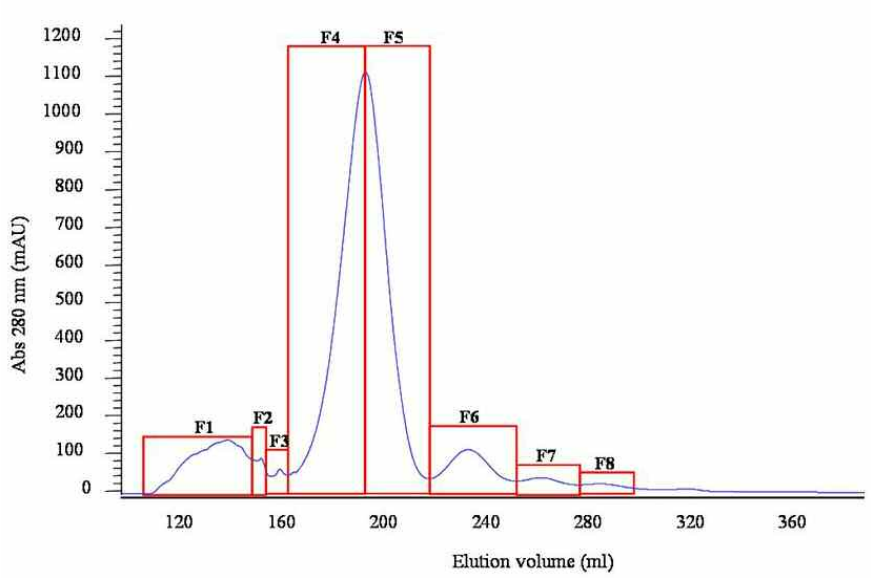


Figure 0.1 *Fractionation of Manzoni Bianco wine proteins (≈ 400 mg) by Size Exclusion Chromatography. Collected fractions (F) are indicated by numbered boxes.*

The protein fractionation was easily distinguishable, with the appearance of three main peaks that were gathered into 8 separated fractions. These results agreed with those obtained by gel filtration of Chardonnay wine proteins by Okuda and co-workers (2006). After protein content determination of each fraction (not shown), a SDS-PAGE analysis was performed only for fractions 1 to 6 (fig. 4.2) because fractions 7 and 8 had a protein content too low to be analysed by SDS-PAGE.

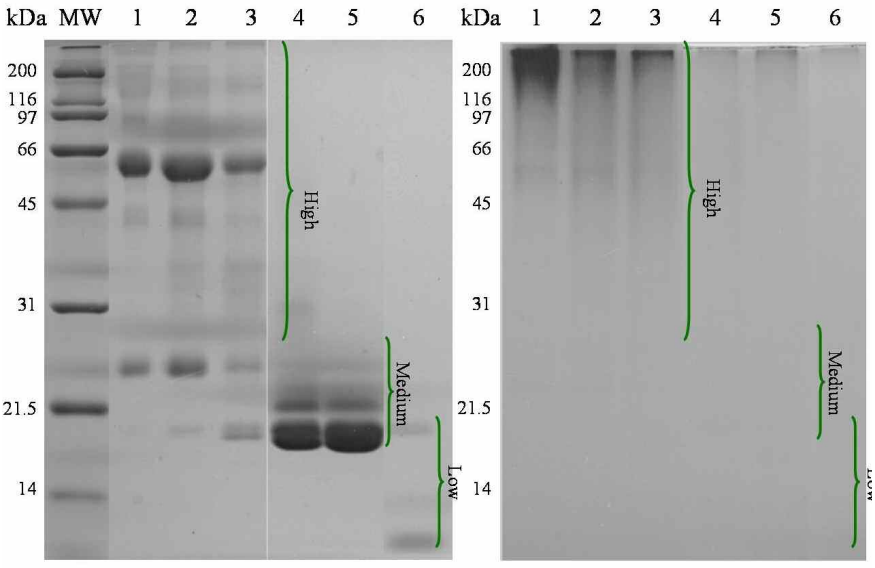


Figure 0.2. *SDS-PAGE analysis (T = 14%; C = 3%) in non-reducing conditions of the*

fractions from Size Exclusion Chromatography (see fig.4.1). Left panel: fractions stained for proteins with Coomassie brilliant blue R-250. Right panel: fractions stained for sugars with Periodic Acid-Schiff (PAS) procedure).

As expected, all the high molecular weight proteins were contained in the firsts three fractions, in which a major band at about 65 kDa appeared. The PAS staining showed that these factions contained almost all the glycosilated high MW compounds (>200 kDa) of the wine. Besides, bands at about 65 kDa were visible in both gels and for that reason were assumed to be an invertase (Porntaveewat *et al.*, 1994; Kwon *et al.*, 2004; Okuda *et al.*, 2006).

Fractions 4 and 5 displayed only bands between 32 and 17 kDa. From the literature data (Tattersall *et al.*, 1997; Waters *et al.*, 1998; Davies and Robinson, 2000; Pocock *et al.*, 2000) it was reasonable to suppose these proteins to belong to the chitinase and thaumatin-like protein classes. Moreover, fractions 4, 5 and 6 did not give any signal when stained with PAS, indicating the absence in the wine of glycosilated proteins with low molecular weight. In fraction 6 only two classes of proteins were detected. The upper band showed a MW similar to that of the thaumatin-like proteins, while the lower had an apparent MW of ≈ 10 kDa. The presence of LTP hydrolysis products with MW of 9.6 kDa instead of 11.6 kDa (the MW estimated from the cDNA sequence) has been suggested (Okuda *et al.*, 2006), and this occurrence matches with the SDS-profile of HIC fraction 6.

SEC separation allowed to “clean” the proteins with intermediate MW from those with MWs higher than ≈ 32 kDa and lower than ≈ 17 kDa. This fact has been considered of interest for a multi-step protein chromatography finalised to purify PR-proteins for their characterisation in relation to haze development in white wines.

Fraction 4 and 5 were considered suitable for a two-step protein purification achieved by coupling a preparative SEC with a Hydrophobic Interaction Chromatography (HIC). To this aim, ammonium sulphate (761 mg/mL at 25°C) was added to fraction 4 until reaching the 99% of saturation to promote a complete protein salting out. After centrifugation, the collected pellet was dissolved with 50 mM Na-Phosphate buffer pH 5.0 to achieve the clarity and the salt concentration required (1.25 M) for HIC fractionation. The sample so

prepared was loaded into a HIC analytical column (fig. 4.3).

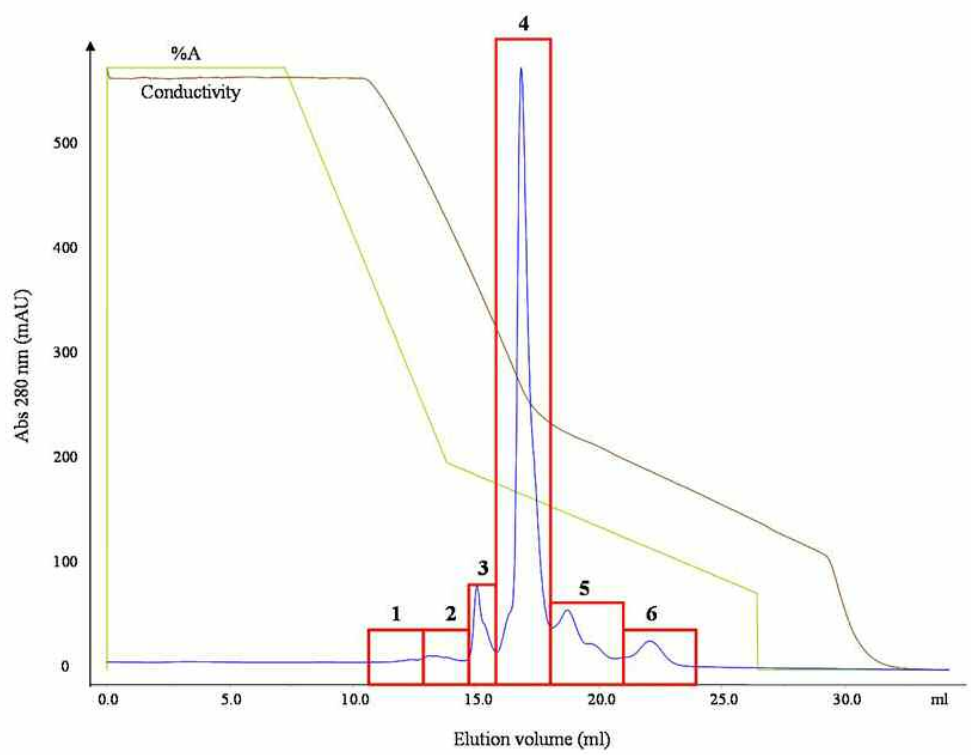


Figure 0.3. *Hydrophobic Interaction Chromatography of fraction 4 from SEC (see fig.4.1) Collected fractions (F) are indicated by numbered boxes*

The HIC column showed a good protein separation ability with the achievement of 5-6 peaks from a fraction that in SDS-PAGE presented only three protein bands (fig. 4.2).

This two-step chromatography gave promising results in terms of protein purification. However, the method here proposed showed an analytical nature more than a preparative one because of the low volume of sample loadable in the SEC column, the high dilution of fractions collected and the necessity of a further protein precipitation step before the HIC fractionation.

1.1.42 STUDIES ON PROTEIN FRACTIONATION BASED ON THEIR HYDROPHOBICITY

The wine proteins separated by HIC were studied in relation to their potential in haze production. HIC chromatography was utilised on a preparative scale to fractionate proteins according to their hydrophobicity level. For this aim, a 500-times concentrated Manzoni Bianco wine was used (fig. 4.4).

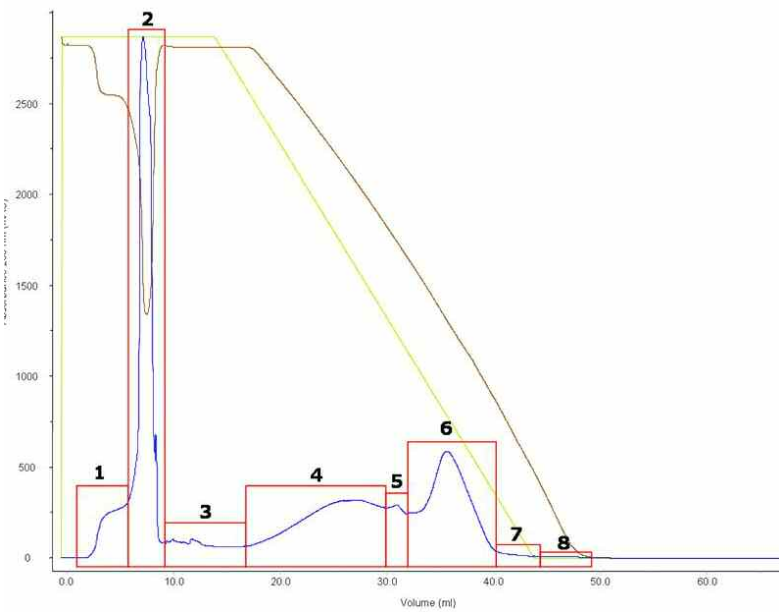


Figure 0.4. *Hydrophobic Interaction Chromatography of the proteins of Manzoni bianco wine proteins. Collected fractions (F) are indicated by numbered boxes. Green line: buffer A percentage; Brown line: conductivity.*

Due to the large quantity of protein loaded on the column, the separation was not as good as that obtained with other HIC fractionation experiments (see fig. 4.13). However, 8 separated fractions were collected and analysed. The very high peak of fraction 2 (fig. 4.4) (unretained fraction) contained all the unbounded proteins that did not interact with the resin. Besides, fractions 4, 5 and 6 were eluted at a conductivity level similar to that previously observed for the elution of the Semillon chitinases and VvTL proteins (see tab. 3.1 and fig. 3.12). After dialysis and protein content determination (not shown), each fraction was analysed by SDS-PAGE (fig. 4.5).

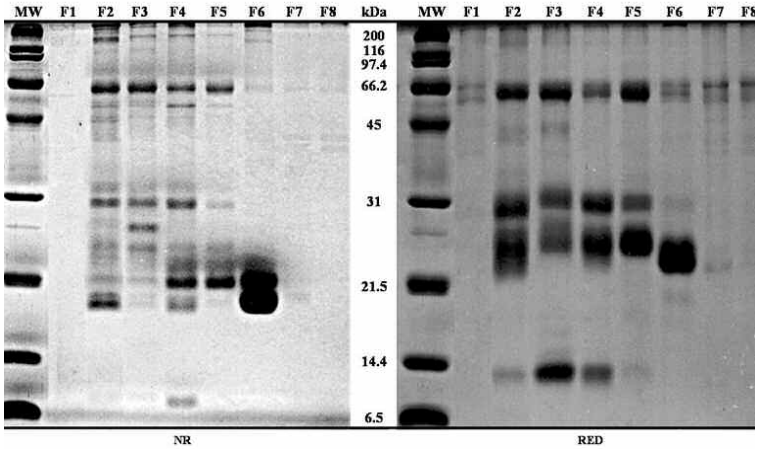


Figure 0.5. *SDS-PAGE (T = 14%, C = 3%) of the 8 fractions separated by HIC in non-*

reducing (left) and reducing (right) conditions. 30 µg of protein was loaded in each lane and gels were stained with Coomassie brilliant blue R-250. F1-F8 corresponds to fractions indicated in fig 4.4. MW standard proteins are on the left of each gel.

The gels showed a wide diversity among wine proteins, both in non-reducing and reducing conditions. A protein with apparent MW of ≈ 65 kDa (probably invertase) was detectable from fraction 2 to fraction 5 and this occurred also for the band at ≈ 30 kDa. It is generally assumed that grape and wine proteins with a SDS-PAGE mobility corresponding to approximately 30 kDa MW are the grape chitinases (Derckel *et al.*, 1996; Pocock *et al.*, 2000; Van Sluyter *et al.*, 2005). Moreover, in every fraction except for F1 (the column flow through which was rich in polysaccharides, not shown), F7 and F8 (containing no detectable peaks A_{280}), bands at 18-21 kDa (non-reducing conditions, left panel) and at 22-27 kDa (reducing conditions, right panel) were observable. Several authors indicated grape and wine proteins with these MWs as belonging to the thaumatin-like (TL) proteins class, which, in grape, can present different isoforms (Peng *et al.*, 1997; Tattersall *et al.*, 1997; Davies and Robinson, 2000). Therefore the observed differences in the HIC retention times, corresponding to differences in hydrophobicity, should be due to the diversities existing among TL-protein isoforms, or to the happening of some post translational modification, likely proteolysis, that resulted in this different behaviour.

Fraction 2 (flow through of the column) contained most of the same protein bands detectable in the following fractions, indicating that the column had been overloaded.

Fraction 3 (in non-reducing conditions, left panel) was the only one in which a band with an apparent MW of ≈ 26-27 kDa was detected. It seemed unlikely that this band was a chitinases because of the absence of activity detected with the chitinolytic activity assay (fig. 4.7), consequently the nature of this band is unknown.

In fraction 4 (in non-reducing conditions, left panel) a protein with an apparent MW of ≈ 10 kDa was detected. This band could be or an isoform of the Lipid Transfer Protein (LTP), a basic protein with MW of 9 kDa (Gomes *et al.*, 2003) or, as suggested by Okuda and colleagues (2006), a LTP hydrolysis

products with MW of 9.6 kDa. However, this protein, which should be very hydrophobic (Blein *et al.*, 2002) was eluted in the middle of the gradient, showing a behaviour similar to that observed during the Semillon juice protein fractionation in chapter 3 (see paragraph 3.4.3.1).

Fraction 6, the last protein peak eluted from the column, seemed the purest among all the HIC fractions, containing two major bands with similar MWs that, from the literature (Tattersall *et al.*, 1997; Ferreira *et al.*, 2007) presumably corresponded to 2 or more TL protein isoforms. Comparing these data to those achieved working with Semillon juice (see chapter 3), the TL-proteins of F6 were confirmed as the most hydrophobic wine proteins.

It is interesting to underline the different migration rate observable for some bands in reducing and non-reducing conditions, as previously observed by Vincenzi and Curioni (2005). Proteins with MWs lower than 40 kDa increased their relative apparent MWs when reduced (compare left and right panels of fig. 4.5). For instance, the 4 bands with apparent MW of ≈ 30 kDa migrated almost equally in the left gel but, after reduction, their apparent MW changed with fraction 3 that moved at ≈ 32 kDa, fractions 4 and 5 at ≈ 31 kDa and fraction 2 at ≈ 30 kDa. These latter result agrees with those observed by Pocock *et al.* (2000), who divided grape chitinases in 4 classes (Chit A, Chit B, Chit C, Chit D) showing different SDS-PAGE MWs in reducing conditions (32, 33, 32, 34 kDa respectively).

Besides, the bands assumed to be thaumatin-like proteins changed their migration rate from an apparent MW of 18-22 kDa (when not reduced) to the apparent MW of 23-27 kDa in reducing conditions. Moreover after reduction of the samples, two bands appeared at ≈ 12-13 kDa in F2 and F4 (right panel), which were absent in non-reducing conditions (left panel). This should indicate that these proteins appeared as a result of the splitting of some disulphide bonds linking together protein aggregates of higher MW. The existence and the significance of such protein aggregates in wines warrant further investigation.

The presence of glycoproteins in the same samples of figure 4.5 was studied by staining the gels with the PAS method (fig. 4.6)

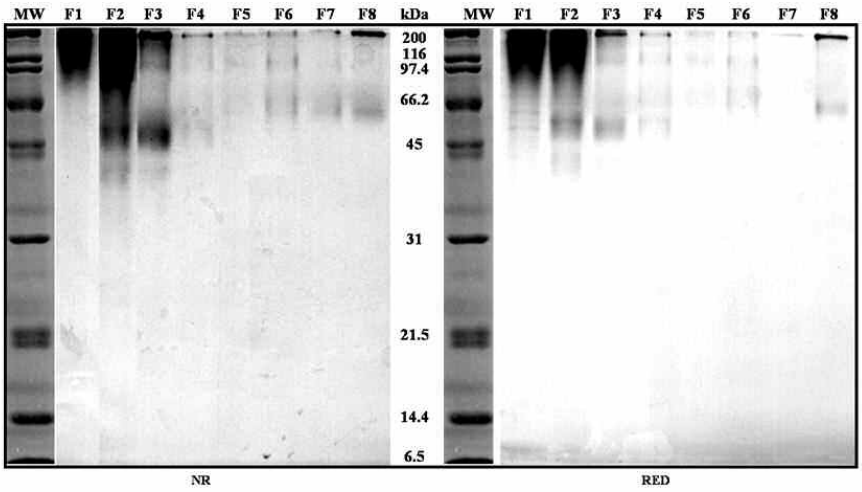


Figure 0.6. *SDS-PAGE (T = 14%, C = 3%) of the 8 fractions separated by HIC in non-reducing (left) and reducing (right) conditions. 2 µg of protein was loaded in each lane and gels were stained with the PAS method for sugar detection. F1-F8 corresponds to fractions indicated in fig. 4.4. MW standard proteins (stained with Coomassie) are on the left of each gel.*

HIC protein fractionation divided the wine glycocompounds differently from what was observable with anion exchange chromatography (Vincenzi *et al.*, 2006) of the same protein preparation (not shown) in which glycocompounds were all eluted in the column flow through fraction, as was also observed by other authors (Dorrestein *et al.*, 1995; Canals *et al.*, 1998). With HIC fractionation, high MW glycoproteins were detected among the first three fractions as well as in F8 in both reducing and not reducing conditions. The total wine glycoproteins pattern was detected in fraction two, confirming the overloading of the column. In detail, fraction 1, which was a shoulder separated from fraction 2 (fig. 4.4) probably due to a size exclusion effect of the column matrix, contained only very high MW compounds stuck on the upper part of the gel (> 200 kDa). Compounds with similar MWs were detected also in fraction 2. Waters and co-workers (1994a) isolated and characterized a high MW mannoprotein of 420 kDa from *Saccharomyces* that could correspond to the high MW bands detectable after PAS staining.

The appearance in fractions 2 (and 3) of a band with a MW of ≈ 50-55 kDa could indicate the presence of an invertase although this MW seems to be too

low for the grape invertase, which has been shown to display a SDS-PAGE mobility corresponding to 60-64 kDa (Nakanishi and Yokotsuka, 1991; Takayanagi *et al.*, 1995; Kwon *et al.*, 2004). However, Porntaveewat *et al.*, (1994) purified an invertase with MW of 72 kDa from grapes (variety Muscat Bailey A) and highlighted that, analysing this glycoprotein by SDS-PAGE three bands appeared at 56, 25 and 24 kDa. This data seemed to agree with the MW of the band detected in lanes F2 (and F3) of fig. 4.6 leading to consider it as a grape invertase.

In fraction 3 the PAS-stained profile resulted similar to that of fraction 2 but with an inverted ratio between the intensity of the two bands.

Fractions 4 and 5 did not show a significant band appearance after staining for sugars, whereas fractions 6, 7 and 8 showed the presence of faint bands at ≈ 65 kDa whose MW could match with that of a vacuolar grape invertase (Davies and Robinson, 1996).

Fraction 8 showed the appearance of a well marked band blocked at the boundary between the stacking and the resolving gels. This band had a high MW as confirmed by further analyses with size exclusion chromatography (fig. 4.9). Due to the nature of the separation, the PAS-stained compound(s) in fraction 8 (eluted with the lowest ionic strength) should present the highest level of hydrophobicity, and so it is unlikely to belong to the wine compounds that are known to have hydrophilic nature, such as polysaccharides (Vernhet *et al.*, 1996; De Freitas *et al.*, 2003). It seemed more likely to suppose that fraction 8 contained a hydrophobic glycoprotein (may be a mannoprotein) deriving from yeast cell walls, whose hydrophobic nature have been largely demonstrated (Iimura *et al.*, 1980; Farris *et al.*, 1993; Masuoka *et al.*, 1997; Martinez *et al.*, 1997; Alexandre *et al.*, 1998).

An additional analysis was performed to better understand the nature of the proteins fractionated on the basis of their hydrophobicity. To this aim, chitinolytic activity on gel was assayed in the 8 HIC fractions (fig. 4.7) according to Vincenzi and Curioni (2005).

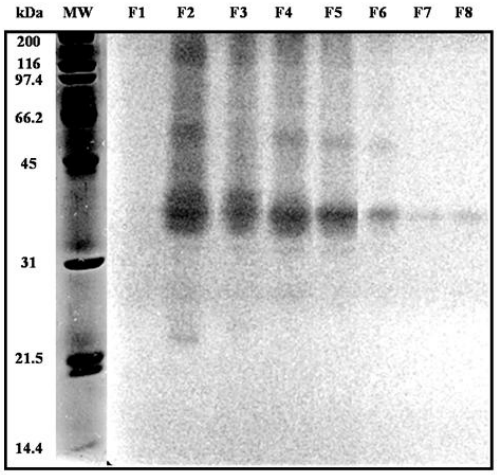


Figure 0.7. *Chitinolytic activity detection on glycol-chitin after SDS-PAGE separation (T = 14%, C = 3%, reducing conditions) of the wine protein fractions obtained from HIC separation. F1-F8 correspond to fractions indicated in fig. 4.4.*

Apart for fraction 1, the chitinase activity was spread among all the HIC fractions collected, although with decreasing staining from fraction 2 to fraction 8. In these fractions, the main chitinolytic activity belonged to the bands at ≈ 35 kDa. These chitinase bands correspond to those detectable at ≈ 30 kDa on the Coomassie-stained gel not containing the glycol chitin (fig. 4.5, right panel), because the presence of the substrate slows down protein bands migration as demonstrated by Vincenzi and Curioni (2005). A fainter chitinolytic band appeared also at ≈ 50 kDa (lanes F2-F6) and also at the top of the gel. The reason of this appearance should be due to the presence of other enzymes acting on glycol chitin, although their precise nature remains to be assessed.

It is noteworthy to focus the attention to fraction 2, in which an additional chitinolytic activity at ≈ 25 kDa was detectable. In the Coomassie-stained SDS-PAGE gel (fig. 4.5, right panel), fraction 2 showed a band at a MW that should be responsible for this activity. Van Sluyter and co-workers (2005) highlighted the presence of active chitinases with MW of 26 kDa in Cabernet Sauvignon and Chardonnay grapes. A confirmation for this hypothesis was achieved after size exclusion chromatography (SEC) analysis of the separated HIC fractions (fig. 4.8 and 4.9). As a matter of fact, the main SEC peak of HIC fraction 2 (53 % of total peak area) had a retention time corresponding to that observed for the chitinases

(tab. 4.1).

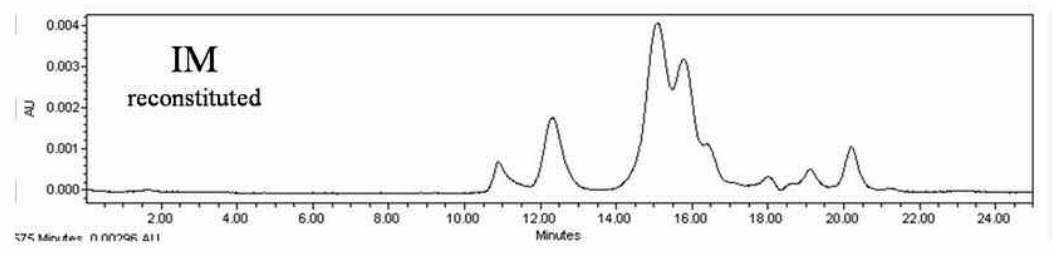


Figure 0.8 *Size Exclusion Chromatography of the reconstituted protein (F1 - F8) after HIC separation of Manzoni bianco wine proteins (see fig.4.4).*

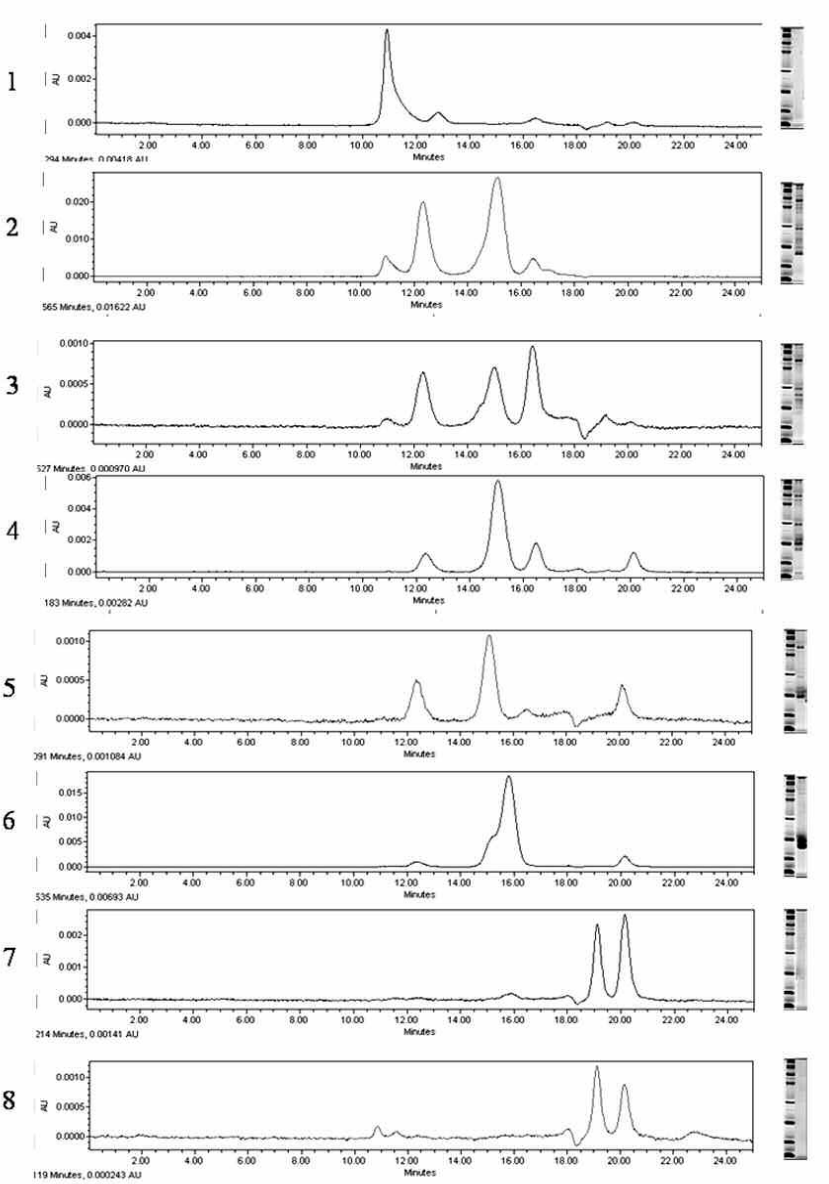


Figure 0.9. *Size Exclusion Chromatography of the single fractions (F1 - F8) obtained from HIC separation of Manzoni bianco wine proteins. Numbers 1-8 on the left indicate*

the HIC fraction F1-F8 (see fig. 4.4), respectively. The SDS-PAGE profile of each fraction is shown for comparison on the right of the corresponding chromatogram.

Presumable MW (kDa)	Peak Retention Time (min)	HIC 1 (Area %)	HIC 2 (Area %)	HIC 3 (Area %)	HIC 4 (% Area)	HIC 5 (Area %)	HIC 6 (Area %)	HIC 7 (Area %)	HIC 8 (Area %)
> 200	10.900	**83.32**	7.45	2.39	n.d.	n.d.	n.d.	n.d.	5.53
? 70	12.330	n.d.	31.24	26.44	10.5	24.56	3.28	n.d.	n.d.
? 64	12.830	9.66	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
35-31	15.010	n.d.	n.d.	35.51	**64.5**	n.d.	n.d.	n.d.	n.d.
30-22	15.120	n.d.	**53.08**	n.d.	n.d.	**57.05**	n.d.	n.d.	n.d.
? 22	15.810	n.d.	n.d.	n.d.	n.d.	n.d.	**91.23***	4.10	n.d.
21-18	16.460	3.33	6.3	35.67	15.06	3.63	n.d.	n.d.	n.d.
< 18	17.040	n.d.	1.92	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
< 14	18.080	n.d.	n.d.	n.d.	1,08	n.d.	n.d.	n.d.	n.d.
< 10	19.150	1.82	n.d.	n.d.	n.d.	n.d.	n.d.	42.64	50.61
Not protein	20.160	1.88	n.d.	n.d.	8.86	14.76	5.49	53.26	43.87
	Total (%)	100	100	100	100	100	100	100	100

n.d: not detected.* a shoulder at 15.1 minutes of RT was detected.

Table 0.1. *Area percent of the SEC peaks of HIC fractions F1 - F8 calculated from the chromatograms of fig 4.9.*

The SEC results are to be compared to those of SDS-PAGE in non-reducing conditions because the HIC fractions were not reduced before loading on the SE-HPLC column. By SEC, HIC fraction 1 showed a main peak with a retention time of 10.9 min. This is the peak with the lowest retention time thus containing the highest molecular weight compounds. These results agreed with those of PAS analyses, indicating the presence of high molecular weight glycocompounds with low hydrophobicity. Moreover, the SEC chromatogram of fraction 1 indicated its low protein content, showing only two small peaks at 12.8 and 16.4 min. of RT, thus confirming the results observed by SDS-PAGE (fig. 4.5).

From the SEC, 4 well defined peaks were visible in fraction 2, the unretained HIC fraction, while fraction 3 gave 4 peaks with RTs very similar to those of fraction 2. Starting from fraction 4, the peak at 10.9 min. of RT disappeared, while a new peak at 18.0 min. of RT was detected, which presumably corresponded to the low molecular weight band observed by SDS-

PAGE. The SEC profile of HIC fraction 5 appeared similar to that of fraction 4. It was interesting to look at the SEC chromatogram of HIC fraction 6 that showed, by SDS-PAGE, the presence of only two bands at apparent MWs of 18 and 20 kDa, presumably two TL-protein isoforms (fig. 4.5). On SEC, the proteins of same fraction behaved differently from the other proteins. Indeed, while all the proteins with MW ≈ 20 kDa had a retention time of ≈ 15.3 minutes, for this fraction the peak at 15.3 was only a shoulder of a bigger peak eluting at 15.8 min, this peak being detectable only in fraction 6 (and marginally in fractions 7 and 8). From this observation and from those made during the TL protein purification from Semillon juice and wine (chapter 3), it seemed that the most hydrophobic wine protein was a particular isoform of TL protein.

Fraction 7 and 8 showed an only protein peak indicating the presence of a very low molecular weight protein with a RT of 19.2 min, whose nature is still under investigation. The appearance of the peaks at 10.9 and 11.5 minutes of RT on fraction 8 confirmed the results of the PAS indicating the presence of high MW glycocompounds eluted only with at low ionic strength.

These results highlighted the good separation achievable with the SEC column for wine protein studies.

In general, it seemed that the hydrophobicity of wine macromolecules was in some way related to their dimension, at least when the level of hydrophobicity was deduced by their chromatographic behaviour on HIC, which showed a lower hydrophobic character for the higher MW compounds. This observation agrees with the statement that generally the bigger the molecule the larger its number of hydrophobic moieties and consequently its hydrophobicity (Wall *et al.*, 2002).

1.1.42.1 Haze potential of wine proteins as related to their hydrophobicity

With the aim to clarify the role of wine protein hydrophobicity on their hazing potential, the first 6 fractions collected from the HIC were subjected to the heat test (fig. 4.10), while fractions 7 and 8 were not tested because of the lack of protein showed.

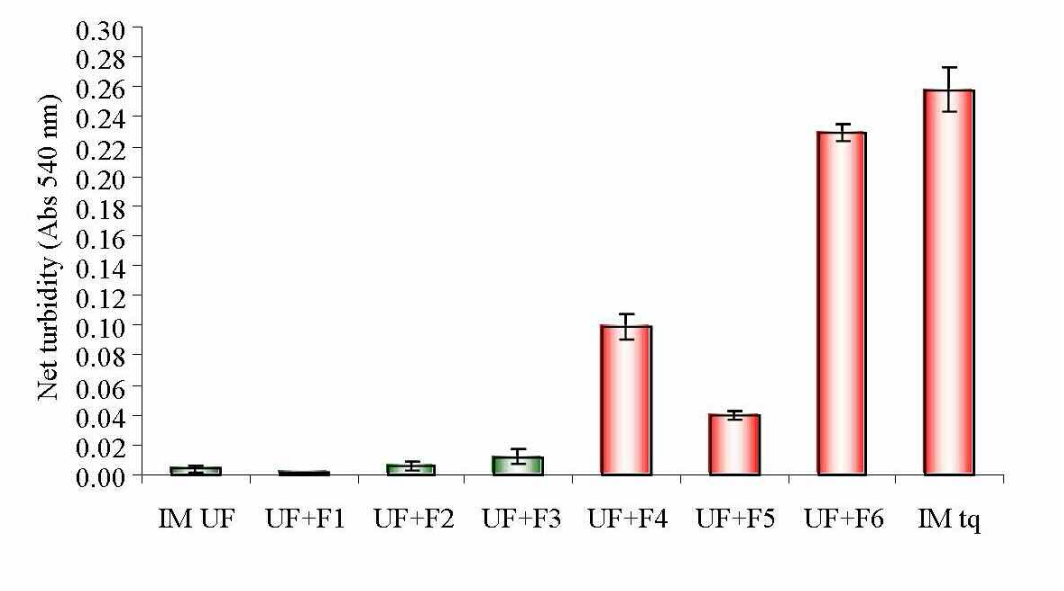


Figure 0.10. *Heat test results for fraction 1 to 6 (F1-F6) collected from HIC of a Manzoni Bianco wine. Proteins were precipitated from each fraction by ethanol addition a re-dissolved at 200 mg/L in Manzoni bianco ultrafiltered wine (UF). Green and red bars are the stable and instable samples (turbidity lower and higher than 0.02), respectively. Results for both the Ultrafiltered Manzoni bianco wine without protein addition (IM UF) and the original (unfined) Manzoni bianco wine (protein content: 200 mg/L) (IM) are also shown.*

Siebert and colleagues (1996) affirmed that protein hazing increased with increasing the protein heating temperature, suggesting that hydrogen bonding was not as important in the interaction between proteins and polyphenols as hydrophobic interaction was. Moreover, other authors have highlighted the role of hydrophobic interactions in the formation of protein-tannin complexes (Oh *et al.*, 1980; Charlton *et al.*, 2002). The results of the heat tests confirmed the data recoverable from the literature. Waters *et al.* (1996) have been the first to identify the proteins that cause haze in wines as PR proteins deriving from grape berries and, in particular, it seemed that the fractions characterized by the highest instability were those containing the grape TL proteins. In our hands, among all the HIC fraction heat tested at the same protein concentration, the fraction mainly composed by TL protein (fraction 6) resulted indeed the most instable, followed by fraction 4 in which both TL and chitinases were contained (with a prevalence of chitinases). Fraction 5 produced a significant turbidity but lower than that of

fraction 4 and 6. From the SEC data, the main peak of all the HIC protein fractions combined was that at 15 min of retention time, which was assumed to be a chitinase. The area of the peak at 16.4 (corresponding to TL protein) was lower in fraction 5 than in fraction 4. This observation suggested that the lower turbidity developed in fraction 5 than in fraction 4 depended from the lower TL protein content of the former. Assuming TL proteins as the main responsible for wine haze formation, this turbidity value scale seemed to be reasonable, with the highest instability of fraction 6 (highest TL protein content), followed by fraction 4 (second TL protein content) and 5 (third TL protein content).

Three first eluting HIC fractions (fractions 1, 2 and 3) resulted stable after the heat test (fig. 4.10), despite they contained proteins apparently belonging to the chitinase and thaumatin-like protein classes. A possible interpretation of this event was based on the data regarding the polysaccharidic content of the different fractions, as determined by combining the information from both UV (protein) and Refractive index (sugar) detection of the SEC analysis for each HIC fraction

To simplify this interpretation, the ratio between protein (as measured at 280 nm) and refractive index peak areas obtained by SEC was calculated (tab. 4.2).

HIC fractions	Retention Time (min)*	Refractive Index (Area µV*sec)	UV Absorbance (Area 280 nm)	Ratio RI/UV
Fraction 1	11.342	688073	128777	**5.34**
	14.896	19912	n.d.	-
Fraction 2	11.344	436981	132813	**3.29**
	12.683	34000	614082	0.06
	15.430	111180	1137353	0.10
Fraction 3	11.319	5307	2492	**2.13**
	12.633	1168	20662	0.06
	15.367	2275	29088	0.08
Fraction 4	11.200	426	n.d.	-
	12.750	2114	37055	0.057
	15.390	20320	210699	0.096
	16.850	2245	48850	0.046
Fraction 5	11.183	466	n.d.	-
	12.633	824	n.d.	-
Fraction 6	16.14	81994	821280	0.10
Fraction 7	n.d.	n.d.	n.d.	-
Fraction 8	11.167	4367	2837	**1.54**

*n.d.: not detected. * RTs of the peaks were delayed of 0.3-0.4 minutes of compared to those of*

table 4.1 due to the passage through the refractometer.

Table 0.2. *Ratio between the Peak areas (µV*sec) detected by the Refractive Index (RI) and UV (A_{280} nm) detector after SEC analyses of the HIC protein fractions*

The RI/UV ratio for each peak suggested the relation between the value of this ratio and the haze produced after the heat test by the different HIC fractions, the higher being the ratio the lower the turbidity. This should indicate that the presence of glycocompounds (detected by refractometry) in a fraction impaired its hazing, also if potentially unstable proteins are present in the same fraction. From the literature, the hypothesis of a stabilising effect of glycocompound, including polysaccharides derived from both the grape berry and yeast (mannoproteins) is generally supported (Waters *et al.*, 1994a; Waters *et al.*, 1994b; Moine-Ledoux and Dubourdieu, 1999; Dupin *et al.*, 2000; Lomolino and Curioni, 2007). It was interesting to note that the protein with RT of 11.1 min of HIC fraction 1 (corresponding to the protein with 10.800 min RT on table 4.1) had the highest RI/UV ratio (table 4.1), confirming the data of sugar detection on gels that were confirmed also for fraction 8 (fig. 4.6).

1.1.43 STUDIES ON WINE PROTEIN REACTIVITY WITH SEED TANNINS

1.1.43.1 Preliminary experiments

In order to study the interactions occurring between wine proteins and grape seed tannins, several tests were performed. As well known for a long time tannins are polyphenolic compounds that form insoluble complexes with proteins (Swain, 1965) and the protein reactivity with these compounds has been studied as a tool for protein removal (Powers *et al.*, 1988). Tannin-protein interactions is important for the sensation of astringency in the mouth, but also for several phenomena occurring during winemaking, including protein haze formation in white wines (Luck et al., 1994; Sarni-Manchado *et al.*, 1999; Sarmento *et al.*, 2000; Mesquita *et al.*, 2001). Therefore, wine protein reactivity with endogenous grape tannins has been extensively studied, although a precise characterisation of the effect of the single wine protein components is scant (Somers and Ziemelis, 1973; Siebert, 1999).

Initially, to confirm the statement that wine proteins can be insolubilised in the presence of tannins (Powers *et al.*, 1988), an experiment was set up by dissolving the total lyophilised wine proteins in model wine and by monitoring the turbidity formation (measured spectrophotometrically at 540 nm and taken as a measure of protein-tannin reactivity) at increasing grape seed tannins dosage (fig. 4.11).

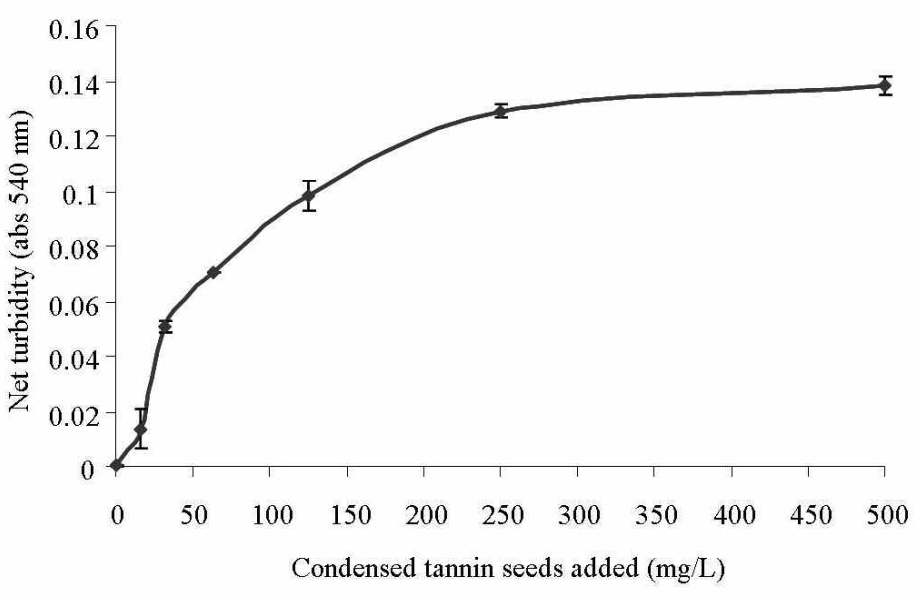


Figure 0.11. *Turbidity produced after reaction at room temperature of wine proteins from an unfined Manzoni bianco wine (200 mg/L in model wine) with increasing seed tannin concentrations. Turbidity was monitored spectrophotometrically at 540 nm against blanks prepared without protein.*

Results showed that the highest haze value was achieved at 250 mg/L of tannins dosage, followed by a plateau effect probably due to the saturation of the protein binding sites.

Furthermore, to determine the effect protein concentration on turbidity, an experiment with both different dosages of tannins and wine proteins was performed (fig. 4.12).

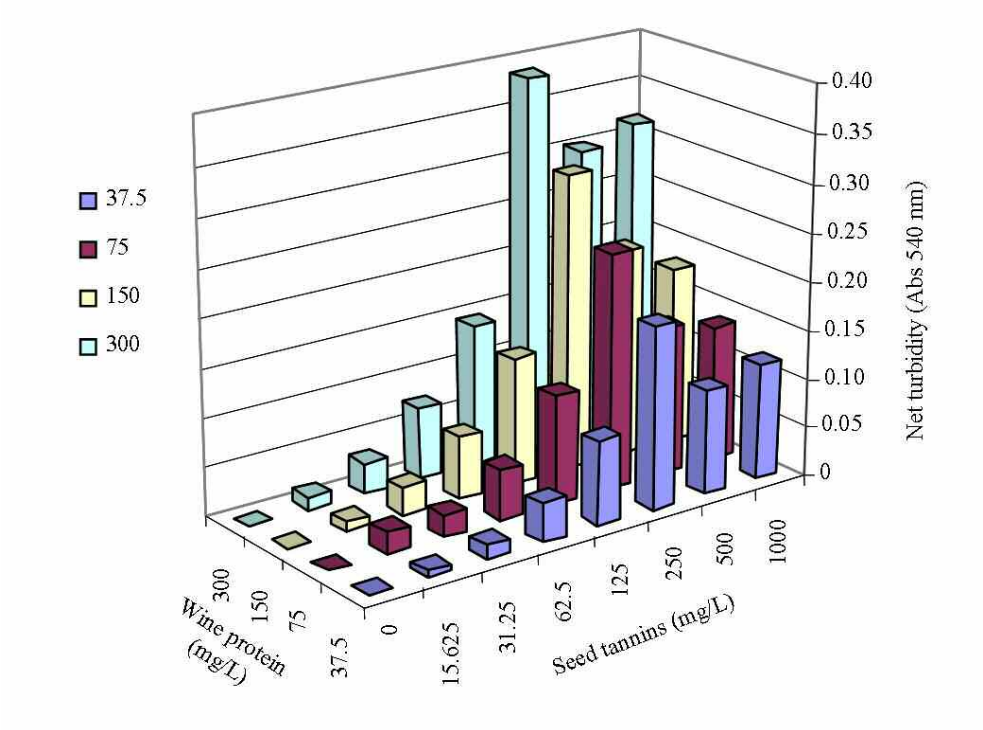


Figure 0.12. *Turbidity produced after reaction, at room temperature, of wine proteins from unfined Manzoni bianco wine at increasing concentrations (from 37.5 mg/L to 300 mg/L) in model wine with increasing seed tannins dosages (from 0 to 1000 mg/L). Turbidity was monitored spectrophotometrically at 540 nm against blanks prepared without protein.*

The results showed that as tannin concentration increased at a fixed level of protein content, the observed turbidity at first rose, then reached a plateau (at 250 mg/L) and then declined. A similar behaviour was observed by Siebert and colleagues (1996) by monitoring the haze formation at different dosages of gelatin and tannin acid.

The relation existing between the hydrophobicity of single wine protein fractions, separated by HIC, and the tannin reactivity was then studied. As a matter of fact, protein hydrophobicity is one of the characteristics that mainly affect tannin-protein interactions (Oh *et al.*, 1980; Siebert *et al.*, 1996).

1.1.43.2 HIC fractionation of wine proteins

In order to obtain protein amounts sufficient to characterise protein-tannin

reactivity of single wine protein fractions differing in hydrophobic character, several new Manzoni bianco wine protein fractionations were performed and fractions pooled.

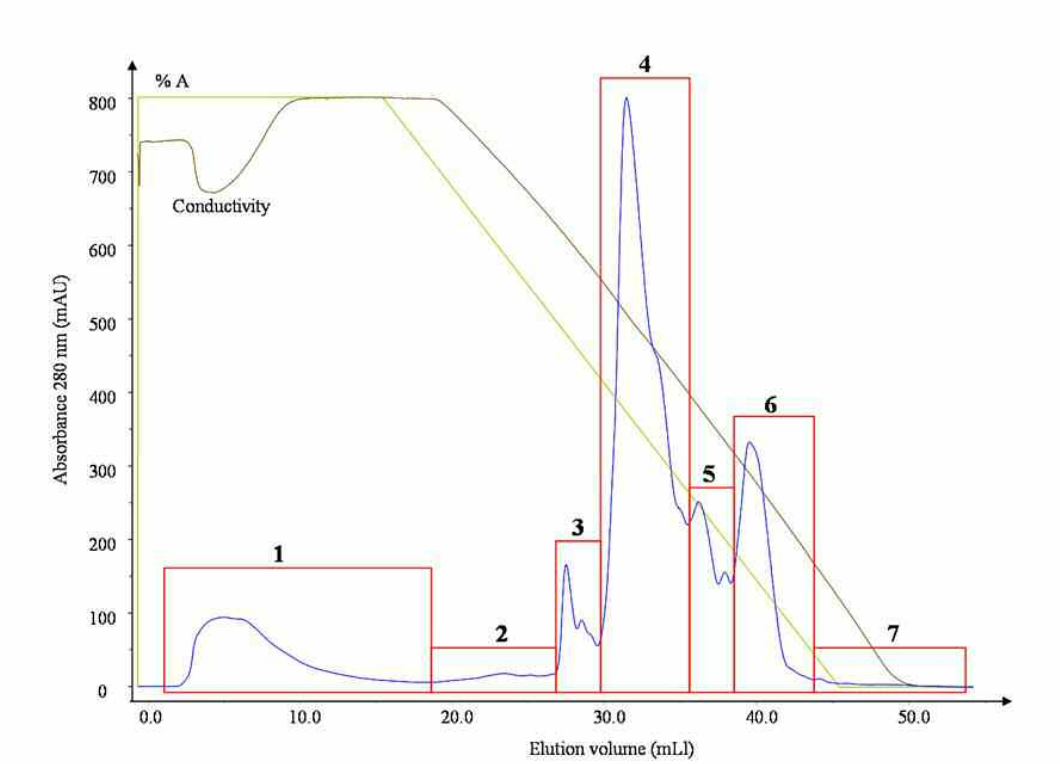


Figure 0.13. *HIC fractionation of Manzoni bianco wine proteins achieved by Bio-suite column (Waters). Collected fractions are indicated by numbered boxes.*

Seven fractions were collected from each HIC separation (fig. 4.13). Fractions were concentrated and dialysed by means of Vivaspin tubing (MWCO 3500 Da). HIC fractions so prepared were stored at -20°C before being studied.

1.1.43.3 Studies on the nature of HIC wine protein fractions

Because the fractions obtained by HIC were those which had to be used for the study of the interactions with tannins, they were preliminarily characterised by electrophoretic and chromatographic methods.

After protein content determination (not shown), a series of experiments was performed starting from SDS-PAGE analysis of the different fractions, which was done in order to visualise the proteins contained in each HIC fraction (fig. 4.14).

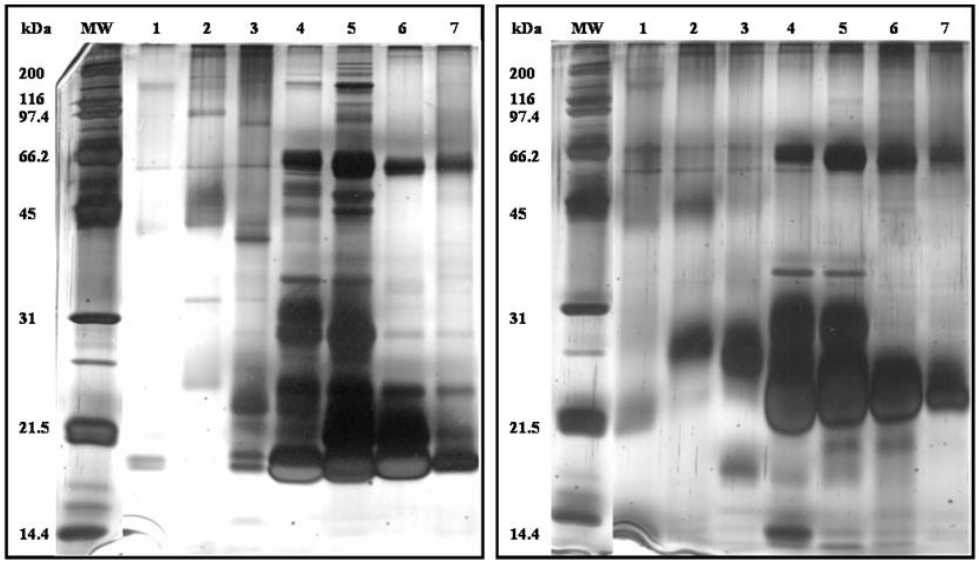


Figure 0.14. *SDS-PAGE (T = 14%) in non-reducing (left) and reducing (right) conditions of the fractions collected after HIC of the wine proteins. Lanes 1-7 correspond to HIC fractions 1-7 (see fig. 4.14). 6 µg of protein was loaded on each lane. Staining was performed with silver procedure. MW standard proteins are on the left of each gel (lanes MW).*

After SDS-PAGE separation in reducing conditions, the same fractions were also stained with the PAS procedure to highlight the presence of glycocompounds (fig. 4.15).

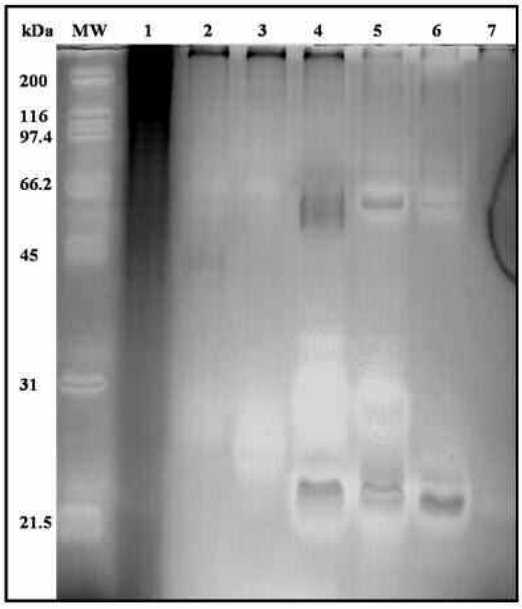


Figure 0.15. *SDS-PAGE (T = 14%, C = 3%) in reducing conditions of HIC of the*

fractions collected after HIC of the wine proteins. Lanes 1-7 correspond to HIC fractions 1-7 (see fig. 4.14). Staining performed with the PAS procedure.

The PAS results confirmed what had been observed on the previous HIC fractionation of the wine proteins (see 4.4.2) showing that the HIC fraction richest in polysaccharides was fraction 1 as confirmed by the total polysaccharide quantification (data not shown).

After staining for both proteins and sugars (fig. 4.14 and 4.15, respectively), fraction 1 showed the appearance of a streaking material resulting in a shadow all along the lane, suggesting that polyphenols might be bound to some wine protein and that, consequently they could disturb the protein interaction with the resin during analyses. Accordingly, Hagerman and colleagues (1998) observed that nonpolar tannins can bind proteins forming a hydrophobic coat around them resulting in their modified solubility. This fact could explain the hiding of the protein interaction capacity with the HIC matrix.

Fractions 2, 3 and 4 showed to contain high molecular weight glycocompounds visible at the top of the gel, whereas fractions 4, 5 and 6 showed a glycosilated band around 65 kDa (fig. 4.15), presumably corresponding to a grape invertase. The PAS staining of bands at low molecular weight (≈ 22 kDa) could be due to the overloading of the gel, which led to detection of glycosilated proteins or protein fragments normally not visible with lower protein loadings. This result was observed other times and always when high protein quantities were loaded on the SDS-PAGE gel (not shown).

Further analyses were done to study the nature of proteins fractionated by HIC by using the method proposed by Peng and co-workers (1997) for RP-HPLC protein identification (fig. 4.16 and tab 4.3).

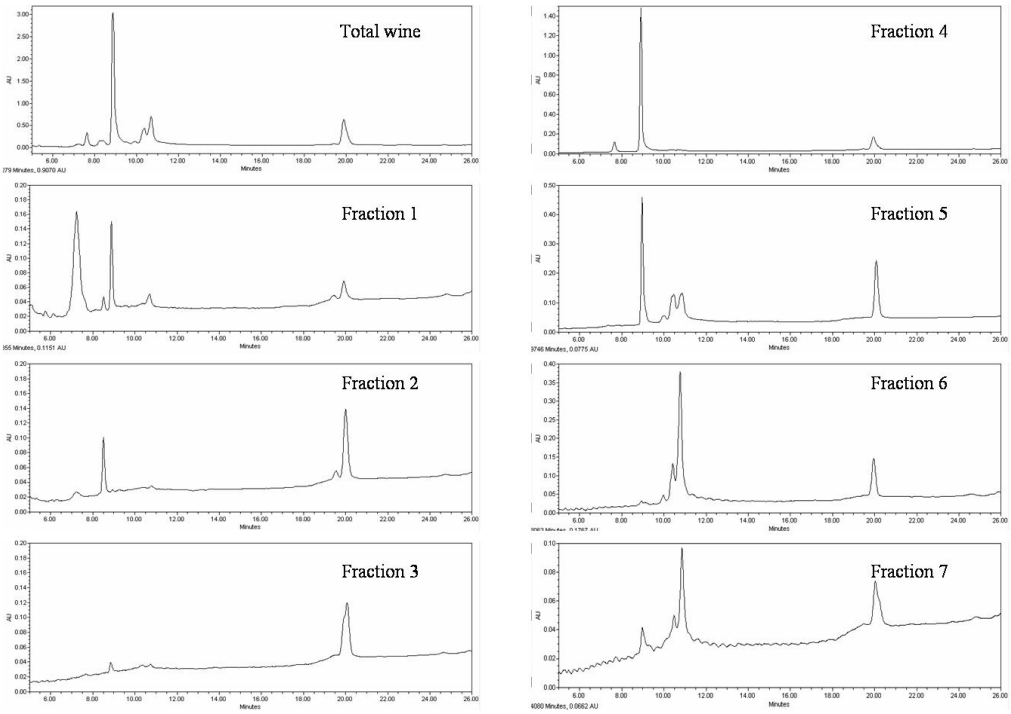


***Figure 0.16. RP**-HPLC chromatograms (C18 Vydac column) of HIC wine protein fractions (1 to 7, see fig. 4.14)) and of the total Manzoni bianco wine proteins.*

Peak RT (min)	HIC 1 (Area %)	HIC 2 (Area %)	HIC 3 (Area %)	HIC 4 (% Area)	HIC 5 (Area %)	HIC 6 (Area %)	HIC 7 (Area %)
5.800	0.96	n.d.	n.d.	n.d	n.d	n.d	n.d
6.100	0.67	n.d.	n.d.	n.d	n.d	n.d	n.d
7.200	**63.34**	8.7	n.d.	n.d	n.d	n.d	n.d
7.700	n.d.	n.d.	n.d.	7.31	n.d	n.d	n.d
8.500	2.75	28.71	n.d.	n.d	n.d	n.d	n.d
8.900	17.57	n.d.	6.45	**77.36**	**37.50**	2.11	15.00
10.000	n.d.	n.d.	n.d	n.d	2.18	1.94	n.d
10.400	n.d.	n.d.	1.93	0.55	17.01	15.05	11.51
10.700	6.45	n.d.	1.53	0.37	17.02	**62.66**	**47.01**
19.400	1.48	5.38	n.d	0.48	n.d	n.d	n.d
19.900	5.70	**57.21**	n.d	13.94	n.d	17.17	n.d
20.100	n.d.	n.d.	**90.09**	n.d	26.29	n.d	26.48
24.800	1.07	n.d.	n.d	n.d	n.d	1.07	n.d
Total (%)	100	100	100	100	100	100	100

***Table 0.3.** Area percent of the RP-HPLC peaks (see fig. 4.16) within the wine protein HIC fractions (1-7, fig. 4.14). Bold numbers indicate the percentage of the area of the main peak of each fraction.*

The RP-HPLC results showed that HIC of wine proteins actually gave fractions differing in protein composition, which had to be related to different hydrophobicity levels.

The total wine protein RP-HPLC profile showed to be the richer of peaks in the first part of the chromatogram (from 7 to 11 minutes of RT), while around 20 minutes of RT only one peak appeared. According to the data collected during Semillon protein identification (chapter 3, fig. 3.11), peaks with this latter RT were considered to be chitinases, while the earlier peaks corresponded to thaumatin like proteins of different classes. These proteins were differently distributed in the individual HIC fraction. This point will be discussed more in detail later.

In order to better characterise the proteins contained in the HIC-separated wine protein fractions, an additional chromatographic analysis were then carried out by, Size Exclusion Chromatography (fig. 4.17 and tab. 4.4).

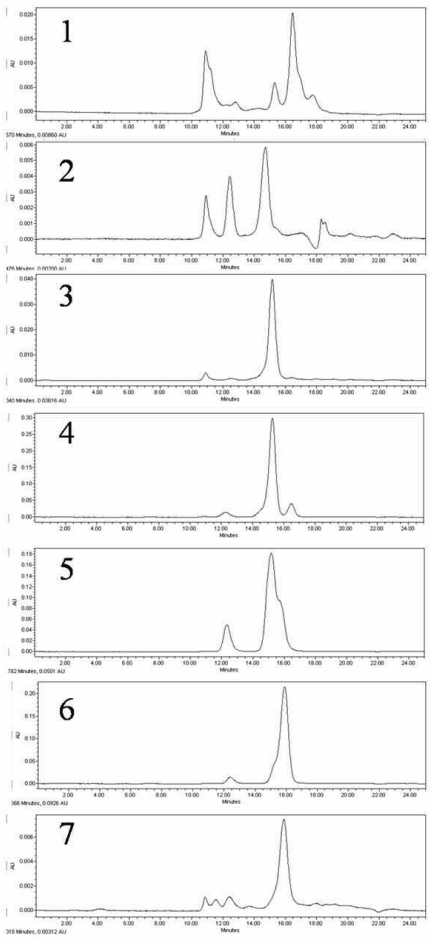


Figure 0.17. *Size Exclusion Chromatography- HPLC (Protein Pak 125, Waters) of the HIC wine protein fractions (1 to 7, see fig. 4.14).*

Peak Retention Time (min)	HIC 1 (Area %)	HIC 2 (Area %)	HIC 3 (Area %)	HIC 4 (% Area)	HIC 5 (Area %)	HIC 6 (Area %)	HIC 7 (Area %)
10.90	28.0	16.4	4.3	0.5	n.d.	n.d.	5.9
11.54	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.1
12.27	2.3	n.d.	n.d.	4.8	14.2	n.d.	n.d.
12.43	n.d.	24.7	1.2	n.d.	n.d.	5.1	9.9
12.81	4.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14.35	3.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14.72	n.d.	**51.2**	n.d.	n.d.	n.d.	n.d.	n.d.
15.16	n.d.	n.d.	**92.1**	n.d.	**85.4**	n.d.	n.d.
15.32	10.4	n.d.	n.d.	**83.8**	n.d.	n.d.	n.d.
15.90	n.d.	n.d.	n.d.	n.d.	n.d.	**94.5**	**74.1**
16.47	**43.1**	n.d.	0.6	10.7	n.d.	n.d.	n.d.
17.77	8.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18.31	n.d.	5.0	n.d.	n.d.	n.d.	n.d.	n.d.
20.14	n.d.	0.6	n.d.	n.d.	n.d.	n.d.	n.d.
22.91	n.d.	2.1	n.d.	n.d.	n.d.	n.d.	n.d.
Total (%)	100	100	97.6	99.8	99.6	99.6	96

Table 0.4. *Area percent of the SE-HPLC peaks (see fig. 4.17) within each wine protein HIC fractions (1-7, fig. 4.14). Bold numbers indicate the percentage of the area of the main peak of each fraction.*

By combining the data collected from SDS-PAGE stained for protein and sugar detection, and HPLC analyses in both Reverse Phase and Size Exclusion modes the following considerations for each HIC wine protein fraction could be made.

HIC Fraction 1. When analysed by SDS-PAGE, fraction 1 showed a poor protein pattern, while resulted the fraction with the highest glycocompound content, as determined by PAS staining. RP-HPLC analysis showed a major peak at 7.2 minutes of retention time. This peak resulted peculiar of this fraction (a minimal amount being detected only in the following fraction (2), and probably corresponded to the first part of the peak at 10.9 min of RT visualised by SEC, suggesting that glycosilated compounds were contained in it. The second peak (in terms of percentage area) detected by RP-HPLC showed RT of 8.9 min, and was

likely to correspond to the 16.47 min RT on SEC that, from the protein identification of chapter 3, could reasonably to be a thaumatin-like protein. This hypothesis was confirmed from the SDS-PAGE analysis in which a band at about 21 kDa (in reducing conditions) was detectable (fig. 4.14, right panel). However, the relative height of peaks in SE- and RP-HPLC was in disagreement with the former discussion. An explanation could be found in the higher polyphenols content of fraction 1 (not shown) which might interfere with the UV signals leading to a misunderstanding of the real quantities of each protein. Other proteins were detectable in HIC fraction 1, which on the basis of their RT in RP-HPLC (RT of 19.4 and 19.9 min) in were classifiable as two chitinases (Waters *et al.*, 1996; Peng *et al.*, 1997).

HIC Fraction 2. Fraction 2 contained a residual amount of the glycosilated compounds recoverable in fraction 1 (RP-HPLC RT 7.2 min; SEC RT 10.9 min) confirming the results showed with PAS staining of the SDS-PAGE gel (fig. 4.15). From the RP-HPLC analysis this fraction resulted to contain only proteins recoverable also in fraction 1, but in different percentages. Indeed, HIC fraction 2 seemed to be mainly composed of chitinases (SEC RT 14.7 min, RP-HPLC RT 19.9 and 19.4 min), in accordance with the SDS-PAGE results (fig. 4.14).

HIC Fraction 3. The RP-HPLC profile of HIC fraction 3 was the easiest to interpret, with the presence of a main peak (90 % of total area) recognised as corresponding to a chitinase (Waters *et al.*, 1996; Peng *et al.*, 1997) and three small peaks appearing in the thaumatin like protein chromatographic position (Waters *et al.*, 1996; Peng *et al.*, 1997).. These results were confirmed by those of the SEC analysis, where a similar profile was identified with a main peak at 15.1 minutes of RT. The differences in the chitinases RTs noted between fraction 2 and 3, (19.9 and 20.1 min respectively) by RP-HPLC and those detected by SEC 14.7 and 15.1 min), together with the different migration rate showed by these bands in SDS-PAGE (Fig. 4.14) led to assess the occurrence of chitinases of different nature in the two HIC fractions, confirming the existence of different chitinase isoforms, as suggested by Pocock and colleagues (2000).

HIC Fraction 4. Fraction 4 showed four main bands in SDS-PAGE (reducing conditions, fig. 4.14, right panel) with apparent molecular weights of

66, 35, 22 and 12 kDa. The 12 kDa band was assumed to be a chitinase fragment deriving from the protein reduction process, because this band was undetectable in non-reducing conditions (fig. 4.14, left panel). However, the escape of this band from the gel in non-reducing conditions, which can result in an increased SDS-PAGE migration rate, could not be excluded. Besides, three peaks have been detected in both RP- HPLC and SEC analyses. The main RP- HPLC peak showed a RT of 8.9 min, which suggested the identification of this protein as a thaumatin like protein (Waters *et al.*, 1996; Peng *et al.*, 1997). This peak accounted for the 77% of the total area though the same peak showed an area percentage of 83 % by SEC analysis (RT of 15.3 min). The RP-HPLC results indicated the presence of a 13% of chitinases in HIC peak 4 (19.900 min RT) (Waters *et al.*, 1996; Peng *et al.*, 1997). The SDS-PAGE band intensity seemed to confirm the RP-HPLC results by showing the highest staining for the TL protein band (22 kDa). Another protein of interest detectable in fraction 4 was that showing a MW of ≈ 66 kDa in SDS-PAGE. This protein appeared also in the PAS-stained gels leading to suppose it as being an invertase (Hsu and Heatherbell, 1987a; Brissonet and Maujean, 1993; Marchal *et al.*, 1996; Dambrouck *et al.*, 2005). The PAS staining of the gel showed this fraction to contain also high MW glycosilated compounds (fig. 4.15). These high MW compounds were detectable by SEC analysis while no trace of them were noted by RP-HPLC, probably due to the chromatographic system adopted, which was specifically developed to distinguish the thaumatin-like protein from the chitinases (Waters *et al.*, 1996; Peng *et al.*, 1997).

HIC Fraction 5. Fraction 5 showed the presence of 4 principal bands in SDS-PAGE with apparent MW of 66, 35, 30 and 22 kDa, showing a profile similar to that of fraction 4 (fig. 4.14). In this fraction, the PAS-stained gel revealed a band at 66 kDa, showing the probable presence of invertase in this fraction too. By SEC analysis it was possible to detect a peak at 12.3 min of RT that indicated the presence of a high MW compounds. This peak was likely to correspond to high MW material stained at the top of the SDS-PAGE gel (fig. 4.14). The other SEC peak had a RT of 15.18 min, though a shoulder was detectable at about 15.9 min of RT. This fact was probably due to the similar MW of the chitinases and thaumatin like proteins contained in fraction 5, resulting in a bad SEC separation also due to the high protein loading. A better understanding of

the nature of the proteins of this fraction was achieved with RP-HPLC analysis, which allowed to distinguish 5 peaks with RT of 8.9 (the same of that in fraction 4), 10, 10.4, 10.7 and 20.1 min. By RP-HPLC, the peak at 8.9 minutes of RT was recoverable in every single fraction except for fraction 2. From previous experiences and from the SDS-PAGE analysis it was possible to deduce that this peak (8.9 RT) corresponded to a protein of about 22 kDa, probably a thaumatin-like protein. The three peaks detected during minute 10 of the RP-HPLC separation were assumed to be different forms of TL proteins. Instead, the RP-HPLC peak eluting at 20.1 min should be a chitinases although it had to be an isoform different from that observed on fraction 3 because of the different hydrophobicity showed by the HIC fractions 5 and 3.

HIC Fraction 6. Fraction 6, which had to contain very hydrophobic wine proteins, showed a SDS-PAGE profile (in reducing conditions) with a band at 66 kDa and a heavy band at 22-23 kDa, that possibly hided other minor bands with similar MW. In non-reducing conditions, the SDS-PAGE pattern appeared less simple with the manifestation of a band with an apparent MW of ≈ 27 kDa (fig. 4.14). This band was supposed to be a chitinase because in the RP-HPLC data 4 detected peaks were assumed to be thaumatin-like proteins (RT 8.9, 10, 10.4 and 10.7 min) while a peak (RT 19.9 min) was assumed as a chitinase (Fig. 4.16). The main percent area (62.6%) was calculated for the peak at 10.7 min of RT, indicating fraction 6 as that containing the main portion of this protein which was similar to that identified as VvTL (gi|33329390) during Semillon grape proteins identification (see paragraph 3.4.2). The SEC analysis confirmed this hypothesis, showing a main peak with a RT (15.9 min) compatible with the mass of a thaumatin-like protein.

HIC Fraction 7. Fraction 7, which was the most hydrophobic one, was only a tail of fraction 6, as appears from the HIC fractionation chromatogram (Fig. 4.13). Actually, the main peak detected in both the chromatographic analyses (fig. 4.16 and 4.17) as well as the SDS-PAGE pattern (fig. 4.14) was the same of fraction 6. Consequently, the protein composition of fraction 7 was similar to that of fraction 6 also if some differences were noticed. In particular, in this HIC fraction three additional small peaks were visible by SEC analysis (RT 10.8, 11.5 and 14.4 minutes) indicating the presence, although in low amounts, of

high MW compounds with high hydrophobicity.

1.1.43.4 Heat stability of wine protein fractions separated by Hydrophobic Interaction Chromatography

The HIC fractions were heat tested to determine their hazing potential (fig. 4.18).

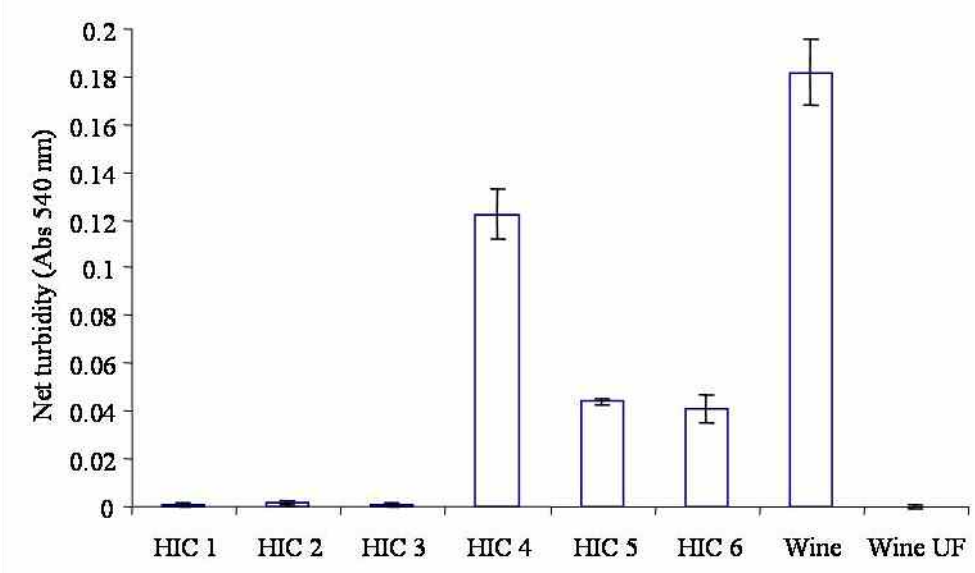


Figure 0.18. *Heat test results of fraction 1 to 6 (HIC 1- HIC 6) collected by HIC fractionation of wine proteins. Each HIC fraction was prepared at 200 mg/L of protein concentration in an ultrafiltered Manzoni bianco wine. The total wine proteins (wine) were tested by dissolving the unfractionated proteins at 200 mg/L in ultrafiltered Manzoni bianco wine. The ultrafiltered wine without protein addition (UF was also tested. Turbidity values (A_{540}) higher than 0.02 means instability.*

The first three fractions eluted from HIC showed the lowest turbidity formation after heating. In fraction 1 and 2, the absence of haze formation could be explained by the large content of high molecular weight glycosilated compounds (see above), which are known to show a protective effect against haze formation. Indeed, the disrupting action of structurally different polysaccharides towards grape seed procyanidin complexation and aggregation by bovine serum albumin has been reported (de Freitas *et al.*, 2003; Mateus *et al.*, 2004). The explanation for the absence of heat-induced haze for fraction three seemed to be different. This fraction, showing a relatively low a hydrophobicity level,

contained mainly chitinases (90% of the proteins, as determined by RP-HPLC) and some glycosilated compounds, as highlighted from PAS staining of the SDS-PAGE gels (see above). Therefore, the reason for the lack of haze formation seemed due to the protective action against chitinases flocculation made by glycocompounds or, less probably, to the low haze potential of chitinases present in this fraction.

In contrast to the first 3 fractions, haze formation (turbidity values higher than 0.02 AU) was observed for HIC fractions 4, 5 and 6, indicating the presence of heat-unstable compounds. Differently from the first heat test experiment performed on HIC fractions (fig. 4.10), the highest haze formation was observed for fraction 4. The reasons for the high turbidity formed by fraction 4 are related to the nature of its proteins, which mainly comprised a thaumatin-like protein (but different from that of fraction 6) and also chitinases, both these PR-proteins being known as the main responsible for haze formation in wines (Waters *et al.*, 1996). Moreover, the SDS-PAGE pattern showed that the bands intensity in fraction 4 was higher than that found for the other HIC fractions (fig. 4.14), leading to hypothesize that protein content determination by KDS-BCA method might lead to a underestimation of the protein content of fraction 4, which actually contained a quantity of protein higher than believed, thus explaining the high turbidity formed after heating.. Fractions 5 and 6 showed similar levels of haze formation. However, the analyses of their protein composition suggested different causes for the turbidity level showed by the two fractions after heating. In particular, fraction 5 contained 4 different TL protein peaks (by RP-HPLC) with the prevalence of the TL protein with 8.9 min of RT, the same observed for the TL protein of fraction 4. Moreover, a chitinases was recoverable in fraction 5 but different from that detected in fraction 6. Therefore these two fractions strongly differed for protein composition although they behaved similarly when heat tested.

For the previous results it seems that the wine proteins eluted after the middle of the HIC gradient were the most heat-unstable while those collected on the first half of the gradient, although containing PR-protein components, showed high heat stability. This should indicate that a certain relation exists between protein hydrophobicity and hazing potential. Taking into account that the heat test experiments were done with protein fractions dissolved in (ultrafiltered) wine, that

contained the original wine polyphenols, and considering haze formation as mainly due to protein-polyphenol interactions (Somers and Ziemelis, 1973; Yokotsuka *et al.*, 1983; Waters *et al.*, 1995) the protein hydrophobicity can be seen as affecting haze formation by determining the degree of protein interactions with wine polyphenols.

1.1.43.5 Haze formation after addition of seed tannins to wine protein fractions differing in hydrophobicity

Due to the importance of tannin-protein interactions in haze formation, the capability of Manzoni bianco wine protein fractions (deriving from the HIC fractionation of fig. 4.13) differing in hydrophobicity in reacting with tannins was studied. Moreover, in order to study the factors involved in protein hazing, the effects of protein heating and sulphate addition were investigated.

Initially, the effects of seed tannin addition on turbidity formation in model wine containing 25 mg/L of protein from the HIC fractions was assayed spectrophotometrically at 540 nm (fig. 4.19). The final tannin concentration was 250 mg/L, according to the preliminary results indicating this dosage as that able to give the maximum turbidity development.

Some turbidity was formed immediately after tannin addition (green bars), but the turbidity strongly increased after boiling the samples for 5 minutes (red bars). Moreover, the proteins of HIC fractions were heated (10 minutes, 100°C) in model wine before the tannin addition, obtaining a turbidity (grey bars) similar or even lower than those observed in the other cases.

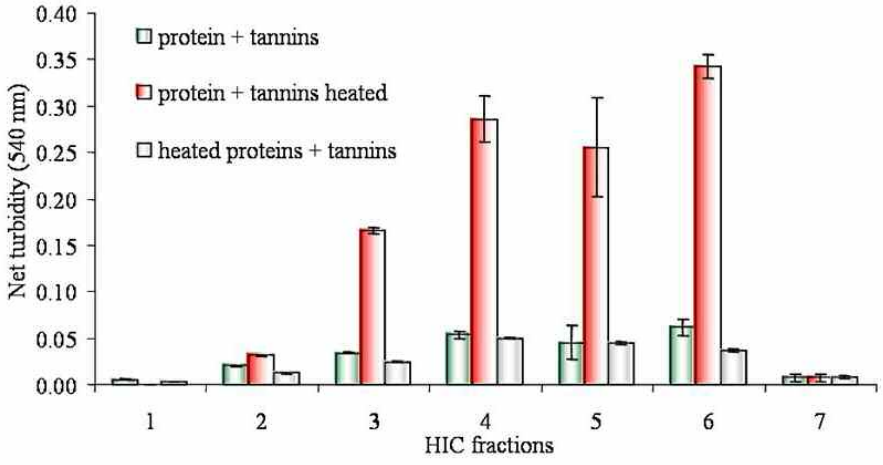


Figure 0.19. *Reactivity of HIC fractions 1-7 (see fig. 4.13) with seed tannins. Reactivity*

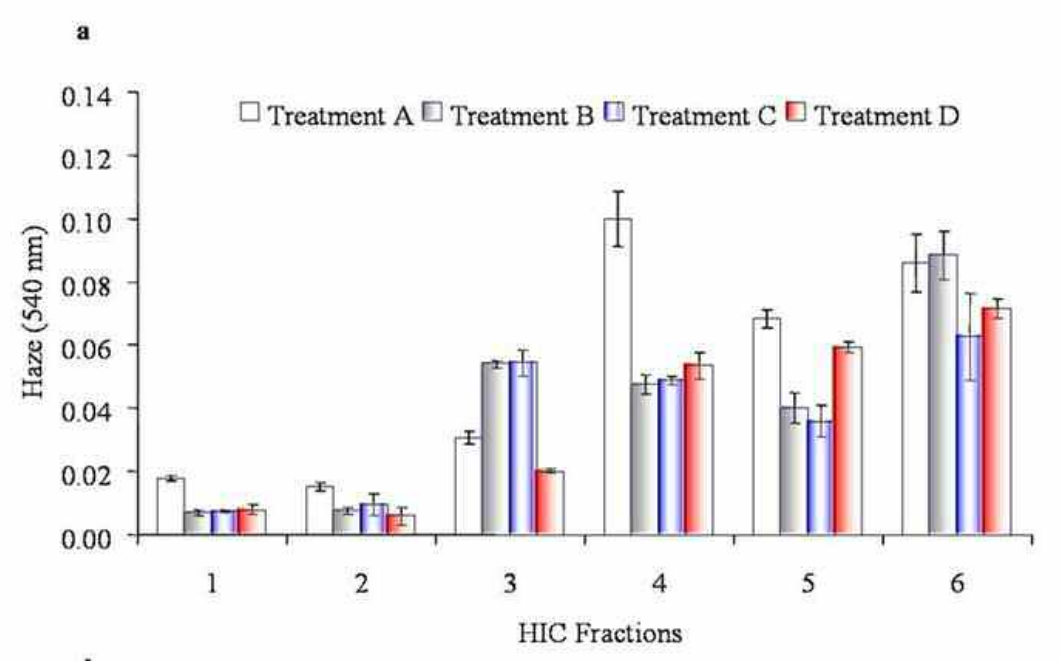
was calculated by measuring the turbidity developed immediately after tannin (250 mg/L) addition to model wine containing 25 mg/L of protein from each HIC fraction. Blanks were prepared without protein. Green bars: reaction at room temperature; Red bars: reaction after heating the mixture (100 °C, 10 minutes); Grey series: reaction at room temperature with proteins heated (100 °C, 10 minutes) before tannin addition.

The results showed in each of the cases the lack of protein-tannin reaction, as measured by the development of turbidity, only for both fraction 1 and 7 in which absorbance values close to zero were detected. In contrast, fractions 2 to 6 immediately developed turbidity when seed tannins were added (fig. 4.19, green bars), although at different extent. By boiling the same samples, the turbidity dramatically increased following the same ranking of the unheated samples. In particular, the turbidity values tended to increase with the fraction number (from 2 to 6), suggesting a relationship between wine protein hydrophobicity and tannin reactivity. As suggested by Oh and co-workers (1980), tannins have a hydrophobic nature and protein-tannin interaction occurs with the involvement of hydrophobic bonding. The results here showed agreed with this hypothesis, confirming that the level of protein hydrophobicity affects their reactivity with tannins, supporting the idea that the level of protein hydrophobicity is a major factor affecting tannin-protein interactions (Oh *et al.*, 1980; Siebert *et al.*, 1996).

Moreover, the higher turbidity resulting from heating the samples indicated the role of the temperature in inducing haze formation. Apart from the effect on the reaction rate, heating the protein-tannin mixture should increase the extent of interaction by inducing protein denaturation and exposition of a high number of tannin binding sites, leading to an increase of haze formation (Koch and Sajak, 1959; Somers and Ziemelis, 1973; Yokotsuka *et al.*, 1991; Waters *et al.*, 1995). Siebert and co-workers (1996) also reported that during protein heating the polyphenol binding sites became exposed because hydrogen bonds are broken. This modification of the protein structure could probably lead to higher tannin-protein reactivity.

To verify whether protein denaturation by heating could be responsible for the increased tannin reactivity, the HIC fractions were heated in model wine (without tannins) before the tannins addition. After cooling the fractions at room

temperature tannins were added and the formed turbidity measured. The results showed a turbidity much lower than that obtained after heating the mixture and even slightly lower than that developed by the unheated fractions in the same reaction conditions, although the same trend was maintained. Therefore, protein heating in model wine without tannins did not allow a turbidity formation after tannin addition. A possible explanation of this fact is that protein denaturation occurring during heating in the absence of tannins is a reversible process. Heating should result in breaking of protein hydrogen bonds at high temperature (Siebert *et al.*, 1996) but these bonds are re-formed upon cooling at room temperature, thus restoring a protein structure with an affinity for tannins even lower than that of the original (un-heated) proteins, which actually showed slightly higher turbidity values after tannin addition (fig. 4.19). Consequently, it seems that protein reactivity with tannins is enhanced only when both the compounds are heated together, with the tannins binding to the protein during its presence in the heat-denatured state.



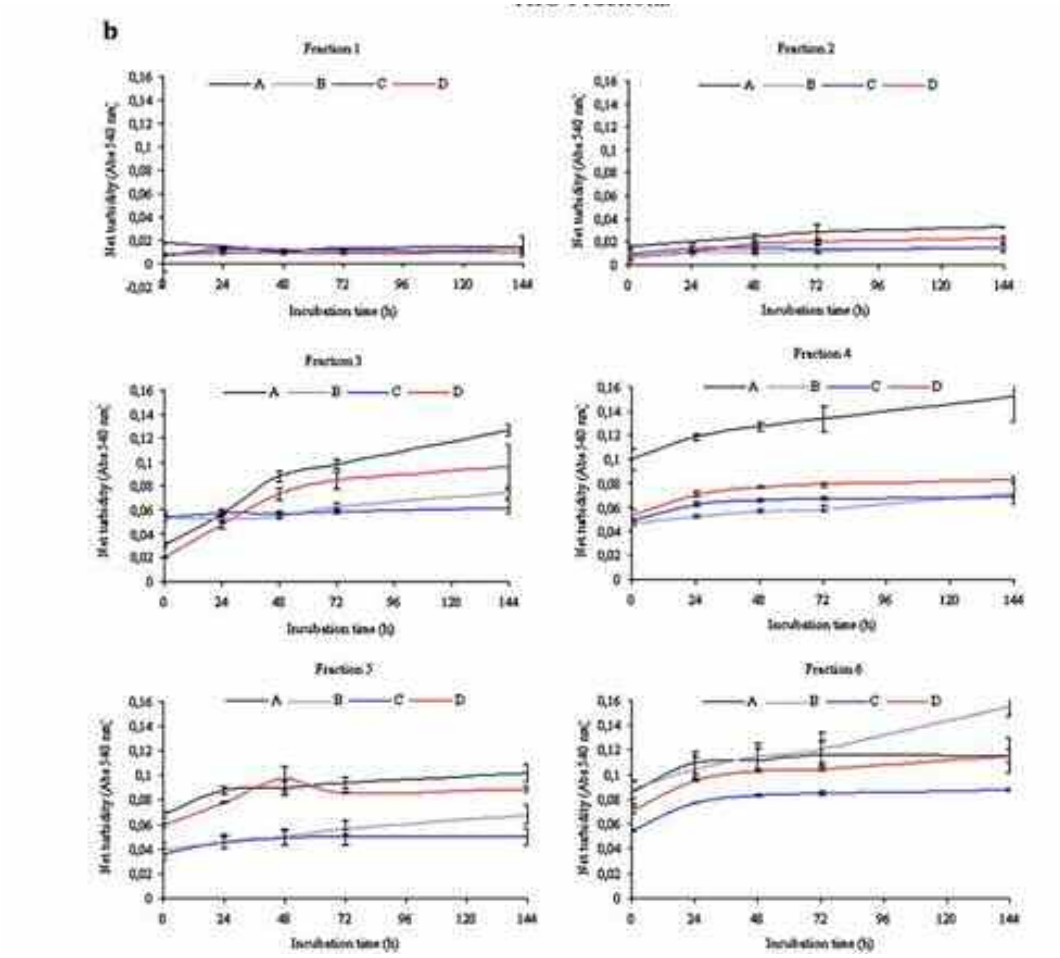


Figure 0.20. *Haze formation of the HIC fractions (25 mg/L protein) 1-6 (see fig. 4.13) after 250 mg/L tannin addition in model wine with 4 different treatments (A-D).* ***A****:, un-heated proteins, incubation at 25°C;* ***C****: pre-heated proteins (100°C, 10 minutes), incubation at 25°C;* ***B****: pre-heated proteins (100°C, 10 minutes) and 0.5 g/L* K_2SO_4*, incubation at 25°C;* ***D****: un-heated proteins and 0.5 g/L* K_2SO_4*, incubation at 25°C.* ***a)*** *Turbidity developed immediately after tannin addition* ***b)*** *Turbidity developed during 6 days after tannin addition.*

Recently, it has been demonstrated that one of the factors involved in protein haze formation in white wine is sulphate (Pocock *et al.* 2007). This compound would contribute to protein denaturation by a sort of salting-out effect promoting protein precipitation and hazing. In order to verify the effect of sulphate, also in combination with that of thermal protein denaturation, tannins were added to both un-heated and pre-heated HIC protein fractions in the presence and in the absence of sulphate. Turbidity was then measured immediately after the

addition (fig. 4.20 a) and during 6 days of incubation of the different mixtures (fig. 4.20 b). Immediately after tannin addition, the samples prepared without pre-heating the proteins and in the absence of sulphate (treatment A) showed results similar, but not identical to those of the previous experiment of fig. 4.19. However, the trend indicating lower haze formation of the less hydrophobic fractions was maintained. Moreover, a lower turbidity formation after pre-heating the proteins (treatments A and C in fig. 4.20, compare with fig. 4.19) was also confirmed for all the fractions and the highest haze formation in all the fractions except for fraction 3 was that obtained with treatment A. Contrary to what was expected, the addition of sulphate to the un-heated proteins-tannin mixtures at a dose claimed to enhance wine protein hazing (Pocock *et al.* 2007) (treatment D) always resulted in lower turbidity values compared to that found in the absence of sulphate, especially for HIC fraction 3 (fig. 4.20). This would indicate that sulphate impairs tannin-protein interactions by changing the ionic strength of the solution, which may increase the burying of tannin binding sites on the proteins probably as a consequence of the reinforcement of the hydrophobic forces in the core of the molecule. Therefore, it can be hypothesized that, in the presence of sulphate, a lower area of hydrophobic sites may be available for the interactions with tannins.

Pre-heating the protein fractions (at 100°C for 10 min in model wine) before tannin addition at 25°C (treatments B and C), generally confirmed to decrease the turbidity compared to the corresponding un-heated samples (treatments A and D), with the exception of fraction 3. In this case the presence of sulphate did not affect the results, except for the most hydrophobic fraction 6 (fig. 4.20).

However, in some HIC fractions, the haze initially formed showed to develop differently according to the different treatments. This was assed by following the variation of the turbidity values for each sample during 144 hours (fig. 4.20 B). The highest long-term turbidity was always detected with un-heated proteins and in the absence of sulphate (treatment A) in all the fractions, except the most hydrophobic fraction 6, which developed the strongest haze when the pre-heated proteins were incubated in the presence of sulphate (treatment B, fig. 4.20). In the other fractions, however, pre-heated proteins generally did not show

a turbidity increment as relevant as that noted for the corresponding un-heated samples. Another exception was HIC fraction 3, mainly made of chitinase (≈ 90 %, as assessed by HPLC) which showed an opposite behaviour, with a turbidity formation when un-heated lower than that observed when pre-heated. Fraction 3, but only when proteins were pre-heated, showed a linear turbidity increase in the first 48 hours, leading to hypothesize that chitinases might play a key role in the mechanism of proteins-tannin haze formation during wine storage. In this case, differently from fraction 6, the presence of sulphate seemed to reduce the long-term turbidity, but only in the pre-heated samples (fig. 4.20 b).

The different behaviour in haze formation of chitinases (mainly present in fraction 3) and thaumatin-like protein (mainly present in fraction 6) has been documented by Pocock and colleagues (2007), which affirmed that 150 mg/L of chitinases required 10 times less sulphate (15 mg/L) than that required by the same amount of thaumatin-like protein to form haze when heat tested. In our experimental conditions the sulphate was added at a dosage (500 mg/L) considered of saturation for the protein content (25 mg/L). Our data indicated that sulphate did not cause instabilization as observed when sample were heated, suggesting a different role played by the sulphate on un-heated proteins. Besides, a sulphate effect was detected in fraction 6 in which the B series showed the higher turbidity increase during the time. The sulphate presence during the heating of protein of fraction 6 (mainly thaumatin-like protein) seemed to affect the long-term protein-tannin reactivity. A theory is that tannins affect the particle size of denatured aggregated proteins, possibly through cross-linking. Several researchers have suggested a hydrophobic mechanism for the interaction between phenolic compounds and proteins, in which the protein has a fixed number of phenolic binding sites (Oh *et al.*, 1980; Siebert *et al.*, 1996). More of these sites are exposed when the protein is denatured, but this exposition should be in some cases a transient phenomenon occurring only at high temperature and influenced by the composition of the solution. However, the behaviour of the different wine protein fractions separated by HIC is clearly different when they are mixed with tannins, as also demonstrated by the different effects caused by heating and sulphate addition. Therefore the aspects related to both the protein structure and hydrophobicity in relation to their interactions with tannins leading to haze

formation warrants further investigation.

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CHAPTER 5

Selection of fungal proteases for the degradation of grape proteins

ABSTRACT

Currently bentonite is still required for the stabilisation of white wines, exploiting the ability of this adsorbent to remove the heat-unstable grape proteins from wine. The utilisation of proteolytic enzymes is widely considered an ideal replacement for bentonite because it does not produce the problems that are incurred with bentonite usage, such as lowering wine quality, wine loss in lees, filtration inconveniences, and waste disposal problems. In order to find an alternative to bentonite for haze prevention in white wines, acid proteases produced by four phytopathogenic fungal strains were tested. The ability of these proteases to degrade grape and wine proteins was preliminary evaluated by protease activity assays, residual protein content quantification and SDS-PAGE analyses. Subsequently, chromatographic separation was attempted to purify and concentrate the proteolytic activity and to check the effect on haze reduction of the obtained preparations. *Sclerotinia minor* and *Sclerotium rolfsii* highlighted the possibility to produce proteases active against grape and wine proteins although the purification steps resulted in significant activity losses.

Key words: Acid proteases, grape, wine, PR-proteins, fungi, haze, *Sclerotinia minor*, *Sclerotium rolfsii*.

INTRODUCTION

The quality of white wines can be impaired by the manifestation of sediments and hazes after bottling. Such precipitates are the result of denaturation of wine proteins (Bayly and Berg, 1967; Hsu and Heatherbell, 1987; Waters *et al*., 1992) that have been identified as pathogenesis-related (PR) proteins (in particular thaumatin-like proteins and chitinases) deriving from the grape berry (Waters *et al*., 1996, 1998). These PR-proteins are likely to protect the berry during ripening

against fungal pathogens (Høj *et al*., 2001).

Botrytis cinerea is an important fungal necrotrophic pathogen that infects at least 235 plant species (Jarvis, 1977). Indeed, the secretion of proteases by *B. cinerea* has been shown in culture media (Ten Have *et al*., 2004) and in fruits such as grapes, apples, tomatoes, zucchini, bell peppers and carrots (Touzani and Muna, 1994; Urbanek and Kaczmarek, 1985; Brown and Adikaram, 1983; Ten Have *et al*., 2004; Movahedi and Heale, 1990).

Marchal and co-workers (1998) immunodecteted the presence of *B. cinerea* proteins in a must obtained from highly botrytised (80%) grapes, with some of these proteins possibly having proteolytic activity responsible for the degradation of grape proteins. Similarly, Modra and Williams (1988), using commercial enzyme preparations, indicated that both plant and fungal proteases could significantly alter the chromatographic profile of a must protein fraction. Karmona *et al*. (1990), using haemoglobin as a substrate, showed that *B. cinerea* secreted an aspartic protease.

Damages caused by *B. cinerea* to grape berries have attracted the attention of many research laboratories throughout the world (Donèche, 1993; Marchal *et al*., 1998; Cilindre *et al*., 2007). Recently, it was observed that the levels of most of the soluble proteins recoverable in the free run juice from *Botrytis*-infected grapes were lower than those in the free run juice from healthy grapes (Marchal *et al*., 1998; Girbau *et al*., 2004, Marchal *et al*., 2006; Cilindre *et al*., 2007), as a result of the activity of proteolytic enzymes from the fungus which degrade the grape proteins.

Proteases secretion has been highlighted for many other fungal strains. For instance, Billon-Grand and co-workers (2002) detected the emission of three groups of mechanistically distinct proteases (aspartyl protease, non-aspartyl acid protease and serine protease) from *Sclerotinia sclerotiorum* and *Sclerotinia minor*. These fungi are necrotrophic pathogens that penetrate plant host surfaces, killing the underlying plant cells and invading the surrounding tissues. A common characteristic detected among these fungal strains is their ability to grow at acidic pHs (Billon-Grand, 2002). Consequently, they have been considered suitable for the production of proteolytic enzymes to be used for the degradation of grape and wine proteins, thus representing and alternative to the use of bentonite for white

wine protein stabilization.

MATERIALS AND METHODS

1.1.44 MATERIALS

Grapes and wines (vintages 2005 and 2006, variety Manzoni bianco) were kindly supplied from the "Scuola Enologica G.B. Cerletti" of Conegliano (Italy). Grapes were harvested in the experimental vineyard while wines were produced in the school winery. All the wines, having an average protein content of ≈ 300 mg/L, were not treated with bentonite.

1.1.45 PROTEIN EXTRACTION FROM GRAPES, WINE AND FUNGAL CULTURES

5.1.1.1. Concentration by ultrafiltration

The protein purification was conducted as a multi-step procedure. After sterile filtration of the liquid (grape juice, wine or fungal media) with cellulose acetate filters with pore size of 0.20 µm (Millipore), samples were concentrated by using a stirred ultrafiltration cell system (Amicon) equipped with 3000 Da MWCO membranes. The obtained retentates were dialysed against distilled water in 3500 Da porosity dialysis bags (Spectrapore). When required, a passage on solid phase extraction C-18 cartridge (1 mL resin, Supelco) was performed to "clean" the protein extract from residual polyphenols. Eventually, the obtained preparations were frozen, freeze-dried and dissolved in a small volume of water or citrate buffer (30 mM, pH 3.50) for long-term storage at -20°C.

5.1.1.2. Protein precipitation with potassium dodecyl sulphate (KDS)

In order to be analysed by SDS-PAGE or to be quantified by bicinchoninic acid (BCA) method (Smith *et al.* 1985), proteins were precipitated from the media by using the KDS method according to the procedure proposed by Zoccatelli and co-workers (2003). 10 µL of SDS (10% in water, Bio-Rad) were added to 1 mL of sample which was then heated (5 min, 100°C). 250 µL of 1M KCl (Carlo Erba) were added to the samples and, after at least two hours of incubation, the formed pellets were collected by centrifugation (15 min, 4°C). Further washes with 1 mL

of 1M KCl were required to completely eliminate the polyphenols from the collected proteins. Every measure was the average of at least three replicates.

1.1.46 GRAPE AND WINE PROTEIN CONTENT DETERMINATION

The protein content determination was performed according to Vincenzi *et al.* (2005). Firstly, proteins were precipitated from 1 mL of wine with the KDS method (Zoccatelli *et al*. 2003). The pellets were dissolved into 1 mL of distilled water and quantified by using the BCA-200 protein assay kit (Pierce). The calibration curve was prepared by using serial dilution of bovine serum albumin (BSA, Sigma) in water. The measurements were performed spectrophotometrically at 562 nm (Shimadzu UV 6010).

1.1.47 TOTAL POLYSACCHARIDE CONTENT DETERMINATION

The polysaccharide content was determined colorimetrically according to Segarra and co-workers (1995). After addition of 5 volumes of absolute ethanol (Baker), samples were left at 4°C overnight before centrifugation (30 min, 14000g). The collected pellets were washed twice with ethanol (Baker) and then dissolved in bi-distilled water. 1 mL of sample was then added of 25 µL of 80% phenol (w/w, Fluka) and 2.5 mL of sulphuric acid (Merck). Samples were mixed and the reaction carried on for 30 minutes at room temperature. Absorbance values were spectrophotometrically measured at 490 nm (Shimadzu UV 6010). The calibration curve was prepared by using serial dilution of galactose (Fluka) in water.

1.1.48 ENZYMATIC ASSAY FOR ACIDIC PROTEASES DETERMINATION WITH HEAMOGLOBIN AS SUBSTRATE

As a result of the necessity to measure the enzymatic activity secreted by the fungal strains in liquid cultures, a new enzymatic assay was developed as a modification of the Anson (1938) method for the determination of aspartil proteases activity with haemoglobin as the substrate. 900 µL of substrate (0.5 % w/v bovine haemoglobin in Glycine-HCl buffer, pH 3.20) was added of 200 µL of

sample to be tested for enzymatic activity and the reaction carried out at 37˚ C for 30 minutes. The reaction was stopped by adding 900 µL of 20 % (w/v) Trichloroacetic Acid (TCA, Baker) in bi-distilled water. Blanks were made by adding TCA before starting the reaction. Afterwards, samples were centrifuged (14000g, 15 min) and the absorbance of the supernatants was spectrophotometrically measured at 280 nm. The difference between the sample and blank values gave the net enzymatic activity. Pepsin (EC 3.4.23.1, Sigma) was used as a positive control.

1.1.49 ASSAY FOR ACIDIC PROTEASES ACTIVITY DETERMINATION WITH WINE PROTEINS AS THE SUBSTRATE

An assay for aspartil protease activity determination, based on a modification of the method proposed by Doi *et al*. (1981), was set. 100 µL of sample to be tested for enzymatic activity were added to 200 µL of a 0.5% (w/v) wine protein (purified from Manzoni bianco wine as formerly described) solution in 5 g/L tartaric acid buffer (pH 3.20) and the reaction kept at a 37˚ C for 30 minutes. After this time, proteins were precipitated by adding 1 mL of absolute ethanol. 200 µL of a 2% ninhydrin solution (Sigma) were added to the supernatant collected after centrifugation (14000g, 10 min) and the sample heated at 100°C for 10 minutes to allow the reaction to take place. After cooling the samples at room temperature, the absorbance was spectrophotometrically measured at 570 nm. Blanks were prepared by adding ethanol before the incubation.

1.1.50 HEAT TEST

According to Pocock and Rankine (1973), a heat test was performed to determine grape and wine protein stability. After heating (80°C for 6 hours), samples were chilled (16 hours at 4°C) and, after equilibration at room temperature, turbidity values were measured nephelometrically (Hach 2100P turbidimeter) or spectrophotometrically (Shimadzu UV 6010) at 540 nm (Waters *et al.*, 1991). Net turbidity values lower than 2 NTU (Net Turbidity Unit) or 0.02 AU (Absorbance Unit) indicated sample stability.

1.1.51 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970). Samples to be analysed were dissolved in a Tris-HCl pH 6.8 buffer containing 15% (v/v) glycerol (Sigma) and 1.5 % (w/v) SDS (Bio-Rad) and heated at 100°C for 5 minutes before loading. For SDS-PAGE in reducing conditions, 3% (v/v) of β-mercaptoethanol (Sigma) was also added to the loading buffer. Electrophoresis was performed in a Mini-Protean III apparatus (Bio-Rad) with T = 14% (acrylamide-N, N' metilen-bisacrylamide 29:1; Fluka) gels. The molecular weight standard proteins were: Myosin (200,000 Da), β-galactosidase (116,250 Da), Phosphorylase b (97,400), Serum Albumin (66,200 Da), Ovalbumin (45,000 Da), 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).

After electrophoresis, gels were stained for 18 h with Coomassie brilliant blue R-250 (Sigma) and then destained with 7 % acetic acid for 24 h (Koenig *et al.*, 1970).

1.1.52 GRAPE AND WINE PROTEIN CHROMATOGRAPHY

The chromatographic separations were performed by means of two instruments:

- An ÄKTA purifier FPLC (GE-Healthcare) equipped with an UV detector (λ Absorbance Detector). Collected data were processed by the Unicorn 5.11 software.
- A HPLC (Waters 1525) equipped with a Dual λ Absorbance Detector (Waters 2487) and a Refractive index detector (Waters 2414). Collected data were processed by Waters Breeze™ Chromatography Software (Version 3.30).

Each solution utilised and sample loaded were previously filtered with cellulose acetate filters (Millipore) with a pore size of 0.20 µm and degassed.

Protein separation was achieved by using an anion exchange Resource™Q column (Amersham). Eluent A was 20 mM Tris-HCl buffer pH 8.5 and eluent B

was 1 M NaCl in eluent A. Samples were previously equilibrated in eluent A and, before the loading, the pH was checked with an universal indicator paper (Carlo Erba). The flow rate was 1 mL/min and the eluting gradient was as follows: from 0% to 14% of eluent B in 70 minutes, then to 50% B in 30 minutes and then to 100% B in 1 minute. This latter concentration was kept for 15 minutes. To recover the main wine proteins, fractions were collected by a Frac-920 (GE-Healthcare) collector. Fractions were dialysed against bi-distilled water on tubes with porosity of 3500 Da (Spectrapore). These preparations were finally frozen, freeze-dried and dissolved in a small volume of water or citrate buffer (30 mM, pH 3.50) for the long-term storage at -20°C.

1.1.53 REVERSE PHASE (RP)-HPLC

The protein composition of wine fractions was determined by HPLC, according to the method proposed by Peng *et al.* (1997).

100 µL of sample was loaded at 1 mL/min onto a semi-preparative C18 column (4.6 x 250 mm, Vydac 218 MS 54, Hesperia, CA) fitted with a C18 guard column (Vydac 218 MS 54, 4.6 x 5 mm, Hesperia, CA) equilibrated in a mixture of 83% (v/v) solvent B [0.1% trifluoroacetic acid (TFA) in 92% Acetonitrile] and 17% solvent A [80% Acetonitrile, 0.1% (v/v) TFA] and held at 35°C. Proteins were eluted by a gradient of solvent A from 17% to 49% in the first 7 minutes, 49% to 57% from 7 to 15 minutes, 57% to 65% from 15 to 16 minutes, 65% to 81% from 16 to 30 minutes and than held at 81% for 5 minutes before re-equilibrating the column in the starting conditions for 6 more minutes. Peaks were detected at 220 nm.

1.1.54 FUNGAL CULTURES: EXPERIMENTAL DESIGN

The fungal cultures utilised in this work were helpfully supplied from the Plant Pathology laboratory of Prof. Francesco Favaron, University of Padova Agripolis, Legnaro (Italy).

Four fungal strains, belonging to *Botrytis cinerea* (BC), *Sclerotinia sclerotiorum* (B 24), *Sclerotium rolfsii* (SR) and *Sclerotinia minor* (SM), were cultured in a medium containing only purified wine proteins as the nitrogen

source. After filtration, the media were assayed for proteolytic activity, residual protein content and SDS-PAGE, in order to screen the fungi for their ability of protein degradation. Some chromatographic steps were attempted to purify the enzymes by using anion exchange chromatography. The effects on musts and wine protein stabilisation of the enzymatic preparations were finally heat tested.

5.1.1.3. Fungal cultures in liquid media

After a period of growth at 25°C in potato dextrose agar (PDA, Difco), *B. cinerea* was transferred into a liquid medium (potato dextrose broth, PDB, Difco) for 5, 10 or 15 days of incubation at 25°C on a rotary shaker (200 rpm).

Besides, *Botrytis cinerea* (BC), *Sclerotinia sclerotiorum* (B 24), *Sclerotium rolfsii* (SR) and *Sclerotinia minor* (SM) were inoculated in a liquid medium (Billon-Grand *et al*., 2002). The four fungal cultures were grown in Erlenmeyer flask on a rotary shaker (200 rpm) at 25°C. To induce proteases emission, the medium had a pH of 3.20. All the essential elements were supplied to the fungi and nitrogen was added as protein purified from wine (Manzoni bianco) at a concentration of 300 mg/L. After the growth period, the media were filtered (0.20 µm) and analysed.

1.1.55 STATISTICAL ANALYSIS

Data were analysed by one-way completely randomized ANOVA with the CoHort Software (CoStat version 6.311, Monterey, CA) and data significativity assessed by Student-Newman-Keuls test.

RESULTS AND DISCUSSION

1.1.56 PRELIMINARY RESULTS ON PROTEASES EMISSION BY FUNGAL STRAINS

The ability of *Botrytis cinerea* to modify and degrade grape and wine proteins has been widely demonstrated (Marchal *et al*., 1998; Cilindre *et al*., 2007). This part of the project, focused on the acidic proteases from *Botrytis cinerea*, started by checking its proteolytic ability against grape proteins.

After a growing period in PDB media, a mixture of different *Botrytis*

cinerea strains isolated from grapes was inoculated on Prosecco and Manzoni bianco (two varieties from the Veneto region) berries in sterile conditions. After 7 days of growth, the resulting must protein profile was analysed by electrophoresis.

As expected, the SDS-PAGE profile resulted simplified, highlighting the disappearance of some protein bands probably due to the action of the acidic proteases secreted by the fungus (fig. 5.1).

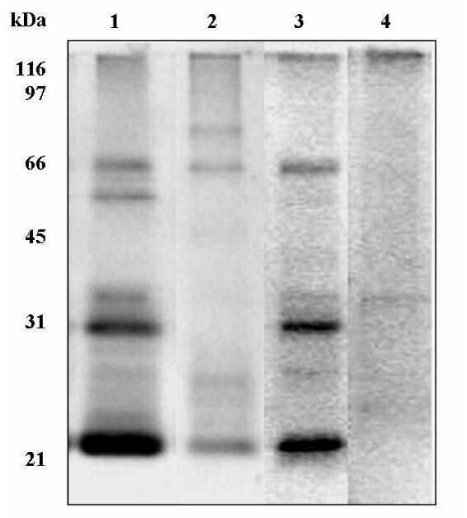

Figure 0.1. *SDS-PAGE in non-reducing conditions (T = 14%, C = 3%) of Manzoni Bianco (lanes 1 and 2) and Prosecco (lane3 and 4) juice. Juices were obtained from healthy (lanes 1 and 3) and botrytised grapes (lanes 2 and 4).*

In accordance with what was observed previously by Marchal and co-workers (1996), *Botrytis* infection seemed to cause the disappearance of the protein bands with an apparent molecular weight of 60, 35, 31 (possibly invertase and chitinases) and a reduction of the intensity of the 22 kDa protein band (probably a TL-protein). Moreover, a new band showed up with an apparent MW of 80 kDa in the sample deriving from botrytised Manzoni bianco grapes (lane 2). These results are in agreement with those reported by Marchal *et al*. (1998) which indicated the appearance of high MW proteins in highly *Botrytis*-infected musts, leading to assume this protein as produced by the fungus. The Prosecco juice, probably because of its lower protein content compared with Manzoni bianco (Vincenzi *et al.*, 2005), seemed to have proteins more sensitive to degradation, leading to a total protein disappearance except for a faint band at about 35 kDa.

These preliminary results seemed to confirm the idea of several authors,

although the possible effects of other fungal enzymes, such as laccase, should be considered. Actually, in these experimental conditions, there were some evidences that laccase activity can cause some modifications of the grape proteins, which could lead to their insolubilisation resulting in a simplification of the SDS-PAGE profile (data not shown) (Zamorani *et al*., 1993).

The degradation and disappearance of the grape proteins from the juice is generally considered as a modification improving wine stability. For this reason, the juices produced from infected berries were heated to measure their hazing potential in comparison to that of the healthy juices (fig. 5.2).

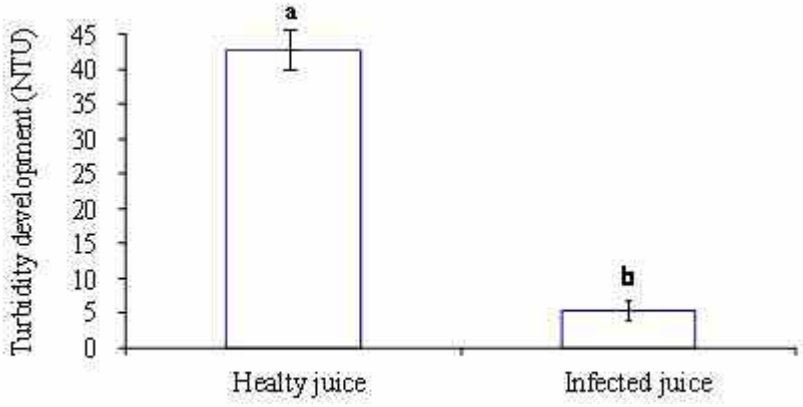

Figure 0.2. *Heat test on Manzoni bianco juices deriving from healthy- and* Botrytis cinerea- *infected grapes. Different letters means significant differences among values for* $P \leq 0.01$ *(Student-Newman-Keuls test).*

The heat test results showed that the infection dramatically reduced juice hazing, although the turbidity level reached by the infected grapes juice was still not sufficient for its complete stabilisation, which should correspond to turbidity values lower than 2 NTU after the heat test (Pocock and Rankine, 1973).

After these firsts experiments to confirm the presence of a proteolytic activity secreted by *B. cinerea*, an attempt to obtain preparations enriched in protease activity was made. To this aim, the fungus was inoculated in PDB liquid medium and incubated for 5, 10 and 15 days.

The collected preparations were initially assayed by SDS-PAGE. The 15 days-incubated medium was left for 24 h at 20°C in contact with the juice obtained from Manzoni Bianco healthy grapes (fig. 5.3).

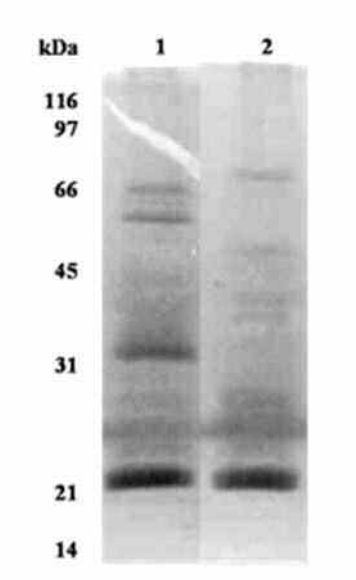

Figure 0.3. *SDS-PAGE of the juice deriving from Manzoni bianco healthy grape analysed before (lane 1) and after 24 hours of incubation with the growth medium (15 days) of* Botrytis cinerea *(lane 2).*

The SDS-PAGE results were similar to those observed in figure 5.1, thus supporting the idea that *B. cinerea* produces proteases able to degrade the grape proteins.

In order to establish the effect of the enzymatic activity also against the proteins of Manzoni Bianco wine, an incubation of the wine for 4 hours with the growth medium (15 days) was made before heat testing samples (fig. 5.4).

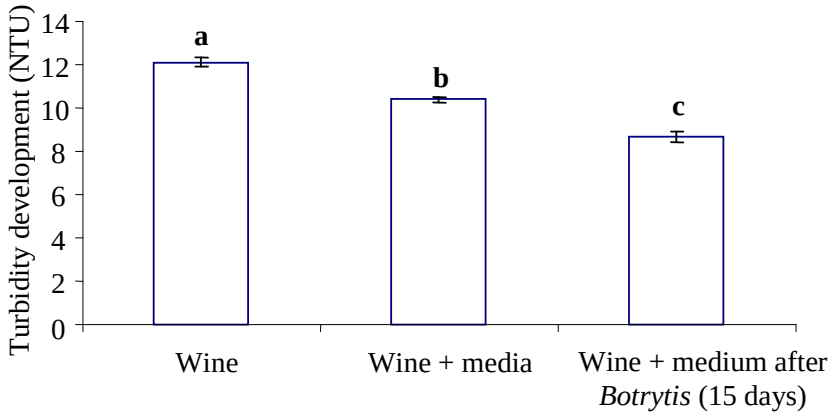

Figure 0.4. *Heat test of untreated Manzoni Bianco wine (wine), wine added of PDB medium (wine + medium, control) and wine after 4 hours of incubation with the PBD medium in which* Botrytis cinerea *was grown for 15 days (Wine + medium after Botrytis).*

Different letters means significant differences among values for P ≤ 0.01 (Student-Newman-Keuls test).

Also in this experiment, the treatment with the growth medium of *B. cinerea* led to a decrease of haze development after the heat test. However, the shortness of the incubation did not allow a large haze reduction. A certain haze diminution occurred also in the control (wine + media). This phenomenon might be due to the polysaccharides present in the medium, which can have a stabilising effect on haze formation similar to that of yeasts mannoproteins acting as a protecting factor (Waters *et al*., 1994; Lomolino and Curioni, 2007).

From the literature it was hardly possible to find an enzymatic assay suitable for acidic protease activity detection. The substrate indicated for this aim, especially azo-albumin and azo-casein, did not give good results because of their precipitation at acidic pH (not shown). The only methods that seemed to work properly were those proposed by Anson (1938) and Castillo-Yañez (2004), both assays using haemoglobin as the substrate. After some adjustments, the method showed a good repeatability and so was adopted to screen the activity of the fungal media.

Firstly, this method was used to check the activity produced by *Botrytis cinerea* growth in PDB medium for three different times (fig. 5.5).

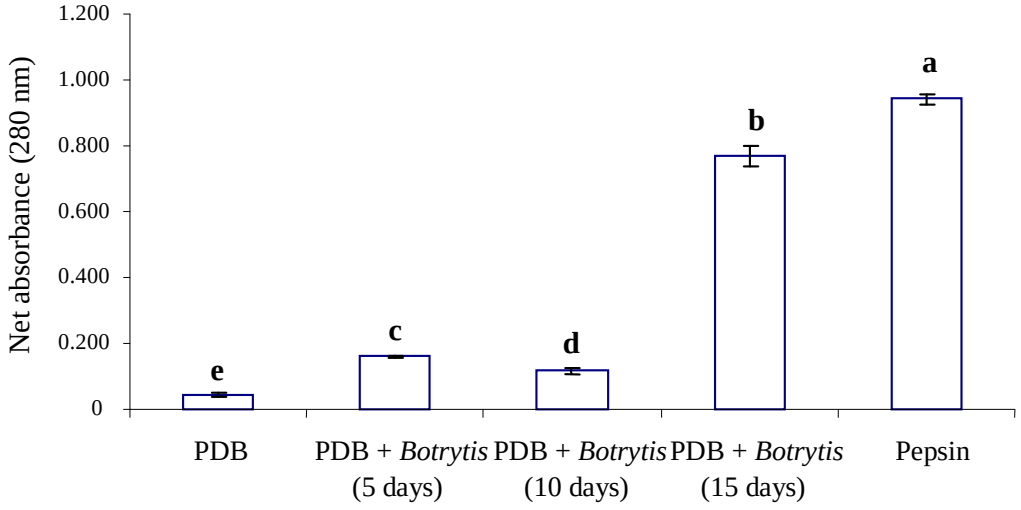

Figure 0.5. *Enzymatic assay of the medium (PDB) and the medium after 5, 10 and 15 days of growth of* Botrytis. *0.01% Pepsin (w/v) was used as the positive control. Different*

letters means significant differences among values for $P \leq 0.01$ (Student-Newman-Keuls test).

This experiment has been replicated several times with different conditions of incubation, storage, substrate concentration. The obtained results (not shown) showed a proteolytic activity in the medium that was always significantly higher after 15 days of incubation, suggesting this length as the best for a proteases emission and for the study of their ability to degrade the grape and wine proteins.

An attempt to separate and concentrate the proteases was made by adding ammonium sulphate (80% saturation) to the media in which *Botrytis* was grown for 15 days. The results showed the complete loss of proteolytic activity after this precipitation step (data not shown).

This first batch of results confirmed the simplification of the SDS-PAGE profile of juices made from grapes infected by *Botrytis*. The heat test on juice and wine showed the possibility of a reduction of haze formation for samples treated with the enzymatic preparation. However, the effect of the PDB medium composition has to be taken into account because of its polysaccharide content that can interfere with the process of turbidity development. The very preliminary attempts of proteases concentration by protein salting out showed a total activity loss, therefore further trials needed to be done to better concentrate the protease fraction and to get rid of the PDB media.

To these aim, additional experiments were planned. *B. cinerea* was inoculated in a medium containing Manzoni bianco wine proteins as only nitrogen source to stimulate proteases emission from the fungus.

The new culture was assayed for proteases activity on haemoglobin as the substrate (fig. 5.6).

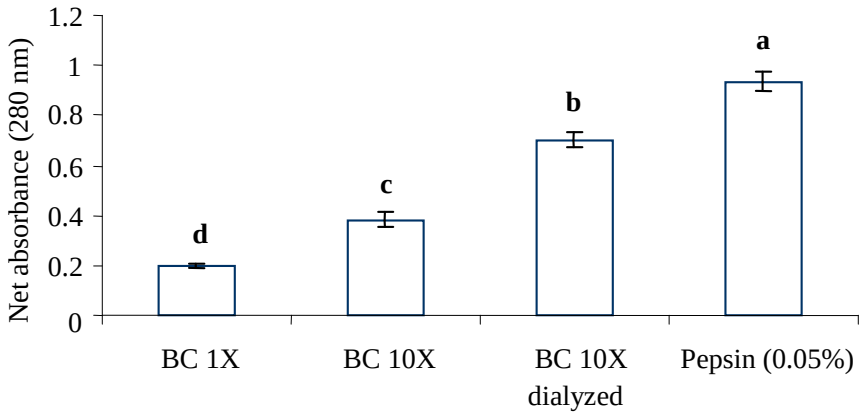

Figure 0.6. *Protease activity of the new* B. cinerea *preparation cultured with wine protein as the nitrogen source. BC 1X: sterile-filtered culture medium; BC 10X: sterile-filtered culture medium concentrated 10 times (MWCO 3000 Da); BC 10X dialysed: sterile-filtered culture medium, concentrated 10 times (MWCO 3500 Da) and dialysed against water; Pepsin was used as the positive control. Different letters means significant differences among values for* $P \leq 0.01$ *(Student-Newman-Keuls test).*

Botrytis cinerea emitted active proteases also in the medium containing 300 mg/L of wine protein as the sole nitrogen source. By concentrating 10 times the fungal medium, the proteolytic activity revealed increase in haemoglobin degradation that was not proportional to the concentration factor. A better result was obtained after dialysis of the concentrated medium. This result was probably due to the removal of interfering compounds acting as inhibitors and belonging to the medium or secreted by the fungus.

Therefore, by using basically PR-proteins as the nitrogen source, that have been shown to possess an antifungal activity *in vitro* (Tattersall *et al.*, 2001), *B. cinerea* demonstrated its ability to grow and it seemed that the proteases emission was stimulated in these conditions.

The number of strains under investigation was enlarged by using the medium so prepared.

Three new fungal strains were cultured in addition to *Botrytis cinerea* (BC): *Sclerotinia minor* (SM), *Sclerotinia sclerotiorum* (B24) and *Sclerotium rolfsii* (SR). Unlike *Botrytis cinerea*, these fungi are not specific grape pathogens but they are known to be able to grow at acidic pH as pathogens of juicy fruits and

plants (Billon-Grand, 2002). For these reasons they have theoretically been considered suitable to release acidic proteases active at the pH of must and wine.

Initially, after a growth period on a medium prepared with purified wine protein as the sole nitrogen source, the fungi were tested for their enzymatic activity against haemoglobin in comparison to *Botrytis cinerea* and pepsin, both used as positive controls (fig. 5.7).

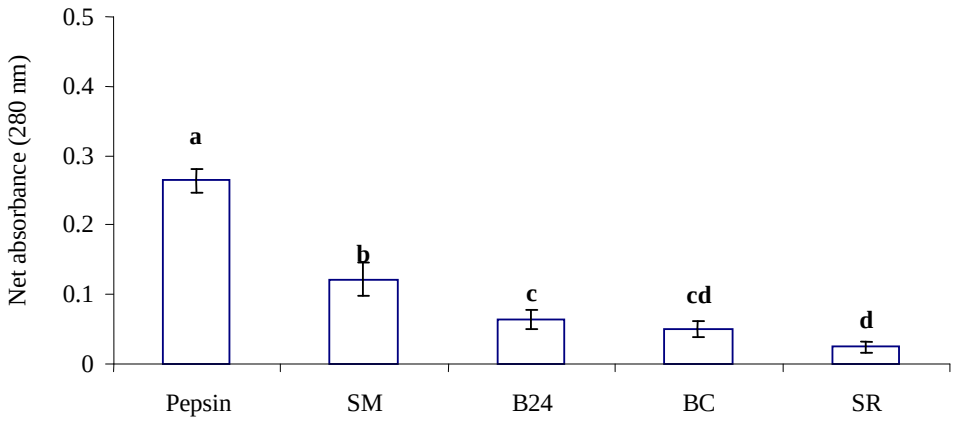

Figure 0.7. *Proteolytic activity assayed on haemoglobin of four fungi after a growth period in a medium containing 300 mg/L of Manzoni bianco wine proteins. BC:* Botrytis cinerea*; SM:* Sclerotinia minor*; B24:* Sclerotinia sclerotiorum*; SR:* Sclerotium rolfsii. *Different letters means significant differences among values for* $P \leq 0.05$ *(Student-Newman-Keuls test).*

The four fungi showed a different degradation aptitude against haemoglobin. In particular, the fungus that showed the largest substrate degradation was *Sclerotinia minor*, the other fungi showing a proteolytic activity significantly lower than that of SM but not very different to that of BC, that was assumed as the reference.

In order to better define the growth conditions able to stimulate the best protease emission into the medium, the four fungi were daily checked for protease activity by the haemoglobin assay starting from the inoculation moment (fig. 5.8).

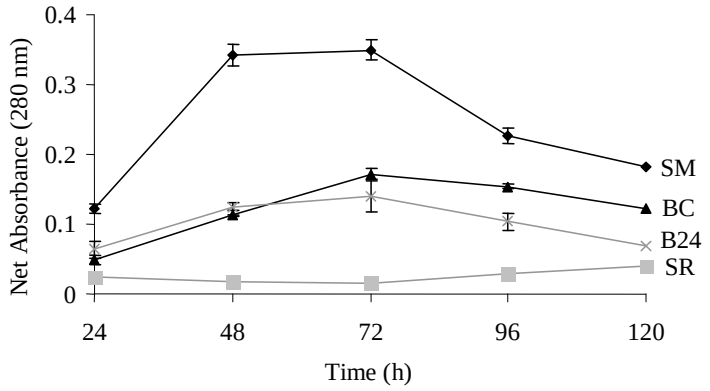

Figure 0.8. *Protease activity assayed on haemoglobin of four fungi during 5 days of growth. BC:* Botrytis cinerea*; SM:* Sclerotinia minor*; B24:* Sclerotinia sclerotiorum*; SR:* Sclerotium rolfsii.

A different evolution of the esocellular protease activity was detected for the different fungi. SM showed a better activity throughout all the period of observation, with a maximum between 48 and 72 hours after the inoculum, its activity being two times higher than that of BC and B24.

It is interesting to observe how SR behaved, with no apparent activity until 120 hours of incubation although a mycelium growth was visually observed.

In the same samples previously analysed for acidic proteases emission, the residual wine protein content was measured as an indirect proof of protein degradation (fig. 5.9).

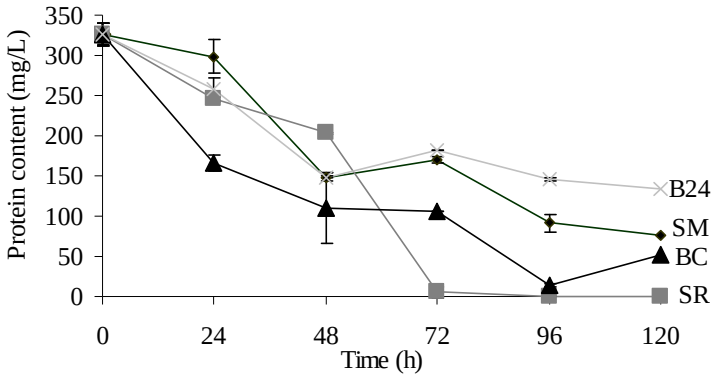

Figure 0.9. *Residual protein content in the medium during the incubation period. BC:* Botrytis cinerea*; SM:* Sclerotinia minor*; B24:* Sclerotinia sclerotiorum*; SR:* Sclerotium rolfsii.

During the period of growth, a diminution of the protein content of the medium was detected for all the strains tested. In particular, SR seemed to be the fungus with the best degradation potentiality reaching the total protein degradation after 72 hours. The other strains showed similar behaviour, reaching a diminution of 58.5% for B24, 76.5% for SM and 83.9% for BC.

Quantitatively, these data did not match with the protease activity observed on haemoglobin for the different fungi. This could be due to a different specificity of the proteases for haemoglobin and wine proteins. By combining the information collected in the two previous screening tests, it seemed that SR and BC were the most active fungi in wine protein degradation, although they did not show a good proteases activity on haemoglobin (especially SR).

To resolve the doubts concerning the suitability of a protease assay with a substrate (haemoglobin) different to that we looked at (grape and wine proteins), a new enzymatic assay was set up by using purified wine proteins as the substrate and by staining the proteolysis products with ninhydrin as suggest by Doi *et al*. (1981) (fig. 5.10).

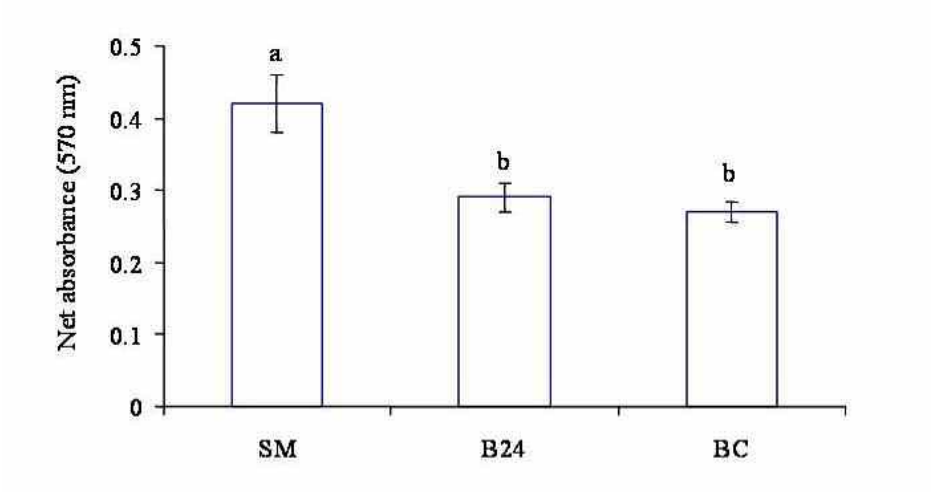

Figure 0.10. *Comparison between proteolytic activity of* Sclerotium minor, Botrytis cinerea *(BC) and* Sclerotinia sclerotiorum *(B24) after 72 hours of growth by using the ninhydrin assay. Different letters means significant differences among values for* $P \leq 0.01$ *(Student-Newman-Keuls test).*

The results confirmed that the highest proteolytic activity was that of SM also when the wine proteins were used as the substrate.

SDS-PAGE analyses of the samples were then performed (fig. 5.11, 5.12, 5.13 and 5.14).

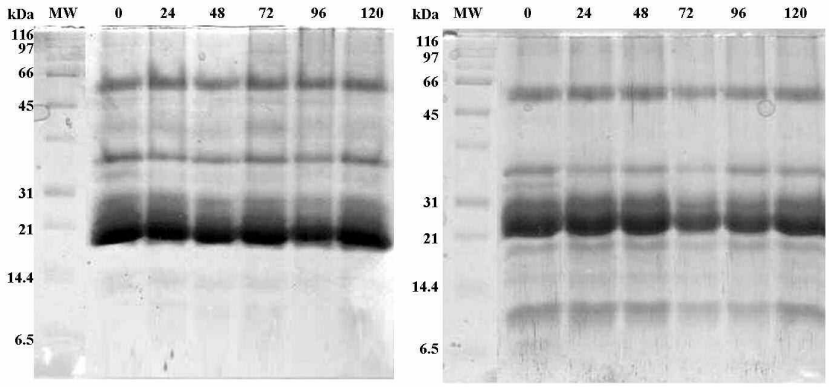

Figure 0.11. *SDS-PAGE analysis of the proteins of the medium during the period of incubation with* Sclerotinia sclerotiorum *(B24) in non-reducing (left) and reducing (right) conditions. MW = molecular weight standards; 0, 24, 48, 72, 96 and 120: hours of incubation.*

B24 was considered not to be a fungus suitable for grape protein degradation because of its low protease emission and protein content decrease in the medium. The SDS-PAGE analyses confirmed these data, highlighting a generalised but low decrease of band intensities not showing any specificity for particular protein bands.

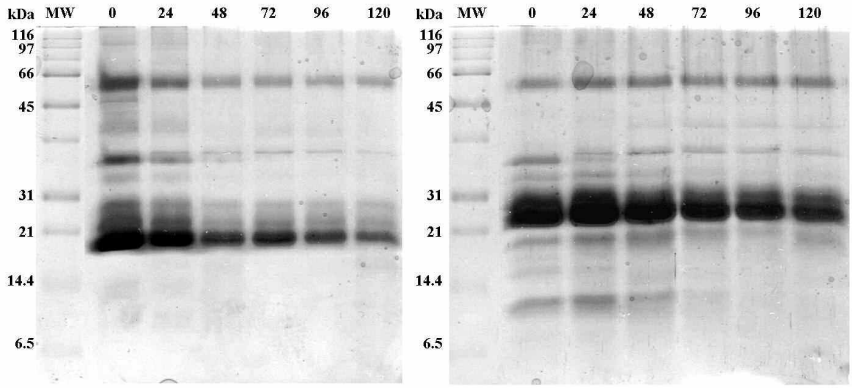

Figure 0.12. *SDS-PAGE analysis of the proteins of the medium during the period of incubation with* Botrytis cinerea *(BC) in non-reducing (left) and reducing (right) conditions. MW = molecular weight standards; 0, 24, 48, 72, 96 and 120: hours of incubation.*

BC confirmed only in part the results of residual protein quantification,

showing only a generalised diminution of band intensities that did not led to the disappearance of any protein. This fact seemed to be due firstly to the shorter incubation period of BC in this culture in comparison to those obtained from PDB media. Secondarily, the incubation of this fungus with the wine proteins seemed not to stimulate a proteases emission as expected, at least not for the incubation times tested.

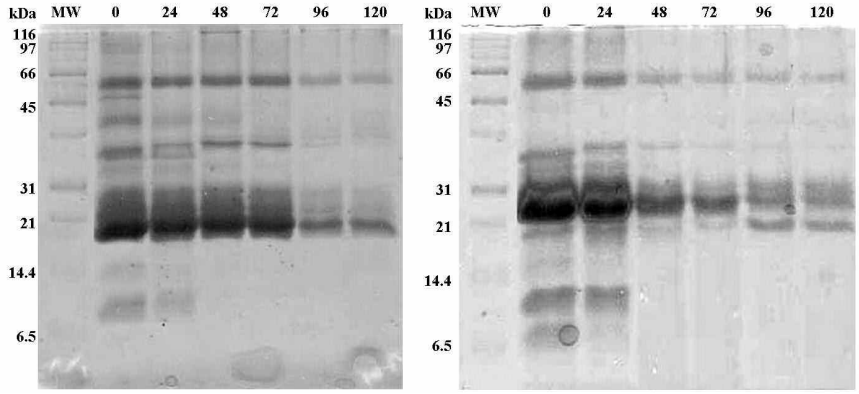

Figure 0.13. *SDS-PAGE analysis of the proteins of the media during the period of incubation with* Sclerotinia minor *(SM) in non-reducing (left) and reducing (right) conditions. MW = molecular weight standards; 0, 24, 48, 72, 96 and 120: hours of incubation.*

The largest protein degradation was observed for SM starting from the 2nd day of growth, with a generalised protein decrease and with the complete disappearance of protein bands with an apparent molecular weight of 35 kDa (probably chitinases) and less than 20 kDa. Besides, a general decrease of band intensities was detected.

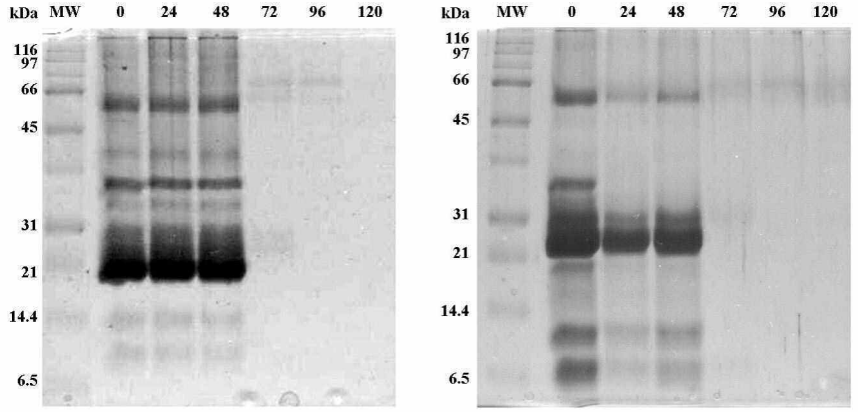

Figure 0.14. *SDS-PAGE analysis of the proteins of the media during the period of*

incubation with Sclerotium rolfsii *(SR) in non-reducing (left) and reducing (right) conditions. MW = molecular weight standards; 0, 24, 48, 72, 96 and 120: hours of incubation.*

SR showed the most interesting behaviour, leading to a complete protein disappearance between the second and the third day of culture, confirming the data of protein quantification but contrasting with those of the haemoglobin assay. This sudden change of protein profile was very different from those observed for the other fungi. Taking into account the very low protease activity of SR on haemoglobin (Fig. 5.8) and the rapidity of protein disappearance in the growing medium, it seemed unlikely that a protease could lead to a similar result, although the activation of a protease after an unidentified step of growth could not be excluded.

Sclerotium rolfsii is a fungus commonly used for the production of scleroglucan (Fariña *et al*. 1998), a polysaccharide that the fungus releases in the medium and whose presence was assumed as a possible reason of protein disappearance. Indeed, the role of scleroglucan in protein sequestration has been demonstrated with further experiments (see chapter 6; Vincenzi *et al*., 2007). Consequently, it was assumed that a protease activity emission into the medium had to be present, because the fungus grew, but this activity was very low. Therefore wine proteins were only minimally degraded, but they disappeared from the medium as a result of the action of the scleroglucan produced by the fungus. Taking into account the antifungal activity of the PR-proteins, a possible reason of this unexpected phenomenon is that the production of scleroglucan acts as a sort of defence mechanism that the fungus adopts to trap proteins and inactivate their toxicity.

An attempt to grow the fungi (BC, SR and SM) on a media containing proteins from dialysed Manzoni bianco juice instead that from wine was made. In this case, a partial precipitation of grape proteins added to the medium occurred, resulting in a 5 times lower protein content after 24 hours (about 50 mg/L instead of 250 mg/L added). This phenomenon seemed to depend on the emission fungal laccase in the media, causing polyphenols oxidation and their reaction with the unfermented must proteins that are believed to be more reactive in this situation

than after fermentation (Zamorani *et al*., 1993). Because of the too low final protein level, no significant proteolytic activity was detectable (data not shown), although a generalised protein content decrease was measured in the fungal medium during the incubation period (fig. 5.15).

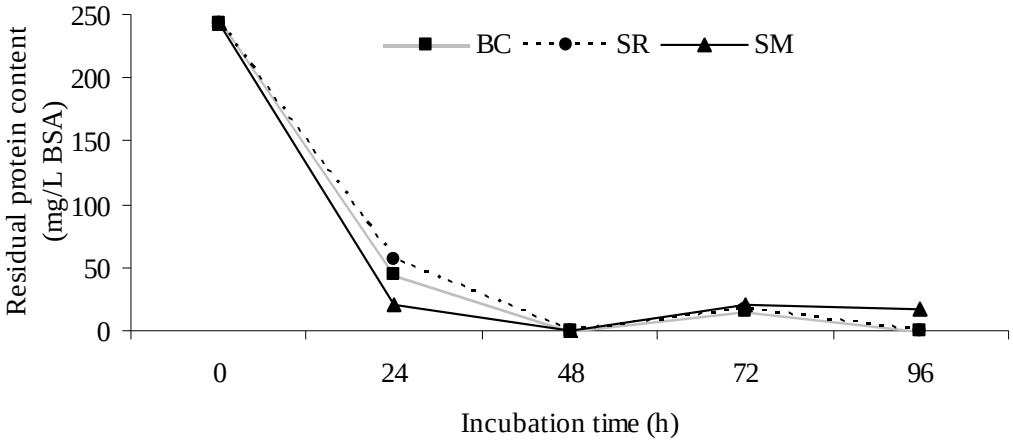

Figure 0.15. *Residual protein content in the medium containing juice proteins as the nitrogen source during the growth of* Sclerotium rolfsii *(SR);* Sclerotinia minor *(SM) and* Botrytis cinerea *(BC).*

The results partially confirmed that SM had the best proteolytic activity on grape PR-proteins, but led to the conclusion that the medium containing proteins from wine as the nitrogen source was more suitable than that prepared starting from juice.

1.1.57 PROTEOLYTIC ACTIVITY OF *SCLEROTIUM MINOR*

Sclerotium minor (SM) was selected because this fungus showed the best proteases emission and good wine protein degradation.

A large quantity of medium was inoculated with SM and the growth was carried out in the conditions considered as optimal after the preliminary trials: 72 h of growth in a liquid media (Billon-Grand, 2002) containing 300 mg/L of proteins purified from wine.

The presence of a good proteolytic activity in this new culture was detected (data not shown).

SDS-PAGE analysis (fig. 5.16) confirmed the degradation of most of the

wine proteins in the medium in which SM was grown, the 66 kDa and 22 kDa bands being the only still visible bands after 72 hours of growth.

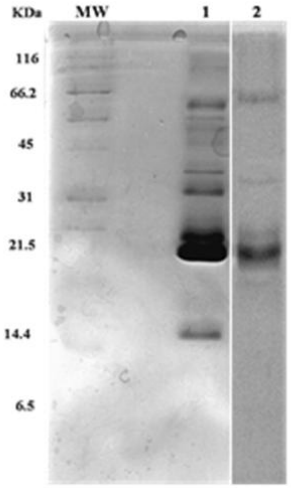

Figure 0.16. *SDS-PAGE analysis of the proteins of the medium before (lane 1) and after 72 hours (lane 2) of growth of* Sclerotinia minor *(SM).*

By comparison with the proteins identified in the Semillon juice (see paragraph 3.4.2), the 66 kDa and 22 kDa bands were assumed to be the grape invertase and a thaumatin-like protein respectively, indicating a certain resistance of these bands to degradation by the fungal protease.

The ninhydrin assay was used to determine the evolution of the proteolytic activity of SM during a 24 h incubation period at 12 and 37°C in a model wine containing 0.5 % (w/v) of wine proteins (fig. 5.17).

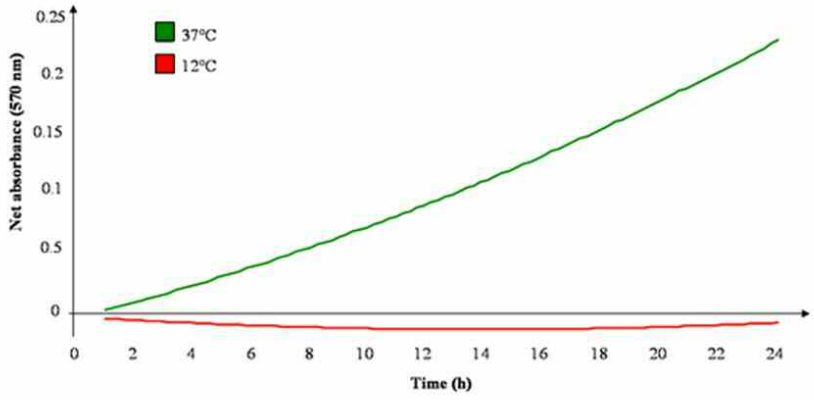

Figure 0.17. *Evolution of the proteolytic activity of SM during 24 h at two temperatures.*

The results showed the total absence of proteolytic activity in the samples kept at winemaking temperature (12°C), while at 37°C a linear increase of the activity was detected. These data confirm that proteolytic enzymes have functional problems in the temperature conditions used during normal winemaking, as previously demonstrated by several authors (Ngaba-Mbiakop, 1981; Waters *et al*., 1992).

The possibility of wine stabilisation against protein haze formation by the use of proteolytic enzymes was checked by adding the SM preparation to an unfined Manzoni bianco wine and by monitoring, after an incubation period, the haze developed after the heat test (Waters *et al*., 1992; Bakalinski and Boulton, 1985; Modra and Williams, 1988; Marchal *et al*., 1998). The wine was not stabilised by the addition of the medium containing the SM protease (not shown). This result might be due to the presence of heat-unstable fungal proteins and/or residual wine proteins deriving from the culture medium added to the wine. To solve this problem, purification of the protease activity from the medium was performed by fractionating it by Anion Exchange Chromatography (AEC) (fig. 5.18).

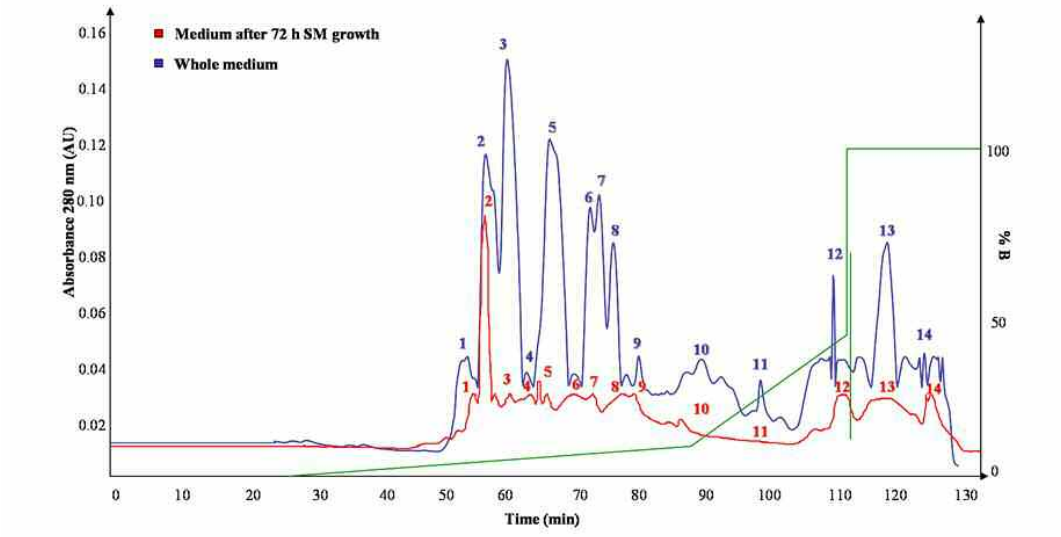

Figure 0.18. *Chromatographic profiles obtained by AEC fractionation of the medium before (blue line and numbers) and after 3 days of fungal growth (red line and numbers). The flow through peak (FT) is not shown.*

The AEC chromatograms confirmed what previously observed by means

of SDS-PAGE. In particular, the peak heights after the fungal growth resulted sensibly modified (red line) in a double way: i) SM growth caused the reduction of the height of all the peaks, showing a generalised effect of protein decrease; ii) most of the peaks were almost completely degraded, with the exception of peak 2, that was previously considered to be a TL-protein with the highest haze potential when heat tested (see chapter 2).

After protein content determination (not shown), each peak was collected and analysed by SDS-PAGE to visualise the effect of the fungal growth on the protein profile (fig. 5.19).

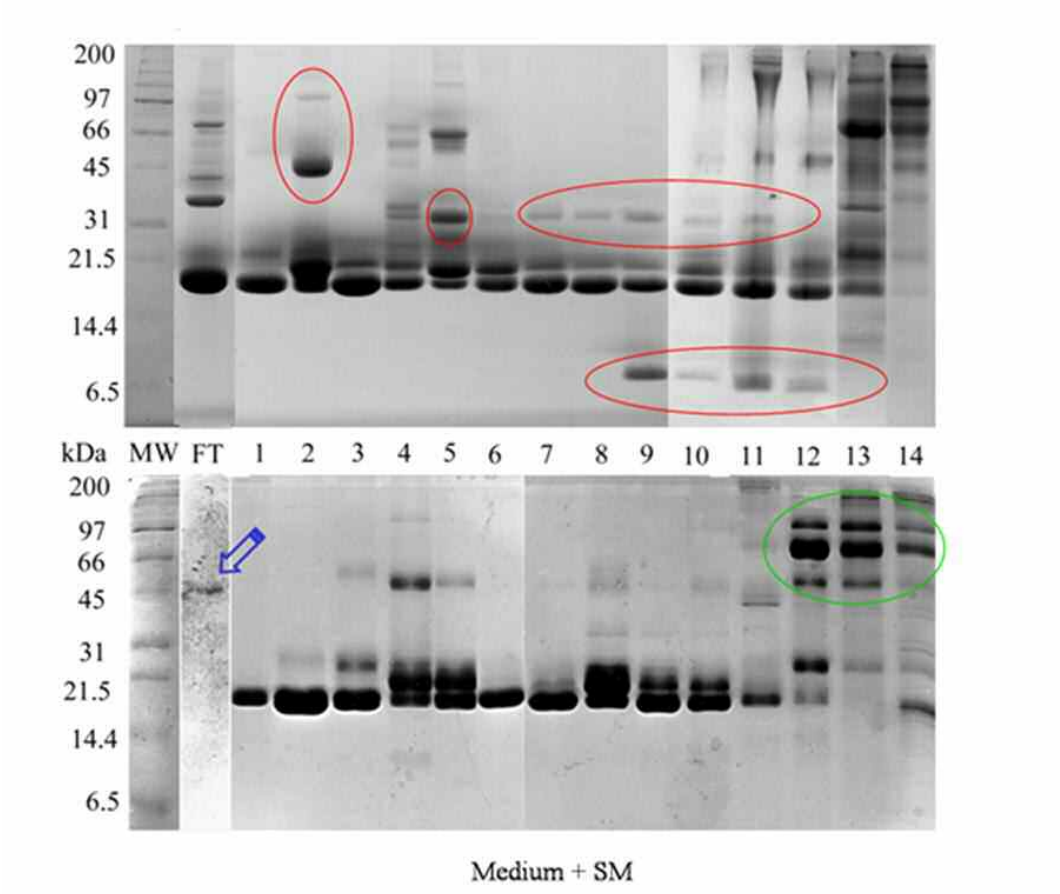

Figure 0.19. *SDS-PAGE (T = 14%; C = 3%) in non-reducing conditions of the peaks (25 µg of protein loaded) collected after anion exchange fractionation of the growth medium (picture above) and after 72h (picture below) of SM growth. Numbers correspond to peaks of Figure 5.18. Red circles indicate the main bands that disappeared after SM growth; the green circle indicates bands appearing after SM growth.*

A band at ≈ 20 kDa was detected nearly in every fraction, confirming that

grape contains a large number of polypeptides with different pI values (affecting the elution from the AEC column) but similar apparent molecular masses in SDS-PAGE (Monteiro *et al.*, 2001). The results showed the degradation of several proteins (Fig. 5.19, red circles) and the appearance of new bands (green circle). This indicates that SM growth degraded proteins with apparent MWs of 31 (probably chitinases) and ≈ 10 kDa. The flow through of the starting medium was characterised by a large number of proteins (Fig. 5.19, FT), but, after the fungal growth, its SDS-PAGE profile was radically altered, showing, in addition to an almost complete disappearance of the original bands, also a new band with an apparent MW of ≈ 50 kDa (Fig. 5.19, blue arrow). Further experiments indicated this band as the protein showing the main proteolytic activity against heamoglobin (fig 5.20 and 5.21).

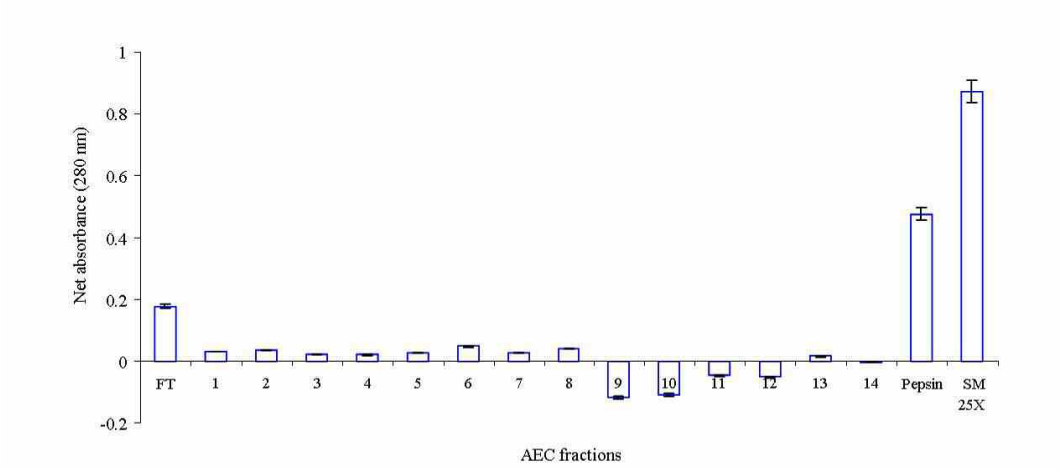

Figure 0.20. *Proteolytic activity on haemoglobin of the peaks collected from AEC fractionation of the SM medium. Pepsin (0.05% w/v) and unfractionated SM medium (25 fold concentrated) were used as the positive controls.*

Among the AEC peaks, FT resulted to be the most active fraction, although in comparison with the starting material (the 25X unfractionated SM medium) it was evident that the proteolytic activity decreased following AEC fractionation. The causes of this fact need to be investigated but it seemed likely that a partial enzyme denaturation occurred during chromatography or that the enzymatic activity resulted divided into more than one fraction.

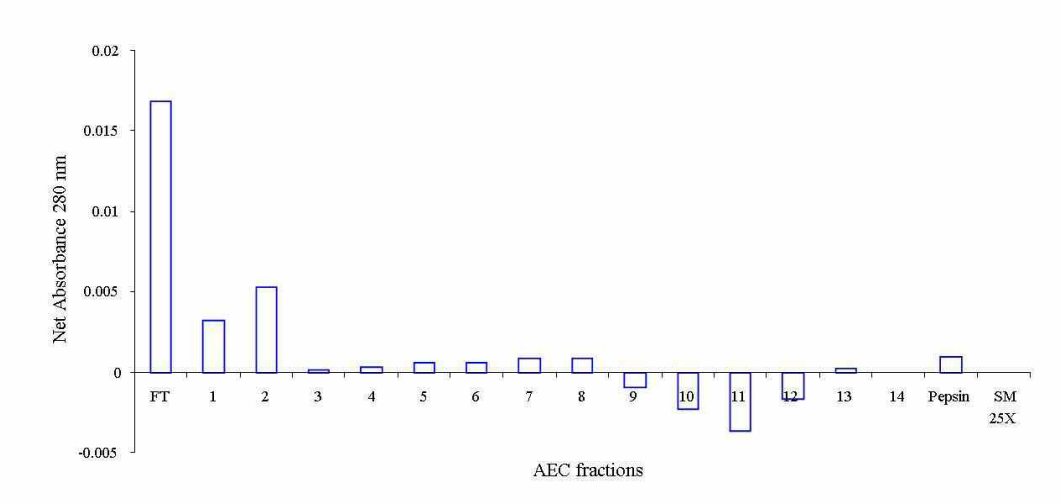

Figure 0.21. *Specific activity (Abs 280 nm/µg protein) of the peaks collected after AEC fractionation of the SM medium.*

By expressing the previous data in terms of specific enzymatic activity (ratio between the activity and protein content) of each fraction (fig. 5.21), FT resulted the most active although two of the following peaks (1 and 2) showed also fairly good specific activities, indicating the proteolytic activity as divided into the first three fractions. The specific activity of FT was much higher than that of both the unfractionated SM medium and pepsin, because of the abundance of protein in these latter.

Once established that FT was the fraction containing the main proteases activity, a wine stabilisation test was performed by incubating fraction FT at 23°C for 7 days with an unfined wine (fig. 5.22).

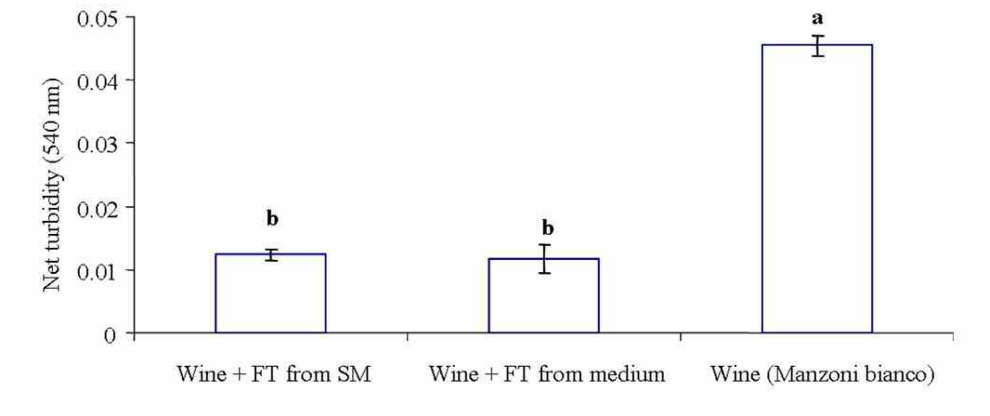

Figure 0.22. *Heat test after incubation (23°C, 7 days) of an unfined Manzoni bianco wine (protein content ≈ 200 mg/L) with the FT fraction from SM medium AEC fractionation.*

An incubation with the FT fraction of the un-inoculated medium was executed as the control. Different letters means significant differences among values for $P \leq 0.01$ (Student-Newman-Keuls test).

The obtained results highlighted the possibility to reach wine stabilisation (turbidity values lower than 0.02 AU). Unexpectedly, addition to the wine of FT from SM and from the starting (un-inoculated) culture medium gave similar results. This phenomenon was attributed to two different mechanisms of action: for the FT fraction deriving from the SM medium, the stabilisation could be ascribed to the wine protein degradation, whereas in the case of the FT fraction from the un-inoculated medium the stabilisation could be due to its high content of polysaccharides (225.6 g/L for the FT from Manzoni bianco wine compared to 37.9 g/L for FT from SM) that acted as protective factors against haze formation, as pointed out by several authors (Waters *et al*., 1991, 1994; Pellerin *et al*., 1994).

These results show the possibility to utilize *S. minor* as a source of proteolytic enzymes for grape and wine protein degradation. From SDS-PAGE analyses, a wine protein degradation was detected although not complete. Two protein bands seemed not to be affected by the action of *S. minor* enzymes, these proteins probably being thaumatin-like proteins and invertases. This information reveals a main drawback because thaumatin-like proteins are considered the major responsible for heat-induced haze development in wines (Waters *et al*., 1993, 1996) as confirmed in chapter 4 of this thesis.

First attempts of wine stabilization by addition of the whole enzymatic preparation highlighted some problems for the passage from laboratory trials to the real conditions of winemaking, the proteolytic activity of the whole enzymatic preparation being totally inhibited at normal winemaking temperatures (12°C).

A certain purification level was achieved by using Anionic Exchange Chromatography, although a loss on activity was observed during the fractionation process, probably due to the distribution of the enzyme in several chromatographic fractions. Besides, the quantities of purified enzyme were too low to plan a large-scale experiment also starting from a quite high volume of sample. Consequently, the purification process should be improved by using other

chromatographic techniques or, the expression of the proteolytic activity of SM in etherologous systems, such as in yeast, could be attempted.

In conclusion, the reported preliminary results indicate the possibility to use phytopathogenic fungi to produce proteases, active at the wine pH, which are able to degrade the grape proteins responsible for haze formation in white wines, although further studies are necessary to make this approach suitable for a practical use in winemaking.

1.1.58 PROTEOLYTIC ACTIVITY OF *SCLEROTIUM ROLFSII*

Scletorium rolfsii (SR) was studied in order to induce a proteolytic activity that was not detected in the preliminary results above described. To this aim, SR was inoculated in a medium (Czapeck) containing 0.26 g/100 mL of Yeast Extract and incubated (at 24°C) for 7 days. After this incubation, the medium was centrifuged and tested for its proteolytic activity with the haemoglobin assay. In these conditions a certain protease emission was detected (0.255 AU), in contrast to the results of the screening experiments. An incubation of this preparation with grape proteins led to the disappearance of some of them as showed by SDS-PAGE analysis (fig. 5.23).

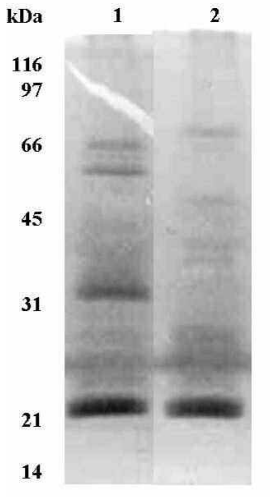

Figure 0.23. *SDS-PAGE analysis of the Manzoni bianco juice proteins (270 µL of dialysed juice) before (0) and after 48 hours of incubation with the SR growth medium (150 µL) (48). MW standard proteins are on the left (MW).*

A general decrease in band intensity was detected with the complete

disappearance of protein with MWs higher than 31 kDa. This experiment was replicated twice to confirm the reported results (not shown).

Because of the presence of a large amount of polysaccharides (scleroglucan) in the medium after the fungal growth, an attempt of purification of the proteases activity was performed by AEC (fig. 5.24), the main aim of this step being to separate the scleroglucan (neutral polysaccharide) from the proteins, that were assumed to be negatively charged at the fractionation pH (8.5). Consequently, the salt for protein elution from the column was applied without using a gradient.

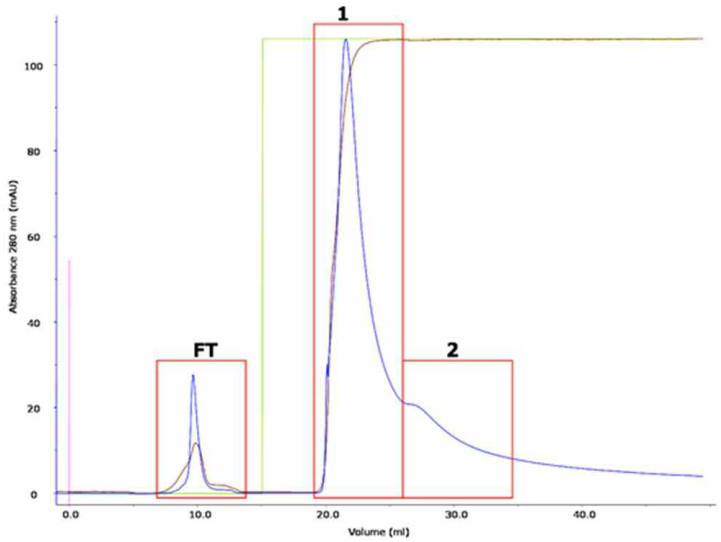

Figure 0.24. *Fractionation of* S. rolfsii *medium by anion exchange chromatography. Green line: % of eluent buffer B. 2 ml/min, 2.5 mL sample loaded.*

The chromatogram showed the presence of a flow trough fraction (FT, containing the scleroglucan) and of two peaks that were collected and dialysed against water. The protein content of each fraction was determined (not shown). The three collected peaks were then incubated (48 h, 25°C) with an unfined Manzoni bianco juice and proteins analysed by SDS-PAGE (fig. 5.25).

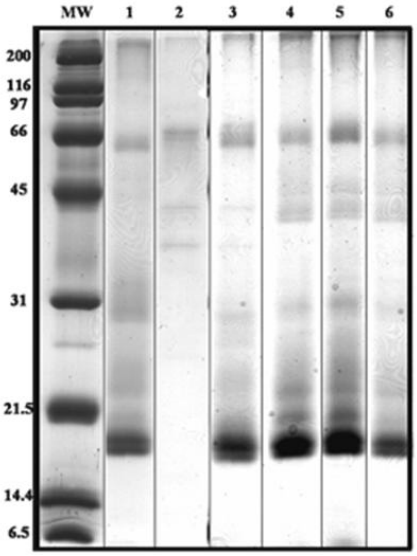

Figure 0.25. *SDS-PAGE in non-reducing conditions of samples of Manzoni bianco juice (Mbj) incubated for 48 h with the three chromatographic peaks obtained by AEC fractionation of the SR medium (fig. 5.24). Un-treated Mbj (lane1); SR medium alone (lane 2); Mbj incubated with un-fractionated SM medium (lane3), FT (lane 4), F1 (lane 5) and F2 (lane 6).*

The protein bands belonging to the fungus showed apparent MWs of 66, 40 and 35 kDa (lane 2). Incubation of the grape juice with the total SR medium caused a generalised band intensity decrease (lane 3) whereas incubation with the AEC FT fraction of the SR medium (lane 4) led to a protein decrease only for chitinase bands and the presence of the fungus proteins were still detectable. The AEC Fraction F1 of the SR medium seemed not to lead to a protein decrease, while new bands appeared (probably belonging to the fungus) (lane 5). AEC Fraction F2 led to a general bands intensity decrease, while probably fungal proteins appeared (lane 6). As observed previously with the *S. minor* medium, these data confirm the loss of proteolytic activity after AEC fractionation. An enzymatic assay confirmed these results (fig. 5.26).

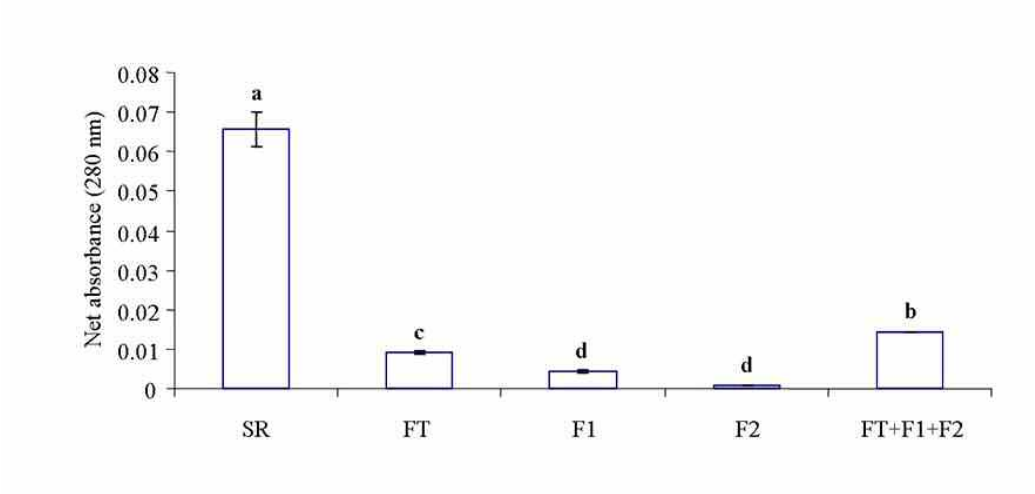

Figure 0.26. *Proteolytic activity on heamoglobin of the total (un-fractionated)* S. rolfsii *medium (SR) and of the fractions (FT, F1 and F2) collected after anion exchange chromatography. Different letters means significant differences among values for* $P \leq 0.01$ *(Student-Newman-Keuls test).*

The un-fractionated SR medium showed the highest protease activity on haemoglobin. It seemed that an activity loss occurred after AEC separation, which gave fractions showing the highest activity in the flow trough (FT; in which the scleroglucan should be). The total activity loss resulting from fractionation was confirmed from the amount of the activity measured after combining the three fractions that resulted sensibly lower (-78.2%) than that of the unfractionated preparation.

Taking into account that haemoglobin could not be suitable substrate for the SR protease, a further attempt to establish if *S. rolfsii* really possessed a useful proteolytic activity was performed by developing a zymography method for the detection of proteolytic activity directly on grape and wine proteins. To this aim, lyophilised Pinot grigio proteins were dissolved into a polyacrilamyde solution that was then polymerized. In this way, a gel matrix containing 0.5 % of purified wine proteins was obtained and, after making a hole in the gel, 25 µL of total SR preparation were applied. After an incubation at 25°C for 72 hours, the proteins were treated with Coomassie stain and the proteolytic activity detected as a clear background.

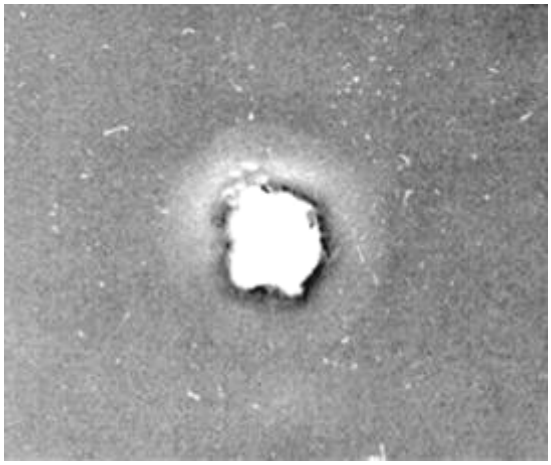

Figure 0.27. *Detection of the proteolytic activity of the unfractionated* S. rolfsii *medium in a gel containing 0.5 % lyophilized wine proteins.*

The presence of a clear background around the point in which the SR medium was applied confirmed the proteolytic activity on the wine proteins (fig. 5.27). In order to establish which proteins were affected by the SR protease, a new incubation test was carried out using the wine proteins (fig. 5.28).

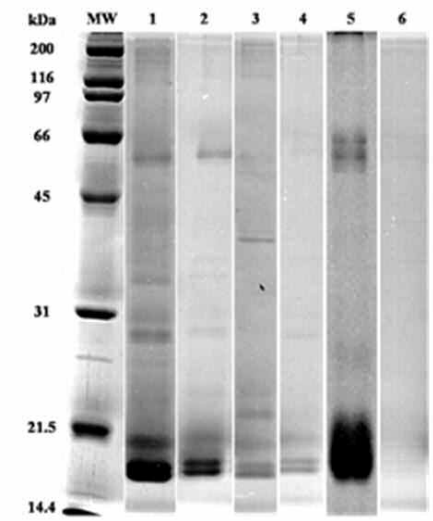

Figure 0.28. *SDS-PAGE in non-reducing condition of the products of different incubations (6 days, 25°C) of the un-fractionated SR medium with grape and wine proteins. Manzoni bianco wine un-treated (lane 1) and after incubation with SR medium (lane 2); Manzoni bianco juice un-treated (lane 3) and after incubation with SR medium (lane 4); Manzoni bianco wine fraction 6 from hydrophobic interaction chromatography (see paragraph 4.4.2) un-treated (lane 5) and after incubation with SR medium (lane 6). Each incubation was carried out in presence of $NaNO_3$ (0.01% final concentration). MW standard proteins are on the left (lane MW).*

Comparing the pattern of the un-treated wine with that of the wine incubated with the fungus (lanes 1 and 2, respectively), it was confirmed that a certain protein reduction occurred, especially for the bands at ≈ 30 kDa and for the TL-protein bands (≈ 21 kDa) that both resulted less intense after the treatment. A similar result was observed when the samples of grape juice were analysed (lane 3 and 4). In this case, a major protein decrease occurred, with the disappearance of two bands at ≈ 40 and ≈ 24 kDa. The wine protein fraction showing the highest hazing potential, deriving from HIC separation (fraction 6, see fig. 4.13, chapter 4) was also used to test the proteolytic activity of SR. From the comparison of lane 5 (un-treated HIC fraction 6) and 6 (the same fraction 6 but incubated with SR), it was possible to observe an almost total disappearance of this band.

Generally, from this experiment it seemed that the total SR preparation had effects on wine chitinases (lanes 1 and 2) and on juice 40 and 24 kDa proteins (lanes 3 and 4). Moreover, the study of *S. rolfsii* effect on the HIC fraction 6 showed its degradative ability on a very heat-instable TL-protein. These results indicate the active role of SR on the degradation or subtraction of the wine proteins. However, the presence of scleroglucan excreted by the fungus need to consider its particular effect on wine proteins.

In order to better understand if the scleroglucan interfered on the obtained results, three cultures of *S. rolfsii* were achieved using different media prepared as follows:

- SR1 = 7 days cultures on Czapeck + 2.6 g/L of Yeast extract (same conditions of the first experiments);
- SR 2 = 9 days cultures on Czapeck without $NaNO_3$ + Manzoni bianco juice;
- SR 3 = 9 days cultures on Czapeck without $NaNO_3$ + Yeast extract.

The three enzymatic preparations were assayed for proteolytic activity with the haemoglobin assay. To have a further proof of the involvement of an enzymatic activity in SR preparations, samples boiled before to be assayed were also tested (fig. 5.29).

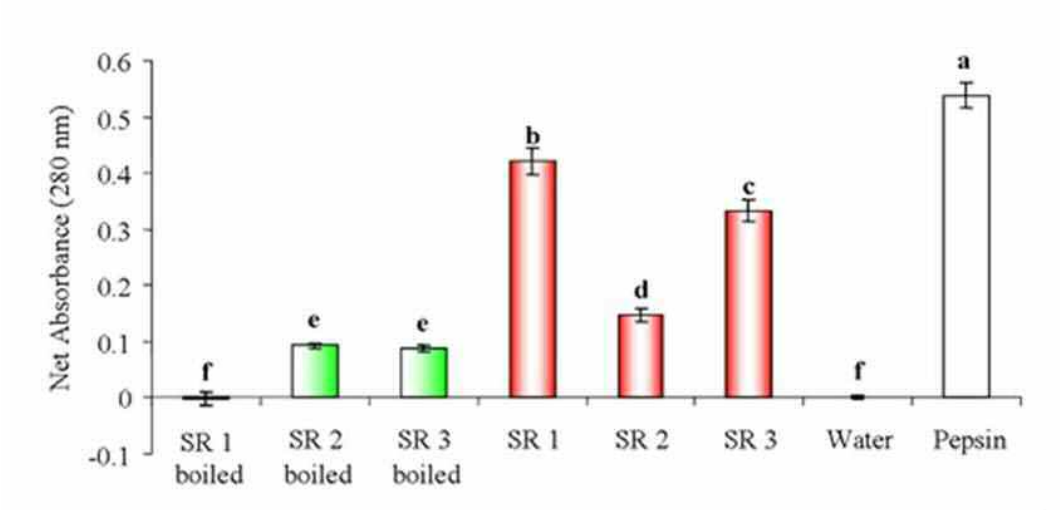

Figure 0.29. *Proteolytic activity (haemoglobin assay) of the three SR preparations (see text). A series of samples was boiled (100°C, 10 min) as negative controls for the enzymatic activity. Pepsin 0.05% (w/v) tested as the positive control. Different letters means significant differences among values for* $P \leq 0.01$ *(Student-Newman-Keuls test).*

The presence of proteases in *S. rolfsii* medium was confirmed by the loss of activity occurring after boiling the samples, although the samples showed different behaviours. SR 1 (a replicate of the sample described in the previous part of the results), showed the best protease activity, which was completely inhibited by heating. As expected, the sample SR 3, also prepared with Yeast extract, behaved similarly to SR 1. In contrast, the activity of SR 2, which was actually very low, was not significantly affected by boiling. An explanation of this unexpected result may be that the conditions of culture (prepared with grape juice instead of yeast extract as the nitrogen source) stimulated scleroglucan emission (indicated by a very high density of the culture medium, not shown). It was supposed that, the sterile filtration applied to all the preparations tested caused retention on the filter of the SR 2 proteases because of the complexation of these latter by the scleroglucan. These results were supported by testing the proteolytic activity of non-filtered cultures of SR 2, that in this case was similar to that of SR 1 and SR 3 (not shown).

All these data indicated the stimulating effect of the grape proteins on scleroglucan emission by *S. rolfsii*. A hypothesis could be made to explain this phenomenon: due to the toxicity of the PR-proteins against fungi, *S. rolfsii*

defended itself by subtracting proteins from the medium by trapping them into the scleroglucan, rather than by using a proteolytic action. This theory would explain the low protease activity detected for SR and lead to hypothesize a cooperative action of scleroglucan and proteases in defending the fungus from the antifungal activity of the grape PR-proteins.

The fungal samples formerly described were analysed also by zymography for proteolytic degradation of the wine proteins (fig. 5.30).

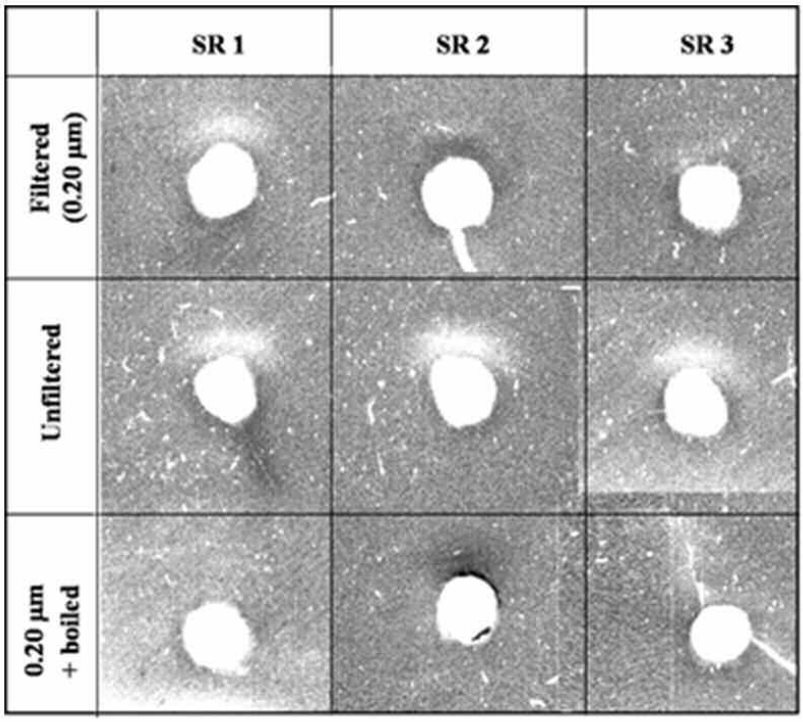

Figure 0.30. *Detection of the proteolytic activity of unfractionated* S. rolfsii *media in a gel containing 0.5 % lyophilized wine proteins. 25 µL of SR 1, SR2, SR3 (see text) filtered, unfiltered and boiled were incubated at 25°C for 120 hours. After incubation the gel was stained with Coomassie and de-stained with 7% acetic acid.*

SR 1 confirmed its activity against the wine proteins both before and after being filtered. SR 2 and SR 3 showed larger protein degradation when not filtered. Boiled samples did not show any protein degradation, confirming the heamoglobin assay results. Once more, the hypothesis of a *S. rolfsii* protease activity against grape and wine protein seemed to be confirmed because no scleroglucan action could interfere with protein disappearance in these experimental conditions.

The three new cultures of SR were added to an unfined Manzoni bianco wine (protein content 300 mg/L) and incubated to assess their effects on protein profiles. After 4 days of incubation, samples were tested by RP-HPLC in order to establish variations in the protein profiles. The RP-HPLC peak areas were analysed (Peng *et al.*, 1997) at the beginning (T0) and after 4 days (T4) of incubation (fig. 5.31, 5.32 and 5.33).

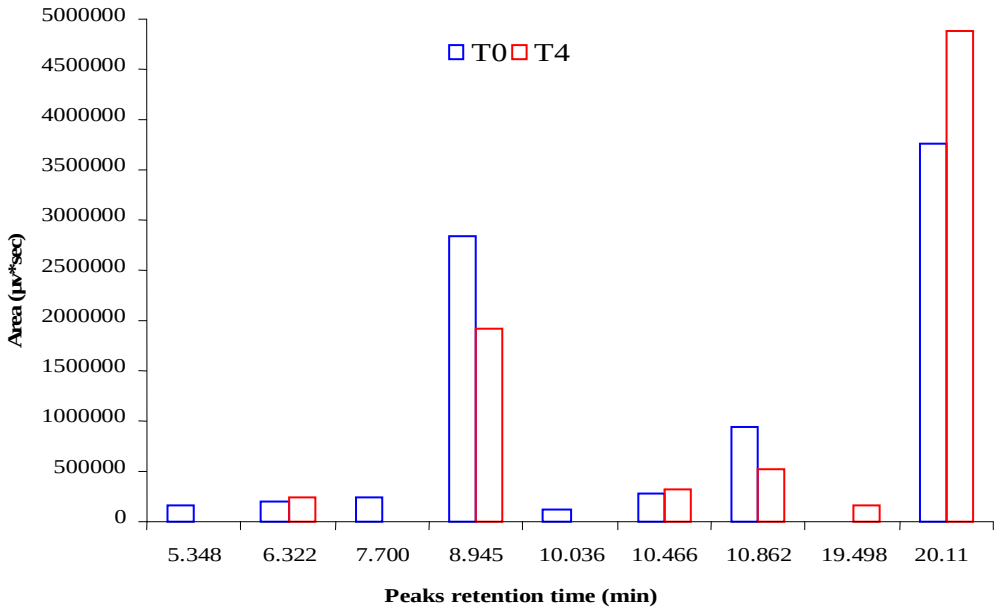

Figure 0.31. *SR1 RP-HPLC peak areas at the beginning (blue bars) and after 4 days of incubation (red bars) with Manzoni bianco wine. The incubation was conducted by adding 100 µL of SR medium to 1 mL of wine.*

Based on the data reported in table 3.1 and in figure 3.11 (chapter 3), the peak retention time detected by RP-HPLC indicated the presence of TL-proteins (from 8.9 to 10.9 min of retention time) and chitinases (from 19.4 to 20.5 min RT). Therefore, the proteolytic activity of the fungus affected the TL-protein content as shown by the sensible decrease for three of the four TL-protein peaks. Surprisingly the same effect did not occur for chitinases that, on the contrary, resulted surprisingly increased. Although it is possible that some degradation products deriving from the action of the fungus elute from the column with the same retention time of the grape chitinases, this occurrence need to be further investigated.

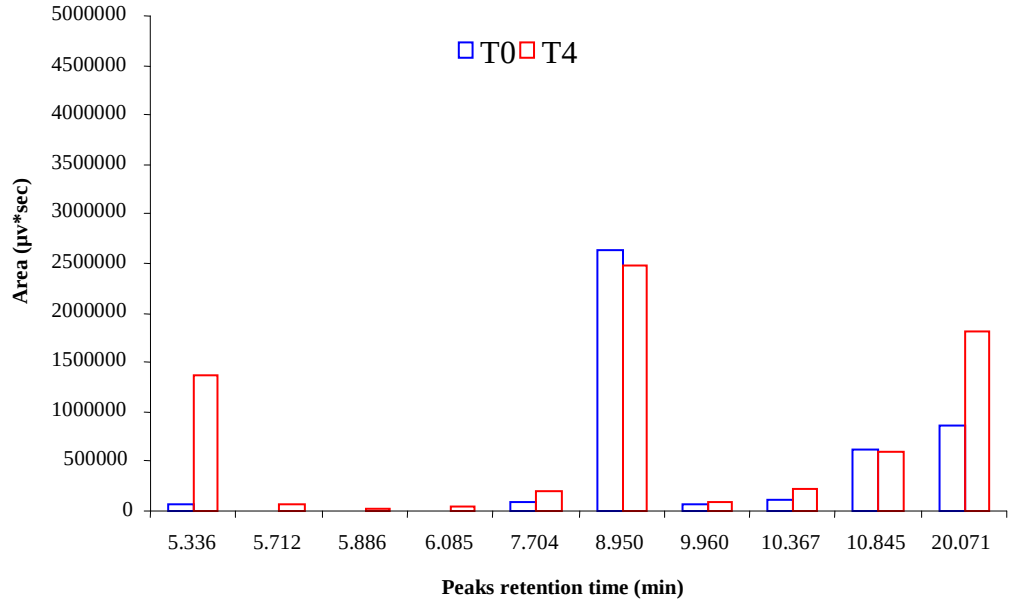

Figure 0.32. *SR2 RP-HPLC peak areas at the beginning (blue bars) and after 4 days of incubation (red bars) with Manzoni bianco wine. The incubation was conducted by adding 100 µL of SR medium to 1 mL of wine.*

SR2 confirmed its lack of proteases activity of the filtered media. Indeed, none of the detected peaks showed diminution, apart for that at 8.9 min of retention.

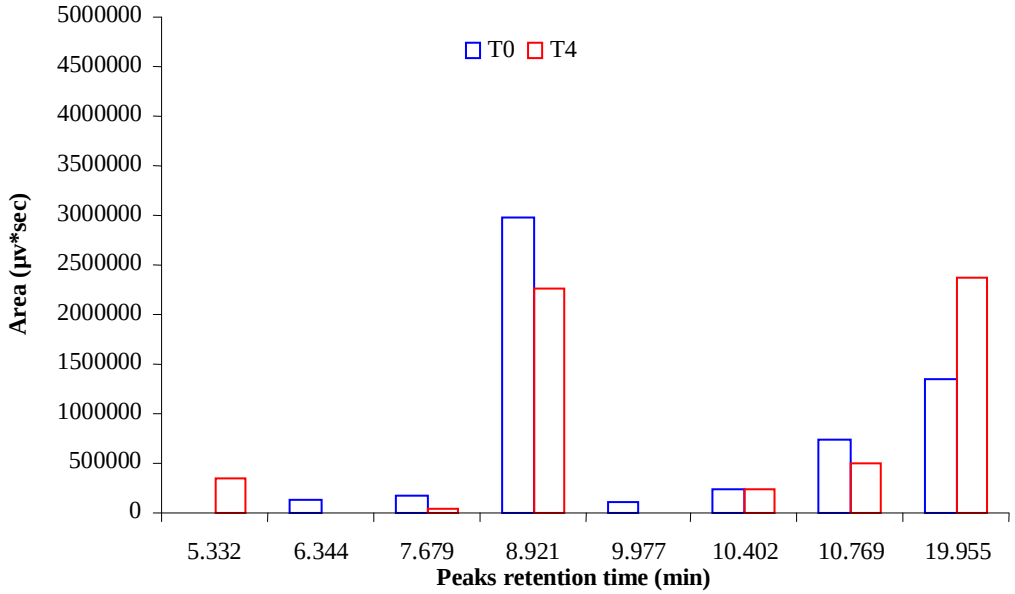

Figure 0.33. *SR3 RP-HPLC peak areas at the beginning (blue bars) and after 4 days of incubation (red bars) with Manzoni bianco wine. The incubation was conducted by adding 100 µL of SR medium to 1 mL of wine.*

Confirming the data previously shown, SR 3, showed behaviour very similar to that of SR1. This result is obvious, since the only difference of these two preparations was the incubation time (7 days for SR 1, 9 for SR3).

The final trial to assess the actual effect of *S. rolfsii* media in wine stabilisation was done through heat testing the wine treated with the culture media (fig. 5.34).

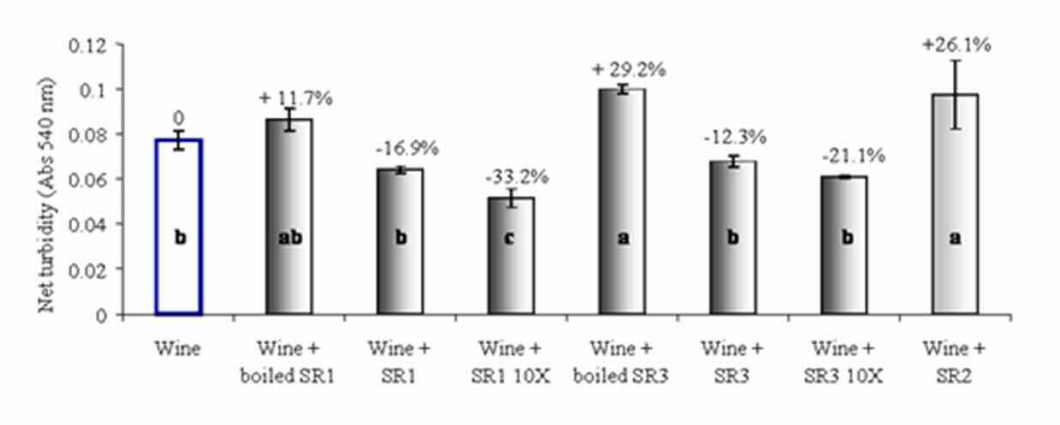

Figure 0.34. *Heat test of an unfined Manzoni bianco wine added of the three SR preparation (SR1, SR2, SR3, see text) in a ratio 1:20 (50 µL/ 1 mL of wine). 10X: enzymatic preparation concentrated 10 times by Vivaspin tubes (3000 MWCO). Incubation was carried out at 25°C for 90 hours before heat-testing the samples (80°C, 6 hours). The turbidity developed was measured as the difference between the absorbance at 540nm before and after the heat test (Pocock and Rankine, 1973; Waters et al., 1991). Different letters means significant differences among values for* $P \leq 0.01$ *(Student-Newman-Keuls test).*

Boiling the fungal preparations before their addition to the wine resulted in the absence of stabilisation and the turbidity was even higher than that of the untreated wine. In contrast, the non-boiled preparations (except SR2) were able to diminish the wine turbidity formed after heat test. In particular, samples SR1 10X and SR3 10X showed a certain stabilizing effect although it was not enough to fully stabilize the wine.

In summary, *Sclerotium rolfsii* has been shown to be a fungus very active on protein removal from grape juice and wine. This removal was due to the action of the scleroglucan emitted by the fungus in the presence of grape proteins. Besides, a certain proteolytic activity has been detected in the SR culture media,

although the presence of scleroglucan interfered with the experimental results. The hypothesis formulated is that a cooperative action between scleroglucan and proteases exists as a protection mechanism exerted by the fungus against grape PR-proteins toxicity. However, attempts to purify the protease/s by AEC and HIC (not shown) brought to a significant enzymatic activity loss and impaired the possibility to experimentally confirm this idea.

In conclusion, the data collected on the fungi studied in this chapter (*B. cinerea, S. rolfsii and S. minor*) indicated the potentiality of their use as a source of proteases active against grape and wine proteins, *S. minor and S. rolfsii* being potentially more suitable than *B. cinerea* for this aim. However, further analyses are required to definitely assess the possibility of the practical application of proteolytic enzymes from the studied fungal sources in the actual conditions of winemaking to stabilise white wines against protein haze formation.

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CHAPTER 6

Scleroglucan-Protein interaction: a tool for protein removal from wine?

ABSTRACT

Many fungi are able to form esocellular polysaccharides. Scleroglucan is a water-soluble polymer produced by fermentation of the filamentous fungus *Sclerotium rolfsii*. This polysaccharide yields only glucose on complete hydrolysis, its molecular structure consisting of beta-1,3-D-glucose residues with one beta-1,6-D-glucose side chain every three main residues. Dissolved scleroglucan chains assume a rod-like triple helical structure, in which the glucose residues are on the outside, thus preventing the helices from coming close to each other and aggregating.

Interaction of scleroglucan with proteins has never been studied. When dissolved in water at a 0.5% concentration, this polysaccharide (used in the form of commercial product Actigum CS) showed no interaction with a standard protein (Bovine Serum Albumin), even after 2h of incubation at room temperature under agitation. However, when the scleroglucan was dissolved at the same concentration in a wine model solution (12% ethanol, 5g/L tartaric acid, pH 3.3), BSA (added at a final concentration of 300mg/L) disappeared completely after only 10 minutes from the addition.

Scleroglucan interacted with proteins also when used to treat a real wine, containing ≈ 350 mg/L of proteins. The interaction appeared slower than in model solution and protein removal was never completed, leaving 15% of the original protein even after a 30h incubation. The interaction with polyphenols was very low, with a maximum removal of 15%. The interaction of scleroglucan with standard proteins and with purified wine proteins and the effects of the solution composition were extensively studied. However, being water soluble, scleroglucan dissolved in both water and wine model solution, thus excluding the possibility to use the native polysaccharide for wine stabilisation.

Key words: *Sclerotium rolfsii*, wine, scleroglucan, haze, protein, polysaccharide.

INTRODUCTION

Wine proteins play a key role in determining the white wines quality, mainly because they are involved in haze formation during wine storage. The most common method to stabilise white wines against haze formation is based on bentonite fining. The negatively charged bentonite particles interact electrostatically with wine proteins allowing their removal from wine. However, this treatment has some drawbacks because bentonite adsorption is rather aspecific and, in addition to proteins, removes different molecules or aggregates, including aroma and flavour compounds (Voilley *et al*., 1990). For these reasons, alternative procedures for protein removal from white wine have been developed, including fining with silica sol/gelatine (Millies, 1975), use of immobilized tannic acid (Weetall *et al*., 1984) or proanthocynidins (Powers *et al*., 1988), use of exchange resins (Sarmento *et al*., 2000), adsorption on the surface of metal oxides (Pachova *et al*., 2002), ultrafiltration (Hsu *et al*., 1987) and adsorption on chitin (Vincenzi *et al*., 2005b). Recently the use of negatively charged polysaccharides has been also proposed to stabilize the wine (Cabello-Pasini *et al*., 2005).

Many fungi are able to form extracellular polysaccharides. The phytopathogenic fungus *Sclerotium rolfsii* (the anamorph form of the basidiomycete *Athelia rolfsii*) attacks a wide variety of plants, primarily annuals and herbaceous perennials, but some woody plants are also attacked when they are young (Aycock, 1961). The filamentous fungus *Sclerotium rolfsii* has also been extensively studied over the last years by virtue of its ability to excrete large amounts of β-1,3-β-1,6-d-glucan or 'scleroglucan' (Fariña *et al*., 1998). This exopolysaccharide exhibits interesting rheological properties for different industrial areas (Fariña *et al*., 2001) and it has been recently included as one of the most potent biological response modifiers (Pretus *et al*., 1991).

Scleroglucan is a water-soluble polymer which yields only glucose on complete hydrolysis; its repeating unit consists of beta-1,3-D-glucose residues with one beta-1,6-D-glucose side chain every three main residues. Dissolved

scleroglucan chains assume a rod-like triple helical structure, in which the glucose residues are on the outside, thus preventing the helices from coming close to each other and aggregating. Scleroglucan is a neutral polysaccharide and its interaction with proteins has never been studied.

MATERIALS AND METHODS

1.1.59 MATERIALS

The wine used (vintage 2005, variety Manzoni bianco) was kindly supplied from the "Scuola Enologica G.B. Cerletti" of Conegliano (Italy).

The experiments were performed by adding (under mild agitation) the commercial dried scleroglucan ACTIGUM CS 11 (Degussa) obtained from *Sclerotium rolfsii*, to the protein solution for an adequate incubation time. The insoluble pellets were collected by centrifugation (14000g for 5 min).

1.1.60 GRAPE AND WINE PROTEIN CONTENT DETERMINATION

Protein content determination was performed according to Vincenzi *et al.* (2005a). Firstly, proteins were precipitated from 1 mL of wine with the KDS method (Zoccatelli *et al*. 2003). The pellets were dissolved into 1 mL of distilled water and quantified by using the BCA-200 protein assay kit (Pierce). The calibration curve was prepared by using serial dilution of bovine serum albumin (BSA, Sigma) in water. The measurements were performed spectrophotometrically at 562 nm (Shimadzu UV 6010).

1.1.61 TOTAL POLYSACCHARIDE CONTENT DETERMINATION

The polysaccharide content was determined colorimetrically according to Segarra and co-workers (1995). After addition of 5 volumes of absolute ethanol (Baker), samples were left at 4°C overnight before centrifugation (30 min, 14000g). The collected pellets were washed twice with ethanol (Baker) and then dissolved in bi-distilled water. 1 mL of sample was then added of 25 µL of 80% phenol (w/w, Fluka) and 2.5 mL of sulphuric acid (Merck). Samples were mixed

and the reaction carried on for 30 minutes at room temperature. Absorbance values were spectrophotometrically measured at 490 nm (Shimadzu UV 6010). The calibration curve was prepared by using serial dilution of galactose (Fluka) in water.

1.1.62 TOTAL POLYPHENOLS CONTENT DETERMINATION

The phenolic content in sample was determined colometrically according with the method proposed by Singleton and Rossi (1965) optimised for small sample volumes by Waterhouse (2002). 200 µL of water diluted sample (1:10 v/v) were added with 1 mL of water diluted (1:10 v/v) 2N Folin-Ciocalteau reagent (Sigma). 800 µL of 7.5% (w/v) Na_2CO_3 (Merck) solution were added to the sample and the incubation carried out for 30 min at 40°C. The calibration curve was prepared by using serial dilution of gallic acid (GAE, Fluka) in water. The measurements were performed spectrophotometrically at 725 nm (Shimadzu UV 6010).

1.1.63 HEAT TEST

According to Waters and colleagues (1991), a heat test was performed to determine protein stability. After heating (80°C for 6 hours), samples were chilled (16 hours at 4°C) and, after equilibration at room temperature, turbidity values were measured nephelometrically (Hach 2100P turbidimeter) or spectrophotometrically at 540 nm (Shimadzu UV 6010). Net turbidity values lower than 2 NTU (Net Turbidity Unit) or 0.02 AU (Absorbance Unit) indicated sample stability.

1.1.64 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970). Samples to be analysed were dissolved in a Tris-HCl pH 6.8 buffer containing 15% (v/v) glycerol (Sigma) and 1.5 % (w/v) SDS (Bio-Rad) and heated at 100°C for 5 minutes before loading. For SDS-PAGE in reducing conditions, 3% (v/v) of

β-mercaptoethanol (Sigma) was also added to the loading buffer. Electrophoresis was performed in a Mini-Protean III apparatus (Bio-Rad) with T = 14% (acrylamide-N, N' metilen-bisacrylamide 29:1; Fluka) gels. The molecular weight standard proteins were Myosin (200,000 Da), β-galactosidase (116,250 Da), Phosphorylase b (97,400), Serum Albumin (66,200 Da), Ovalbumin (45,000 Da), 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). After electrophoresis, gels were stained for 18 h with Coomassie brilliant blue R-250 (Sigma) and then destained with 7 % acetic acid for 24 h (Koenig *et al.*, 1970).

1.1.65 STATISTICAL ANALYSIS

Data were analysed by one-way completely randomized ANOVA with the CoHort Software (CoStat version 6.311, Monterey, CA) and data significativity assessed by Student-Newman-Keuls test.

RESULTS AND DISCUSSION

1.1.66 KINETIC OF SCLEROGLUCAN-PROTEIN INTERACTIONS

As discussed in chapter 5, the growth of the fungus *Sclerotium rolfsii*, in a medium containing only wine proteins as the nitrogen source, caused the complete disappearance of these latter after 72 h of incubation (fig.6.1).

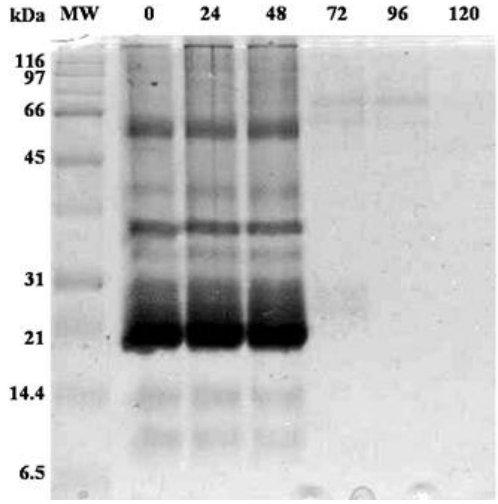


Figure 0.1. *SDS-PAGE analysis of the proteins of the media during the period of incubation with* Sclerotium rolfsii *(SR) in non-reducing conditions. MW = molecular weight standards; 0, 24, 48, 72, 96 and 120: hours of incubation.*

Further analyses indicated that protein disappearance was due to protein sequestration from the scleroglucan produced in large quantities by the fungus, rather than to a protease activity that was seen to be not relevant (see Chapter 5, fig. 5.8).

Grape and wine PR-proteins demonstrate *in vitro* antifungal activity against common fungal pathogens of grapevine (Giananakis *et al*., 1998; Salzman *et al*., 1998; Tattersall *et al*., 2001; Jayasankar *et al*., 2003; Monteiro *et al*., 2003). Consequently, the emission of scleroglucan by the fungus might be part of a defence mechanism against PR-proteins. This mechanism of action is probably necessary to keep the fungi safe from PR-proteins toxicity and to use them as a nitrogen source, probably by means of proteolytic enzymes adsorbed into the scleroglucan (see chapter 5).

Sclerotium rolfsii is a fungus that releases high quantities of esocellular scleroglucan in the culture medium. Preliminary experiments were performed in order to assess the adsorption capability of the scleroglucan towards Manzoni bianco wine proteins. To this aim, a commercial dried scleroglucan was added to an unfined Manzoni bianco wine at dosages between 0 and 10 mg/mL.

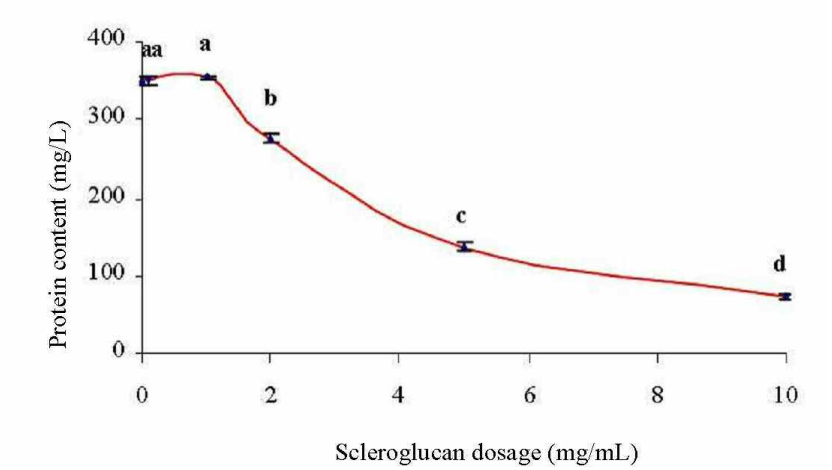


Figure 0.2. *Effect of the addition of scleroglucan at increasing concentration (0.1, 1, 2, 5, 10 mg/mL) on the wine total protein content after 48 hours of incubation with an unfined Manzoni bianco wine (original protein content 351 mg/L). Each data is the mean of at least three replicates. Different letters means significant differences among values for P ≤ 0.01 (Student-Newman-Keuls test).*

A significant protein diminution was detectable with dosages higher than 1 mg/mL, until reaching of a decrease of the 80% at 10 mg/mL. These results confirmed the ability of the scleroglucan to adsorb wine proteins also in real conditions.

The effects of the scleroglucan treatment on some wine parameters were studied. Firstly, a quantification of the total polysaccharide content was performed in the same samples of figure 6.2 (fig. 6.3).

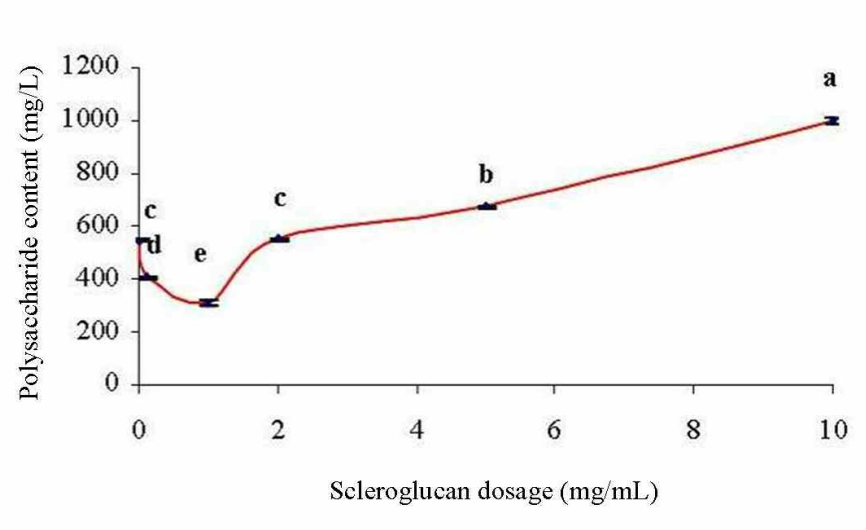


Figure 0.3. *Effect of the addition of scleroglucan at increasing concentrations (0.1, 1, 2,*

5, 10 mg/mL) on the wine total polysaccharide content after 48 hours of incubation with an unfined Manzoni bianco wine. Different letters means significant differences among values for P ≤ 0.01 (Student-Newman-Keuls test).

The results highlighted a partial subtraction of polysaccharides from wine at low dosages of scleroglucan, while starting from 2 mg/mL a release of polysaccharides was detectable. It is known that scleroglucan is soluble in aqueous solutions. Consequently, this release should be due to the release of scleroglucan in the wine, probably deriving from its uncompleted interaction with the wine proteins.

The effect of the treatment with scleroglucan was also assessed for the wine total polyphenol content (fig. 6.4).

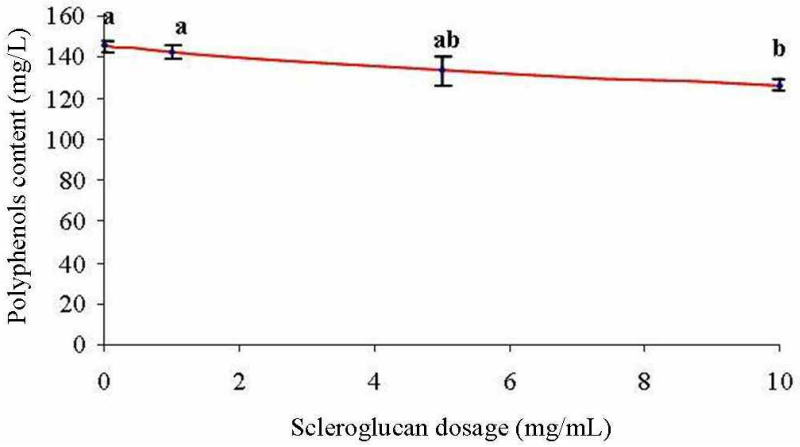


Figure 0.4. *Effect of the addition of scleroglucan at increasing concentrations (0.1, 1, 2, 5, 10 mg/mL) on the wine total polyphenols content after 48 hours of incubation with an unfined Manzoni bianco wine. Different letters means significant differences among values for P ≤ 0.01 (Student-Newman-Keuls test).*

A little effect on polyphenol content was observed, with significant decrement (-13.1%) at the maximum dosage tested. It seems that this reduction depended more on the elimination of polyphenols bound to proteins (Somers and Ziemelis, 1973) than to a direct effect of the polymer on these compounds. This hypothesis was supported by the visual aspect of the pellet obtained after the treatment of a real wine with scleroglucan in comparison to that obtained from a model wine (fig. 6.5).

Figure 0.5. *Left: Pellet of a model wine containing BSA treated with scleroglucan. Right: pellet of a Manzoni bianco wine treated with scleroglucan.*

The yellow colour of the pellet achieved from the real wine (fig. 6.5, right) should indicate the presence of polyphenols, which are absent in model wine (fig. 6.5, left).

To determine the kinetics of scleroglucan-protein interactions, the polysaccharide was added at both four concentrations and incubation times in a model solution (tartaric acid 5g/L, ethanol 12%, pH 3.2) in the presence of 300 mg/L of Bovine Serum Albumin (BSA). The data showed the reaching of complete BSA elimination after only 10 minutes with 5 mg/mL of scleroglucan (fig. 6.6), whereas the presence of little protein was observed at higher concentrations.

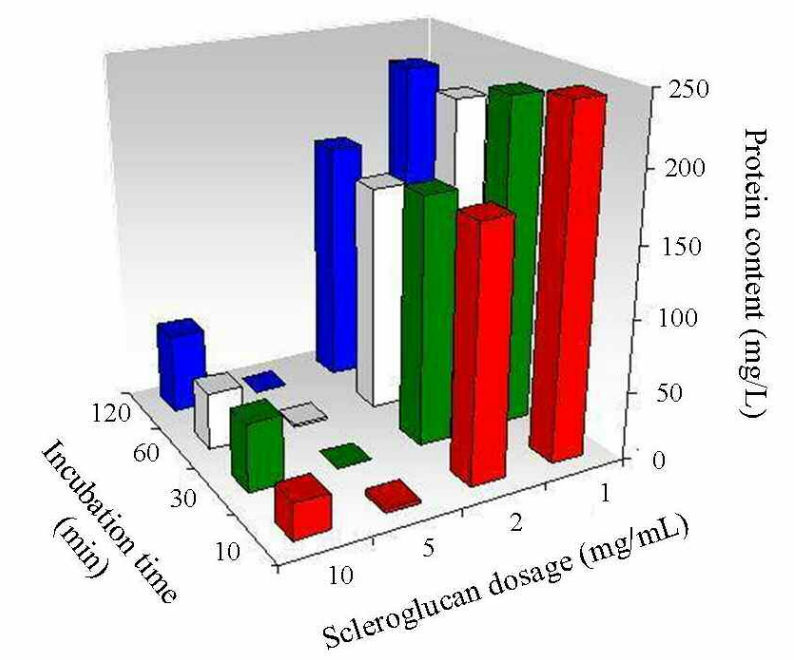


Figure 0.6. *Effect of the addition of scleroglucan at increasing concentrations (0.1, 1, 2, 5, 10 mg/mL) and of the contact time (10, 30, 60 and 120 min) in the total protein content of a model wine prepared with 300 mg/L of BSA. Each data is expressed as an average of at least three replicates.*

The data confirmed that the scleroglucan concentration strongly influences the protein content, with a maximum BSA subtraction at 5 mg/mL. Each scleroglucan dosage significantly influenced the residual protein content for $P \leq 0.01$. In this experiment, a certain release of protein was observed at higher dosages (10 mg/mL). It is noteworthy that the adsorption kinetic resulted linear from 0 to 5 mg/mL of scleroglucan ($R^2 > 0.9$), while no significant effect ($P \leq 0.01$) was observed for the incubation time. In fact, after 10 minutes BSA adsorption resulted complete.

The same samples tested for protein adsorption were analysed to assess the quantity of polysaccharides resulting from scleroglucan dissolution into the model wine (fig. 6.7).

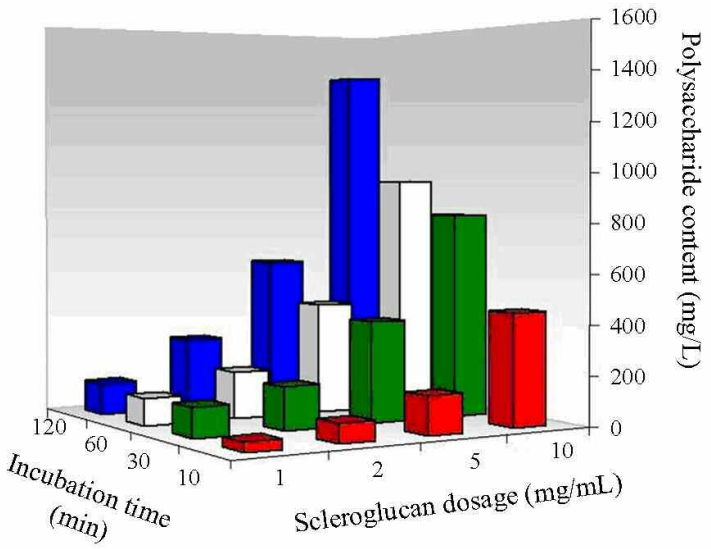


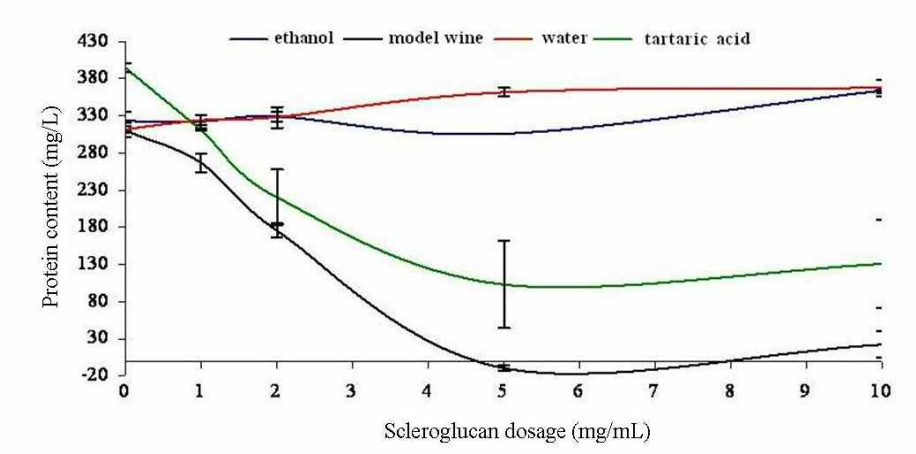
Figure 0.7. *Effect of the addition of scleroglucan at increasing concentrations (0.1, 1, 2, 5, 10 mg/mL) and of the contact time (10, 30, 60 and 120 min) on the total polysaccharide content of a model wine prepared with 300 mg/L of BSA. Each data is expressed as an average of at least three replicates.*

The results confirmed what observed formerly, by highlighting a polysaccharide release that was significantly influenced by both the scleroglucan dosage ($P \leq 0.01$) and the incubation time ($P \leq 0.01$). These data confirmed scleroglucan solubility in aqueous solutions.

1.1.67 SOLVENT EFFECT ON SCLEROGLUCAN-PROTEIN INTERACTIONS

The scleroglucan was incubated at several concentrations for 30' in water, tartaric acid (5 g/L, pH 3.2), ethanol (12%) and wine model solution, all containing 300 mg/L of BSA (fig. 6.8).

In water and ethanol solutions the interaction with proteins was significantly lower ($P \leq 0.01$) than in acidic solutions. The highest protein removal was observed in model wine, suggesting a synergistic effect of pH and ethanol, probably due to a better hydration of the polysaccharide in the presence



of alcohol.

Figure 0.8. *Effect of the solvent composition on the BSA-scleroglucan interaction. Each data is expressed as an average of at least three replicates.*

1.1.68 IONIC STRENGTH EFFECT ON SCLEROGLUCAN-PROTEIN INTERACTIONS

The scleroglucan is a neutral polysaccharide. However, to exclude the possibility of an ionic interaction with proteins, 0.5M NaCl was added to the solutions.

As showed in fig. 6.9, the protein elimination rate was diminished by the salt, though proteins were still completely removed after 24h of incubation.

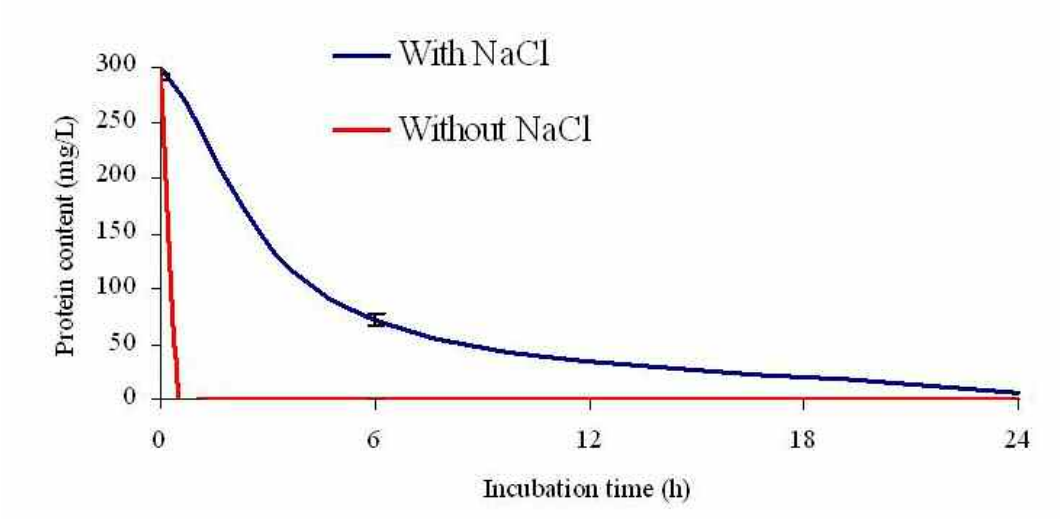


Figure 0.9. *Effect of 0.5 M NaCl on the BSA-scleroglucan interaction. Scleroglucan was added at 5 mg/mL. Each data is expressed as an average of at least three replicates.*

The presence of NaCl produced also a reduction on polysaccharide solubility (data not shown) and this may explain the reduction of the scleroglucan-protein interaction rate.

1.1.69 EFFECT OF THE HIGH MW ENDOGENOUS WINE COMPOUNDS ON SCLEROGLUCAN-PROTEIN INTERACTIONS.

An ultrafiltered wine (3 kDa MWCO) was used to study the effects of scleroglucan addition (5 mg/mL, 30 min incubation) on BSA (added at 300 mg/L) removal in the presence of all the non-macromolecular components of wine, including polyphenols (fig. 6.10).

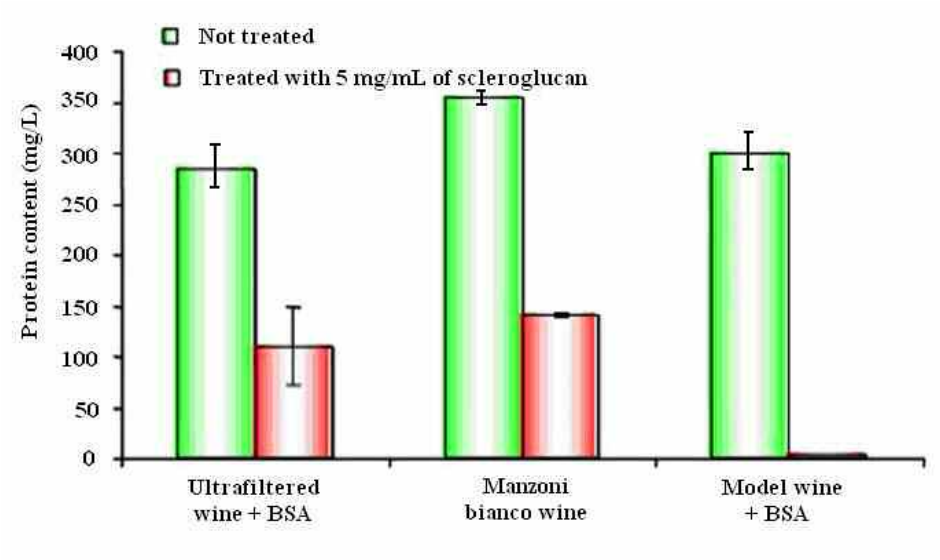


Figure 0.10. *Effect of the removal of macromolecular compounds (> 3 kDa) from wine on the protein-scleroglucan interaction. Each data is expressed as an average of at least three replicates.*

The results showed that the protein removal was complete only in model wine. In the presence of endogenous wine compounds with a MW lower than 3 kDa the protein removal resulted incomplete, with a diminution of 65 % in the protein content, which corresponds to the quantity observed for the whole wine. Therefore, some endogenous compounds with low MW present in the ultrafiltered wine impair to some extent scleroglucan-protein interactions. Although the nature of these compounds has not been elucidated, an involvement of wine polyphenols (fig. 6.4) on this result can be hypothesized.

1.1.70 EFFECT OF PROTEIN TYPE ON THE INTERACTIONS WITH SCLEROGLUCAN

Because in the presence of wine low MW compounds (may be

polyphenols) the protein reduction obtained by scleroglucan addition resulted similar using both a model protein (BSA) and the total wine proteins (fig. 6.10), it could be hypothesized that the compositional characteristic of the solution influences the interaction more than the protein nature. Therefore, to confirm this idea, the effect of scleroglucan addition was studied on solutions prepared with various proteins, differing in both molecular weight and glycosilation degree (fig. 6.11).

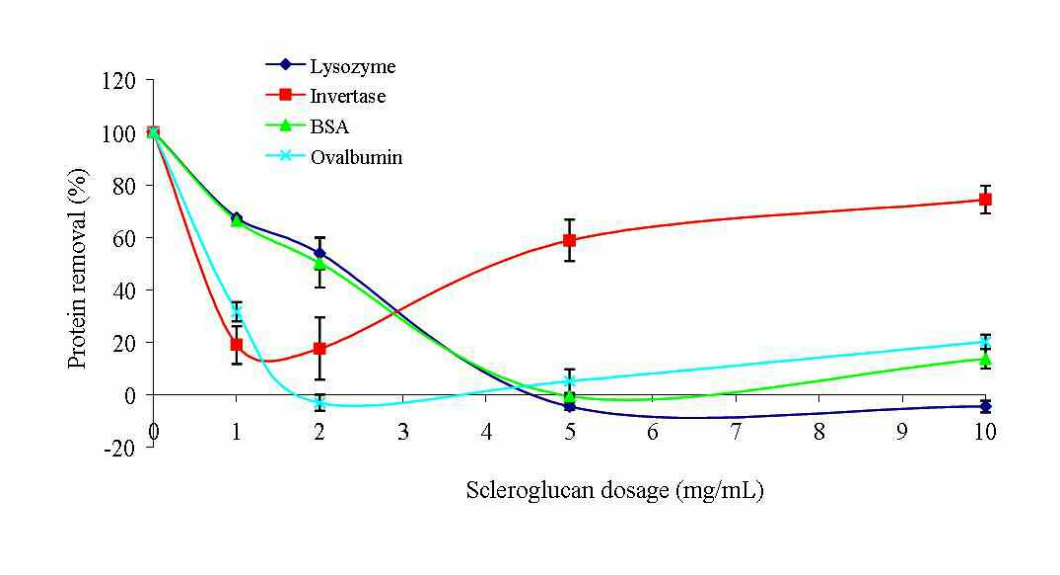


Figure 0.11. *Interaction of different proteins with scleroglucan in a model wine solution. Each data is expressed as an average of at least three replicates.*

The protein removal effect of the scleroglucan appeared to be independent from the protein molecular weight (see the overlapping curves for BSA, 66 kDa, and lysozyme, 14.4 kDa), whereas protein glycosilation seemed to have some effect on the interaction with the polysaccharide. It is to be noted that the ovalbumin, a protein with a MW between BSA and lysozyme but with a high glycosylation degree, needed the lowest dose of scleroglucan to be removed, indicating a strong affinity for the polysaccharide. This effect was observed also with yeast invertase (about 50% of glycosylation), although the interaction of the scleroglucan with this protein was progressively reduced at scleroglucan concentrations higher than ≈ 1.5 mg/mL. Therefore, the effect of the protein type on scleroglucan-protein interactions warrants further investigation.

1.1.71 EFFECTS OF SCLEROGLUCAN ADDITION ON HEAT STABILITY OF MODEL SOLUTIONS

To correlate the effect of protein removal by scleroglucan and the thermal stability of the treated protein solution, heat tests were performed on model wines prepared with 300 mg/L of BSA. In general, the heat stability test (fig. 6.12) revealed a proportional relationship between scleroglucan concentration and haze formation.

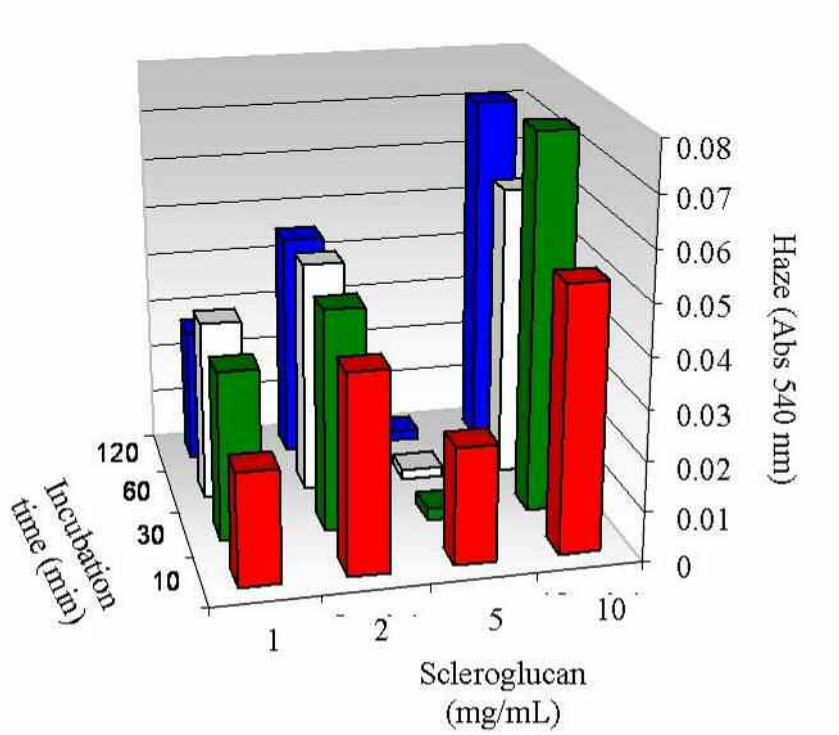


Figure 0.12. *Heat test on model wine samples prepared with 300 mg/L of BSA. Each data is expressed as an average of at least three replicates.*

Consequently, the increase of scleroglucan concentration matched with an increase in heat-induced turbidity, indicating that the polysaccharide can contribute to its formation. However, it is noteworthy that the treatments at 5 mg/mL of scleroglucan made the samples heat stable. This can be explained by the almost total absence of proteins at this polysaccharide dosage (see fig. 6.8). However, also at 10 mg/mL of scleroglucan addition BSA was present in near zero quantities (see fig. 6.8), but in this case the heat-induced turbidity was the highest. Taking into account that heating the scleroglucan alone in model wine did

not result in haze formation (data not shown), this occurrence may be explained as follows. The presence of an excess of scleroglucan with respect to the protein in solution, could result in the formation of heat-unstable complexes different from those formed at the polysaccharide/BSA ratio existing at 5 mg/mL of scleroglucan addition, which were instead heat stable.

Moreover, this experiment indicated also an effect of the incubation time, at least for the samples treated with 5 mg/mL of scleroglucan (fig. 6.12). In particular, after 10 minutes this sample was still instable (Δ Abs > 0.02) and complete stabilisation was achieved only after 30 min of treatment. This would indicate that, despite an almost immediate interaction between scleroglucan and proteins (fig. 6.6), the formation of heat stable complexes needed a longer time to occur.

1.1.72 CONCLUSIONS

Scleroglucan is able to interact with proteins when dissolved in acidic buffer, and the presence of little quantity of ethanol enhances this binding.

This interaction seems to be of non-ionic nature because of the neutral characteristic of the scleroglucan. The presence of polyphenols reduces of only 30% the ability of the polysaccharide to remove proteins. These characteristics would make the scleroglucan a good tool for protein removal from wine. However, this polysaccharide is soluble in aqueous solutions and tends to remain in the wine after the treatment. Consequently, in order to use scleroglucan for wine stabilisation, it seems necessary to overcome this inconvenient by using chemical modifications to make the polysaccharide completely insoluble in the wines to be stabilised.

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CHAPTER 7

Conclusions

This work aimed to improve the comprehension of several grape and wine protein features, such as the effect of fermentation on protein stability, the role of protein hydrophobicity on haze formation and on their reactivity with seed tannins. Moreover, the investigation for novel chromatographic methods for grape and wine proteins fractionation was carried out. Besides, in order to solve the problem of haze formation in white wines, two alternative approaches to remove proteins responsible for this drawback were studied.

Firstly, this thesis aspired to clear the fate of grape proteins during the alcoholic fermentation. In particular, it was highlighted that the soluble proteins of the berry of a white grape variety (cv. Manzoni bianco) vary during and after the alcoholic fermentation in both quantity and relative proportions. Indeed, a protein fraction containing a single 20 kDa band (thaumatin-like protein), obtained with Anion Exchange Chromatography, resulted that with the lowest heat-stability and with the largest quantitative increase throughout the fermentation process, constituting a large proportion of the total wine proteins. These results are consistent with the statement that thaumatin-like proteins are the main responsible for haze formation in white wines, as established in chapter 4. Moreover, it was confirmed that fermenting yeasts release glycocompounds in the wine (probably mannoproteins), resulting in an improved heat stability of the total wine proteins, despite the increase in the relative proportion of their most unstable component.

In chapter 3, a novel method for grape and wine proteins purification is proposed. The chromatographic approach used takes advantage of the different hydrophobicity of the grape and wine proteins, which were fractionated according to this characteristic. The main outcome was that with Hydrophobic Interaction Chromatography (HIC) it is possible to effectively fractionate grape juice and wine proteins, combining a good preparative separation with the attainment of high protein recovery and purity for several protein fractions. In particular, a *Vitis*

vinifera thaumatin-like protein was purified with high yields, from both Semillon grape juice and wine, by exploiting its highest hydrophobicity level among grape proteins. Additionally, this chromatographic system demonstrated the potentiality to purify more than one protein, especially in wine in which the number of proteins is low. For this reason, this knowledge will be very helpful for the purification of other grape and wine proteins. Therefore the application of HIC in enological studies should contribute to improve the understanding on protein characteristics and functionality.

Besides, by using a multi-step purification procedure, involving HIC and RP-HPLC it was possible to separate by SDS-PAGE 26 different protein spots that have been analysed by LC-MS/MS. A large number of grape proteins were thus identified by MS and database searching, with a majority of chitinases, followed by thaumatin like proteins. However, not all the identified proteins presented the highest homology with *Vitis vinifera* proteins, indicating that further investigation are required to establish the complexity of the grape protein composition.

From these sequence results it was possible to create a scheme summarizing the putative hydrophobicity of the grape proteins, that can be useful to interpret their physical-chemical behaviour. Moreover, the precise identification of the single grape proteins allowed to assign them to each RP-HPLC peak and this outcome could be useful to researchers for an unequivocal and rapid identification of grape proteins on the basis of the RP-HPLC retention time.

An example of how HIC could be utilized in protein studies is given in chapter 4, in which an extension of this technique was performed in order to fractionate and study proteins from an unfined Manzoni bianco wine. By combining a preparative HIC fractionation with other analytical techniques (i.e. SDS-PAGE, RP-HPLC, SE-HPLC), it was possible to partially characterize the fractionated Manzoni bianco wine proteins. A relationship between protein hydrophobicity and haze potential after the heat test was found. Furthermore, the thaumatin-like protein content of each fraction resulted strictly related to its hazing potential, confirming the assumption that thaumatin-like proteins are the

main heat-unstable wine proteins. Moreover, by means of Size Exclusion Chromatography it was also confirmed the protective role of glycocompounds against proteins insolubilisation.

From the study of wine protein fractions with different hydrophobicity some conclusions are reached. In particular, it can be stated that the turbidity formed after tannin addition to protein fractions in model wine tended to increase according to their hydrophobicity, suggesting a relationship between this parameter and wine proteins reactivity with tannins. Moreover, temperature plays a key role in the haze induction, since a dramatic increase of tannin-protein reactivity occurs after heating. Heating of the protein-tannin mixture might increase the extent of interactions by inducing protein denaturation and exposition of a high number of tannin binding site. In the protein native state, these sites should be buried in its core, which can be supposed to contain the most hydrophobic portion of the molecule. Therefore a role of hydrophobic interactions, which, on the other hand are favoured at high temperature, should be confirmed. However, pre-heating the proteins in model wine did not increase turbidity development after tannin addition. A possible explanation of this fact is that the change in protein conformation occurring during the heating in the absence of tannins is a reversible process. Therefore, protein reactivity with tannins is enhanced only when protein is present in its heat-denatured state. Moreover, an enhancement of hydrophobic interactions, whose strength increases with the temperature, would be supposed to contribute to tannin-protein interactions at high temperature. Therefore, if the increase in turbidity formation with the increasing of protein hydrophobicity, as demonstrated in chapter 4, is considered, the idea that hydrophobicity plays a key role in determining wine protein hazing seems to be justified.

All these notions will be useful to better define the relationship between the presence different types of protein and tannins, in order to clear their role in white wines hazing.

In the second part of this thesis, two approaches to overtake the still unsolved problem of haze formation in white wines are illustrated.

Taking into account that treatments with proteolytic enzymes are widely

considered the ideal system to remove the heat-unstable proteins from musts and wines, a selection of fungal strains with the potential to produce proteases acting on wine proteins was conducted. Our results confirmed that *Botrytis cinerea* is able to secrete exocellular proteases active against grape proteins. Besides, the suitability of other filamentous phytopathogenic fungi in growing utilizing wine proteins as the sole nitrogen source was demonstrated. In particular *Sclerotina minor* and *Sclerotium rolfsii* seemed more suitable than *B. cinerea* in eliminating grape and wine proteins responsible for haze formation in white wines, highlighting the potentiality to be used as a source of proteolytic enzymes, active at the wine pH, useful to this aim. However the attempts to purify the proteases resulted in significant activity losses and further investigations are required to definitely assess the possibility of a practical application of the studied fungi in the actual conditions of winemaking. In particular, the study has to continue by trying to obtain a high production of active proteases by the fungi and by studying purification techniques more efficient and able to preserve the enzymatic activity. In addition, single purified protein fractions of heat-unstable grape and wine proteins (thaumatin-like proteins and chitinases) should be tested for degradability with the purified fungal proteases. In this way, a better understanding of the well known PR-protein resistance to proteolysis will be achieved, with the obtainment of useful information for the selection of fungal proteases to be used in winemaking.

By studying *Sclerotium rolfsii* for its possible proteases production, it was found that this fungus emits in the culture medium, in addition to a protease, also a polysaccharide (scleroglucan) having the capability to completely remove the wine proteins, which were also seen to act as an enhancing factor for its production. The probable explanation for this occurrence was that a cooperative action between scleroglucan and proteases exists as a protection mechanism exerted by the fungus against grape and wine PR-proteins toxicity. From the characterisation of the ability of the scleroglucan to interact with proteins it was demonstrated that this polysaccharide is able to interact with both a standard (BSA) and wine proteins when dissolved in acidic buffer, and that the presence of little quantity of ethanol enhances this binding. This interaction seems not to

depend on the ionic strength because of the neutral characteristic of the scleroglucan. Besides, the presence of the non-macromolecular fraction (< 3 kDa) of the wine reduces of only by 35% the ability of the polysaccharide to remove proteins. These characteristics would make the scleroglucan a good tool for protein removal from wine. However, this polysaccharide showed a main drawback, because it dissolved in part in the solution. Consequently, in order to use scleroglucan for wine stabilization, it will be essential to overcome this inconvenient by using chemical modifications of its structure (i.e. by cross-linking and/or immobilization) in order to make the polysaccharide completely insoluble in the wines to be stabilized.

In conclusion, main results of this thesis work are the improvement in grape and wine protein fractionation and the study of alternative methods for the removal of heat-unstable protein from wines. A demonstration of how the combination of different chromatographic techniques can lead to a deeper comprehension of the functional and biochemical characteristics of grape and wine proteins is given. These data allowed to clear some grape and wine protein characteristic, such as their hydrophobicity, their peculiar heat-stability and reactivity with tannins which, at date, are not fully understood.

It seems that this line of research can offer useful information to the researchers in finding new strategies to understand and prevent protein hazing in white wine.