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Grape and Wine Proteins: Their Fractionation by Hydrophobic Interaction Chromatography and Identification by Chromatographic and Proteomic Analysis

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A method to fractionate grape and wine proteins by hydrophobic interaction chromatography (HIC) was developed. This method allowed the isolation of a thaumatin-like protein in a single step with high yield and >90% purity and has potential to purify several other proteins. In addition, by separating HIC fractions by reverse phase HPLC and by collecting the obtained peaks, the grape juice proteins were further separated, by SDS-PAGE, into 24 bands. The bands were subjected to nanoLC-MS/MS analysis, and the results were matched against a database and characterized as various *Vitis vinifera* proteins. Moreover, either directly or by homology searching, identity or function was attributed to all of the gel bands identified, which mainly consisted of grape chitinases and thaumatin-like proteins but also included vacuolar invertase, PR-4 type proteins, and a lipid transfer protein from grapes.

KEYWORDS: Grape juice; wine; HIC; nanoLC-MS/MS; *Vitis vinifera*; thaumatin-like protein; chitinases

INTRODUCTION

The problem of protein haze formation in white wines is still not fully resolved, despite haze being a serious quality defect because consumers perceive hazy wines as faulty. Protein haze is caused by a relatively low concentration, generally varying from a few to several hundreds of milligrams per liter (*1*−*3*), of pathogenesis-related (PR) proteins of grape (*Vitis vinifera*), namely, thaumatin-like (TL) proteins and chitinases. These proteins aggregate, resulting in light-dispersing particles and visible haziness. Currently, haze formation is prevented in commercial wines by removing the proteins by adsorption onto bentonite (*3*). Although bentonite is effective in removing the problem, it is not an ideal treatment because it can adversely affect the quality of the treated wine (*3*). Furthermore, because of bentonite's considerable swelling and poor settling characteristics, 3−10% of the wine volume is taken up by the bentonite and the quality of this "lees" wine is reduced. In addition, handling and disposal of spent bentonite continues to be of concern, because of high labor input and associated costs, occupational health and safety issues, and the wine industry's environmental responsibilities. It was estimated in 2000 that the cost of bentonite fining to the world wine industry was U.S. $300−500 million annually (*4*). A better understanding of protein haze that would allow the development of improved process technologies as compared with bentonite would therefore be highly desirable.

In any study of this problem in wine, researchers need easy and high-yielding protein purification procedures. Previously, techniques such as ion exchange chromatography and gel filtration have been widely used for fractionation of grape and wine macromolecules (*5*−*10*). Other chromatographic methods, such as hydrophobic interaction chromatography (HIC), have been used only infrequently (*11*). HIC takes advantage of the hydrophobicity of proteins promoting separation based on hydrophobic interactions between immobilized hydrophobic ligands and nonpolar regions on the surface of proteins. The adsorption increases with high salt concentration in the mobile phase, and elution is achieved by decreasing the salt concentration of the eluent (*12*−*14*).

During the past 10 years a new research technology, proteomics, has expanded at an astonishing rate. Since 2004, there have been only a few reports of a proteomics approach being used in grape and wine protein studies (*15*−*17*). Although proteomics has the potential to identify the proteins involved in haze formation and allow significant progress to be made, its use is not yet widespread.

We developed a simple and high-yield purification method for grape proteins. The method involved protein salting out followed by direct fractionation through a HIC stationary phase. Moreover, after the grape protein fractionation by hydrophobicity, we identified the proteins in the fractions by a HPLC-MS/MS-based proteomics approach.

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MATERIALS AND METHODS

Materials. Grape and wine proteins were purified from (unfined) Semillon grape juice and wine from the Adelaide Hills region (South Australia) harvested in 2005.

Ammonium Sulfate Protein Precipitation. Grape proteins from Semillon juice or wine were concentrated by ammonium sulfate precipitation. The salt was added to 80% saturation to the grape juice or wine, previously buffered to pH 5.0 (with KOH). After 16 h at 4 °C, the mixture was centrifuged (14000*g*) for 30 min at 4 °C. The pellet was then dissolved in the buffer required for the next step (see below).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoretic analyses were performed according to the method of Laemmli (*18*). The samples were dissolved in a Tris-HCl, pH 6.8, buffer containing 15% (v/v) glycerol (Sigma-Aldrich) and 1.5% (w/v) SDS (Bio-Rad Laboratories Pty. Ltd.), and heated at 100 °C for 5 min before loading for analysis performed by using a Mini-Protean III apparatus (Bio-Rad). All analyses were conducted under nonreducing conditions. The Broad Range molecular weight standards (Bio-Rad) and Ready Tris-HCl Gels [Bio-Rad, 4−20%, 15-well, 15 μL, 8.6 × 6.8 cm (W × L)] were used. Gels were alternatively stained with Bio-Safe Coomassie stain (Bio-Rad) or silver stain procedure for high-sensitivity protein detection, according to the method of Blum et al. (*19*). Band intensities were measured using ImageJ software (http://rsb.info.nih.gov/ij/).

Chromatography Instrumentation. The chromatographic separations were performed on either a low-pressure system or an analytical scale HPLC. The low-pressure system used was an ÄKTA Prime apparatus (Amersham Biosciences) equipped with an UV detector (λ absorbance detector). For analytical HPLC separations, an Agilent 1100 series (Agilent Technologies) equipped with autosampler, fraction collector, and diode array detector was used.

Hydrophobic Interaction Chromatography. A HiTrap Hydrophobic Interaction Chromatography (HIC) kit (Pharmacia) containing columns (1 mL resin each) of 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 was used in preliminary studies. For all other work, Phenyl Sepharose High Performance resin was used (55 mL column, 1.6 cm diameter). The protein pellet after ammonium sulfate precipitation was resuspended in 50 mM sodium phosphate, pH 5.0, buffer until the required ammonium sulfate concentration (1.25 M, starting conditions for HIC fractionations, equivalent to eluant A) was reached. The ammonium sulfate concentration was determined by conductivity. Samples (up to 200 mL) were loaded at 3 mL/min onto the column previously equilibrated in 50 mM sodium phosphate (eluant B) containing 1.25 M ammonium sulfate, pH 5.0 (eluant A = eluant B + ammonium sulfate). After sample loading, proteins were eluted by a linear gradient of decreasing ammonium sulfate to 100% eluant B (from 200 to 800 mL).

Analysis and Quantification of Protein by RP-HPLC. The protein composition of the grape and wine fractions was determined by reversed phase (RP) HPLC (*20*). Samples (100 μL) were loaded at 1 mL/min onto a C8 column (4.6 × 250 mm, Vydac 208TP54) fitted with a C8 guard column kit (Vydac 208GK54, 4.6 × 5 mm) equilibrated in a mixture of 83% (v/v) solvent B [0.1% trifluoroacetic acid (TFA) in 8% 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 min, from 49 to 57% from 7 to 15 min, from 57 to 65% from 15 to 16 min, from 65 to 81% from 16 to 30 min, and then held at 81% for 5 min before re-equilibrating the column in the starting conditions for an additional 6 min (*20*). Elution was followed by absorbance at 210, 220, 260, 280, and 320 nm. From the 210 nm chromatogram, their identity was assigned by comparison of their retention times to those of purified grape PR proteins (*21*) as follows: peaks with a retention time between 9 and 12 min were assigned to the TL protein classes, whereas peaks eluted from 18.5 and 24.5 min were assumed to be chitinases. Protein quantification was through comparison to the peak area of two standard proteins: horse heart cytochrome C (Cyt C) (Sigma) or bovine serum albumin (BSA) (Sigma).

HPLC Protein Analyses by Size Exclusion Chromatography. Protein fractions were analyzed by size exclusion chromatography using the analytical scale HPLC equipped with a BioSep SEC S2000 (7.8 × 300 mm) column (Phenomenex) with guard column at 1 mL/min in 50 mM KH_2PO_4 buffer at ambient temperature. Twenty microliters of sample was injected. Elution was monitored by absorbance at 210 nm.

Sample Desalting. Samples were desalted by using an ÄKTA Prime apparatus fitted with a 20 mL column containing Bio-Gel P-10DG gel (Bio-Rad). The separation was performed in isocratic conditions (50 mM citric acid/NaOH buffer, pH 3.50) at 2 mL/min with a maximum loaded volume of 2 mL.

Protein Identification by NanoLC-MS/MS Analyses. Analyses were undertaken on two occasions. On the first occasion (two samples), grape proteins were excised after electrophoretic separation and reduced (25 mM dithiothreitol/50 mM NH_4CO_3 at 56 °C) and alkylated (55 mM iodoacetamide/50 mM NH_4CO_3 at room temperature in the dark), followed by a 16 h tryptic digestion at 37 °C. Peptides were extracted with 0.1%TFA/2%CH_3CN. Peptides were injected (40 μL each) onto a Michrom peptide Captrap for preconcentration and desalted with 0.1% TFA/2% acetonitrile at 10 μL/min. The peptide trap was then switched into line with the analytical column (SGE ProteCol C18, 300A, 3 μm, 150 μm × 10 cm). Peptides were eluted from the column using an Agilent 1100 nanoflow pump, with 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 600 nL/min over a 60 min period. The LC eluent was subjected to positive ion nanoelectrospray analysis on an Applied Biosystems QSTAR XL mass spectrometer. The QSTAR was operated in an information-dependent acquisition mode (IDA). A TOF-MS survey scan was acquired (*m*/*z* 370−1600, 1.0 s), with the three most abundant 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). Mass data collected during MS/MS analysis were processed, converted into Mascot Generic Format using MASCOT.dll script (Applied Biosystems), and interpreted using a local Mascot server (Matrix Science). Searches were performed against the National Center for Biotechnology Information nonredundant (NCBInr) 20070504 database (4900652 sequences; 1692193060 residues), taxonomy Viridiplantae (Green Plants) (356743 sequences), without any molecular weight or isoelectric point restrictions. Searches were performed with a fragment mass tolerance of ±0.6 Da. One missed cleavage per peptide was allowed, and some variable modifications were taken into account: carbamidomethyl (C), oxidation (M), and propionamide (C). Identifications were validated according to the established guidelines for proteomics data publication (*22*).

In the second series of experiments five selected HIC fractions (4, 5, 9, 11, and 13, see **Figure 2a**) were fractionated by RP-HPLC as above and all peaks collected. Three consecutive HPLC fractionations were undertaken and the collected fractions pooled. In total, 12 different peaks were obtained and, after concentration and equilibration with the loading buffer, SDS-PAGE in nonreducing conditions was performed. RP-HPLC peaks characterized by a high protein concentration as judged by peak area were stained with Coomassie. RP-HPLC peaks with low protein content were stained with silver. The protein with a RT of 24.5 min (sample 23) was not loaded on the gel but analyzed by nanoLC-MS/MS directly in liquid form because its protein content was too low to visualize in the gel. This second batch of analyses (24 samples) was performed by excising protein bands from the SDS-PAGE gel. Proteins were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin, and peptides were extracted in 50% acetonitrile + 2% formic acid. Peptide extracts were dried under vacuum, reconstituted in 10 μL of 1% formic acid, and analyzed by nanoLC-MS/MS using a LCQ-Deca ion-trap mass spectrometer (Thermo) according to the method of Breci et al. (*23*). Reversed phase columns were packed in-house to approximately 7 cm (100 μm i.d.) using 100 Å, 5 mM Zorbax C18 resin (Agilent Technologies) in a fused silica capillary with an integrated electrospray tip. A 1.8 kV electrospray voltage was applied via a liquid junction upstream of the C18 column. Samples were injected onto the C18 column using a Surveyor autosampler (Thermo). Each sample was loaded onto the C18 column followed by an initial wash step with buffer A [5% (v/v) CH_3CN, 0.1% (v/v) formic acid] for 10 min at 1 μL/min. Peptides were subsequently eluted from the C18 column with 0−50% buffer B [95% (v/v) CH_3CN, 0.1% (v/v) formic acid] for 58 min at 500 nL/min followed by 50−95% buffer B for 5 min at

500 nL/min. The column eluate was directed into a nanospray ionization source of the mass spectrometer. Spectra were scanned over the range of 400−1500 amu. Automated peak recognition, dynamic exclusion, and tandem MS of the top three most intense precursor ions at 40% normalized collision energy were performed using the Xcalibur software (ThermoFinnigan). Raw data files were converted to mzXML format with readw.exe and processed through global proteome machine (GPM) software, freely available from www.thegpm.org (*24*, *25*). Peptide identification was determined using a 0.4 Da fragment ion tolerance. Carbamidomethyl was considered to be a complete modification; in addition, partial modifications were also considered, which included oxidation of methionine and threonine and deamidation of asparagine and glutamine. MS/MS spectra were searched against the PlantUnigene database (*Vitis vinifera* and *V. vinifera* genome entries) of the Global Proteome Machine database (GPMDB), and reverse database searching was used for estimating false discovery rates (*26*). Using a minimum of two peptides per protein and a maximum log(e) score of −10, no reverse database hits were found in the search results.

RESULTS AND DISCUSSION

Grape Protein Fractionation by HIC. We screened a range of different HIC resins and chromatography conditions using a HiTrap HIC kit (Pharmacia; data not shown). Phenyl Sepharose High Performance resin was selected due to its ability to separate grape juice proteins into five peaks in a short gradient length (**Figure 1**).

The protein concentration of fractions collected was determined from peak area, and their composition was tentatively assigned from their retention time by reverse phase (RP) HPLC (inset in **Figure 1**) (*20*, *21*, *27*). No protein was detected in fraction 1 (flow through). The other fractions contained the two main classes of grape PR proteins. TL proteins and chitinases were both present at a similar concentration in the minor peaks 2 and 3. The purification potential of this method showed in the other fractions. Fraction 4 contained chitinases but no TL proteins (**Figure 1**). Fractions 5 and 6 contained both chitinases and TL proteins; the former dominated in fraction 5 and the latter in fraction 6.

These conditions were scaled up (**Figure 2a**), and a larger number of peaks were detected than with the 1 mL column (see **Figure 1**). The fractionation was repeated, and the corresponding fractions from both runs were pooled and analyzed by RP-HPLC to determine their protein concentration and tentative identity (**Table 1**). As a comparison, the starting material (total precipitated juice proteins) before HIC fractionation was also analyzed (**Table 1**).

Data in **Table 1** (based on the HPLC separation shown in Supporting Information Figure S2) summarize how grape proteins were fractionated by HIC. Each protein was eluted in adjacent HIC fractions rather than in several different times spanning across the elution profile. This elution of proteins in relatively narrow bands further suggests that Phenyl Sepharose HP resin is appropriate for grape protein fractionation. Some proteins were eluted in the first four fractions before the gradient began, which suggested their interaction with the medium was minimal. Their concentration represented only a minimal part of the total amount of proteins loaded. Once the elution gradient started, the first protein eluted had a RT of 14.1 min on RP-HPLC at low concentration of the elution buffer (fractions 4 and 5), suggesting a very low hydrophobicity for this protein. A HPLC protein peak at 5.5 min was detectable in the next few fractions (6−9) together with a protein with a RT of 19.5 min that was tentatively identified as a chitinase. This latter protein was detected in all of the fractions, showing a not strictly hydrophobic dependent elution. Another protein that showed similar behavior had a RT of 13.3 min and eluted in fraction 7

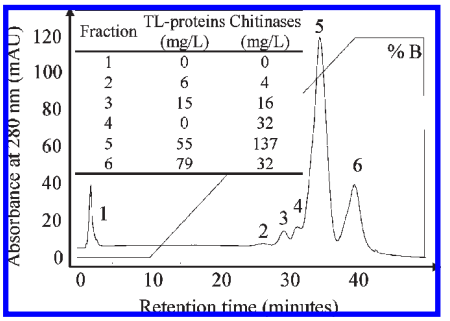

Figure 1. Separation of Semillon juice proteins (approximately 1.65 mg of total protein loaded) by HIC (Phenyl Sepharose High Performance; 1 mL column). The table shows distribution of the TL and chitinase proteins, given as a sum of the different isoforms and expressed in equivalents of cytochrome C, in the HIC fractions. Protein identities were tentatively assigned by their retention times.

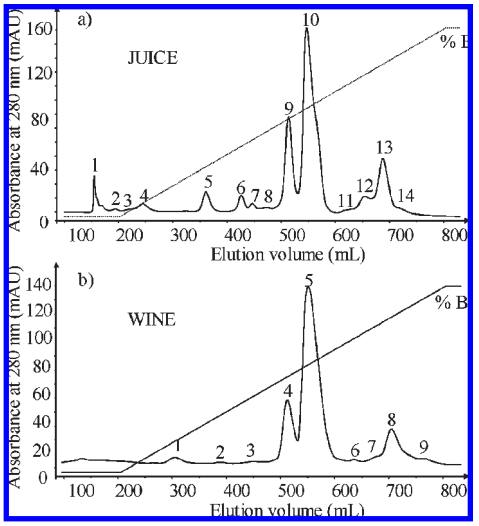

Figure 2. Chromatograms of the protein fractionation by hydrophobic interaction chromatography of (**a**) Semillon juice and (**b**) Semillon wine performed with a column (∅ = 1.6 cm) containing 55 mL of Phenyl Sepharose High Performance. Numbers indicate the fractions collected.

through to the last fraction. Proteins tentatively identified as thaumatin-like (TL; RTs of 9.3, 10.2, and 10.9 min) began to elute from fraction 9 onward and with the majority of TL material in fraction 13. Hence, in our experimental conditions, chitinases tended to behave in a less hydrophobic way than TL proteins, confirming the preliminary results achieved with the small column (see **Figure 1**). Several fractions were enriched in a particular class of protein, as judged by peak area percentage from RP-HPLC analysis. For instance, the chitinase peak area of fractions 6 and 7 was approximately 90% of the total peak area of proteins in those fractions, whereas fractions 11−14 mainly consisted of TL proteins, with a minimum peak area on RP-HPLC of 82% in the fractions (see Supporting Information Figure S2). These data indicated the possibility to highly purify a TL protein from grape juice in a one-step preparative process based on the different hydrophobicity characteristics of grape proteins.

Fraction 13, the fraction for which 92% of the peak area by HPLC was TL protein, was analyzed by SDS-PAGE (**Figure 3**). Unexpectedly, this fraction, which appeared to be pure by HPLC, showed two bands on SDS-PAGE. The band intensity of the fraction at approximately 22 kDa was 75% of the total intensity, whereas the 65 kDa band was 25%. To characterize the nature of these proteins, the two bands

Table 1. Protein Composition and Quantity of Fractions Collected after Semillon Juice HIC[a]

	RT	Starting material	HIC Fraction (#) 1	2	3	4	5	6	7	8	9	10	11	12	13	14
RP-HPLC peak	5.5	X						X	**X**	X	X					
	8.1	X		X	X	X				X	**X***	X				
	9.0	X	X	**X***	**X***	**X***										
	9.3	X*								X	X*	**X***	X			
	10.2	X												**X**	X	
	10.9	X*											X*	X*	**X***	X*
	13.3	X							X	**X**	X*	X	X	X	X	X
	13.9	X						X	X	X	**X**	X				
	14.1	X				X	**X***									
	18.6	X							X	X	X	X				
	19.5	X*	X*	X*	X	X	X	X*	X*	X*	X*	**X***	X	X	X	X
	20.4	X							X	X	X	**X**				
	24.5	X								X	X	**X**				
Protein content (as mg/L BSA)		337.5	1.1	1.1	2.7	6.2	13.6	7.5	5.2	4.2	58.8	98.3	3.0	12.9	35.3	2.8

[a] The RP-HPLC chromatograms from which these data were acquired are shown in Supporting Information Figure S2. Red font, tentatively identified as TL proteins; blue font, tentatively identified as chitinases; X, present in this HIC fraction; **X**, major content(s) in RP-HPLC peak (in that row); **X***, major component(s) of each HIC fraction (in that column).

were excised from the gel and analyzed by nanoLC-MS/MS (**Table 2**).

The higher MW band was identified as vacuolar invertase 1, GIN1 from *V. vinifera* (*28*, *29*), whereas the lower band was classified as a TL protein from *V. vinifera* (*30*). Size exclusion HPLC was used to determine the content of invertase in fraction 13 (**Figure 4**).

The size exclusion chromatography results confirmed the previous RP-HPLC data, with this fraction mostly composed of VvTL protein (91%) at a mass of approximately 20 kDa. It is likely that the Coomassie stain in SDS-PAGE overestimated the invertase "impurity" and underestimated the TL protein content, as difficulties with this stain and TL proteins have been observed by others (*15*).

Semillon Wine Protein Fractionation by HIC. To determine if the proposed HIC method was applicable to wine, proteins from several liters of Semillon wine were precipitated with ammonium sulfate (80% saturation), collected by centrifugation, dissolved in the HIC loading buffer, and fractionated. The resulting chromatograms (**Figure 2b**) showed similarity to those of Semillon juice (**Figure 2a**), with the wine profile lacking six early eluting peaks.

As with the grape proteins, the wine fractions were examined by RP-HPLC (see Supporting Information Figure S1). Data were highly similar to those obtained from the grape proteins, with some differences as follows. For wine, the major TL protein content was observed in fractions 5−8. Particularly, fraction 5 (corresponding to fraction 10 of juice fractionation) had a TL protein eluting at 9.3 min on RP-HPLC, a retention time that was different from that of the TL protein eluted in fraction 8 (RT 10.9 min), indicating that different TL classes were in the two fractions. Although the wine used here was not prepared from the same grapes as the juice fractionated in this study, it appears that, in passing from juice to wine, the main grape TL protein classes were unaffected but the number of chitinase peaks decreased. This supports the observation of others that some proteolysis of chitinases can occur during fermentation (*31*−*33*), possibly through the action of yeast proteases. These results also confirmed the suitability of HIC for purification of protein derived from white wine.

Semillon Grape Protein Identification by NanoLC-MS/MS. As described above, the proteins in the fractions were initially tentatively identified from their retention time on RP-HPLC. To confirm these identities, the proteins in five of the HIC fractions (4, 5, 9, 11, and 13) were collected after RP-HPLC separation and then further fractionated by SDS-PAGE under nonreducing conditions (**Figure 5**). These fractions were chosen because they contained all of the proteins present in the whole juice and in all fractions, as assessed by RP-HPLC (see Supporting Information Figure S2).

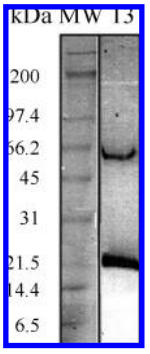

Figure 3. Nonreducing SDS-PAGE of fraction 13 obtained from the HIC separation (see **Figure 2a**).

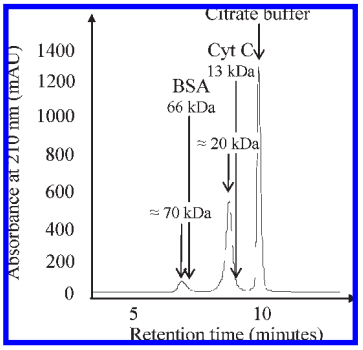

Figure 4. Size exclusion HPLC chromatogram of HIC fraction 13. BSA (20 μL injected at 3 mg/mL concentration in 30 mM citrate buffer, pH 3.50) and cytochrome C (20 μL injected at 2.5 mg/mL concentration in 30 mM citrate buffer, pH 3.50) were utilized as standards (see arrows indicating their retention times during the separation).

Table 3 summarizes the results obtained and the protein identifications associated with each band. Our initial analysis was performed using the Plant Protein sequence database from the *Arabidopsis* Information Resource (TAIR) and showed that 17 samples of 24 contained proteins from *V. vinifera*. However, after the more recent release of the genome of *V. vinifera* (*34*) and its inclusion in the GPM plant Unigene database, all 24

Table 2. Identification from Mascot Search Results (Matrix Science Database) of Proteins from HIC Fraction 13[a]

gel band	protein name [*organism*]	accession no.	theor M_r/p*I*	total no. of peptides matched	score
22	thaumatin-like protein [*Vitis vinifera*]	gil33329390	23866/4.67	37	775
	putative thaumatin-like protein [*Vitis vinifera*]	gil7406714	20108/4.94	34	724
	osmotin-like protein [*Vitis vinifera*]	gil1839046	23859/4.56	32	647
	thaumatin-like protein [*Vitis vinifera*]	gil89242714	23919/4.50	36	494
	thaumatin-like protein [*Vitis vinifera*]	gil89242712	23848/4.67	17	440
65	vacuolar invertase 1, GIN1 [*Vitis vinifera* = grape berries, Sultana, berries, peptide, 642 aa]	gil1839578	71501/4.6	20	567

[a] Individual ion scores of >40 indicate identity or extensive homology ($p < 0.05$).

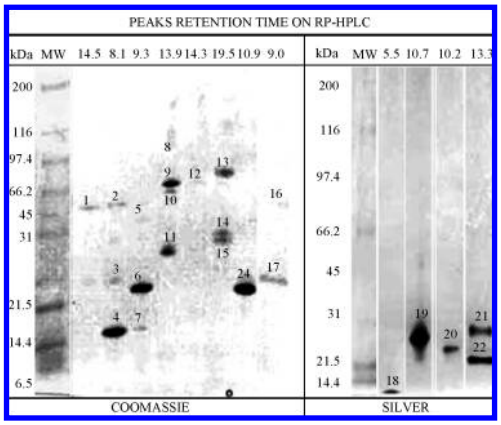

Figure 5. Nonreducing SDS-PAGE of peaks collected by RP-HPLC. Numbered bands were cut and analyzed by nanoLC-MS/MS (**Table 3**). RP-HPLC peaks characterized by a high protein concentration were stained with Coomassie. RP-HPLC peaks with low protein content were stained with silver.

samples were successfully identified as containing *V. vinifera* proteins.

Many of the protein bands were identified as PR proteins such as PR-4 type proteins, TL proteins, chitinases, putative ripening-related protein, lipid transfer protein isoform 1, and vacuolar invertase 1, GIN1. This is consistent with the observation of Sarry and colleagues (*16*) that 19% of the total proteins from grape berry mesocarp belong to the PR protein category. It is worth mentioning the absence of microbial proteins, indicating the healthiness of the grape juice used (*35*). In six gel bands (gel bands 3, 6, 7, 14, 22, and 24) multiple proteins were identified, with this fact attributable to band overlapping in SDS-PAGE for matches resulting from the identification of different peptides (for gel bands 3, 7, and 22) or to the identification of the same peptides that matched different proteins (for gel bands 6, 14, and 24, see Supporting Information Table S1). It is to be noted that in several cases the headers of the protein identified by GPM searches were mRNA sequences present in the *V. vinifera* genome database. This indicates that little work has been done on these proteins so far. For most of these mRNA sequences no information is yet available about what proteins they encode. To give some indication of the protein function, we have reported the protein header of the nearest homologue when available in the GPM search output due to the presence of peptides found in more than one database entry. If no protein homologues were identified by GPM, the identified peptides were searched against the NCBI nonredundant protein sequence database using BLAST (*36*), and the results from the alignment were included in a separate column. Using this approach, the most likely identity and function are reported for each identified protein. This approach can be very useful in validating gene translations and predictions reported in the available genome sequence.

Class IV Chitinase [Vitis vinifera] and WIN1028.C21_P01 Muscat Hamburg Postveraison Pericarp Normalized (WIN10) Vitis vinifera cDNA Clone WIN1028_P01 3′, mRNA Sequence. Among the 24 grape protein samples, 9 bands were recognized as similar/identical to a protein predicted by a mRNA sequence from Muscat Hamburg. Most of these bands were those unidentified during the first analysis of these results with the TAIR database, which means before the improvement of the database quality after the grapevine genome sequencing. Because of the absence of homologues to this mRNA sequence in the GPM database, the real nature of this protein needed to be further investigated by loading the peptide sequences identified into Blast. Doing this, 7 of 9 bands showed high homology with an unnamed protein product from *V. vinifera* having a chitin-binding domain. Using the same approach, the two remaining bands (10 and 12) were recognized, respectively, as a class IV chitinase and a class IV endochitinase. In summary, all of these mRNA sequences related to proteins that are likely to have a chitinase function, confirming our results from RP-HPLC analysis of HIC fractions in the first part of this work (see **Table 1**).

Four bands (13−16) were identified as class IV chitinase proteins despite showing variable MW in nonreducing SDS-PAGE. Examination of the protein sequence and the identified peptides made it apparent that for bands 13−15 most of the sequence was covered, thus excluding the hypothesis of proteolysis being responsible for their different electrophoretic behaviors. Band 16, on the contrary, was identified on the basis of one unique peptide, which was in common with the peptides of the other three class IV chitinases, so it might result from proteolysis of bands 13−15.

Together with the nine samples discussed above, the overall number of bands with chitinase function was 13 of 24, confirming the predominance of chitinases in grape proteins as highlighted by other authors (*27*, *31*, *37*). By monitoring the HIC distribution of chitinases, it was observed that most of these proteins were eluted in the middle of the HIC gradient (HIC fractions 9 and 11). It is interesting to point out the differences of behavior highlighted by these bands. For instance, bands from 8 to 16 did not behave similarly in nonreducing SDS-PAGE in which a great MW variability in a range from approximately 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 (*38*). However, these notions seem insufficient to explain these great differences in MWs; thus, additional investigations are required.

Table 3. Summary Table of the NanoLC-MS/MS Identification of Grape Protein Bands Excised from Nonreducing SDS-PAGE (**Figure 5**)[a]

gel band	HIC fraction	RP-HPLCRT	SDS-PAGE MW[b]	accession no.[c]	protein name [*organism*][d]	total no. of peptides matched[e]	log(e)[f]	protein homologues in X!Tandem[g]	protein homologues in Blast [h]
1	5	14.5	50	gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	61	−192.2		gi|157353734|[CD] unnamed protein product [*Vitis vinifera*]
2	9	8.1	53	gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	55	−222		gi|157353734|[CD] unnamed protein product [*Vitis vinifera*]
3	9	8.1	24	gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	22	−110		gi|157353734|[CD] unnamed protein product [*Vitis vinifera*]
				gi|22010598|gb|BQ795632|3	EST 4570 ripening grape berries Lambda Zap II Library *Vitis vinifera* cDNA clone RB008D02 3′, mRNA sequence	5	−12.8	gi|3511147| log(e) −12.8 PR-4 type protein [*Vitis vinifera*]	
4	9	8.1	15	gi|3511147|	PR-4 type protein [*Vitis vinifera*]	36	−90.9		
5	9	9.3	40	gi|7406716|	putative thaumatin-like protein [*Vitis vinifera*]	17	−58.5		
6	9	9.3	22	gi|7406716|	putative thaumatin-like protein [*Vitis vinifera*]	98	−170		
				gi|2213851|gb|AF003007|-1	*Vitis vinifera* thaumatin-like protein VVTL1 mRNA, complete cds. ORGANISM *Vitis vinifera*	73	−139	gi|2213852| log(e) −139.4 VVTL1 [*Vitis vinifera*]	
				gi|8980665|	thaumatin-like protein [*Vitis vinifera*]	39	−39.3		
7	9	9.3	16	gi|7406716|	putative thaumatin-like protein [*Vitis vinifera*]	11	−44.7		
				gi|22010598|gb|BQ795632|3	EST 4570 ripening grape berries Lambda Zap II Library *Vitis vinifera* cDNA clone RB008D02 3′, mRNA sequence	6	−38.8	gi|3511147| log(e) −38.8 PR-4 type protein [*Vitis vinifera*]	
8	9	13.9	100	gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	33	−153.1		gi|157353734|[CD] unnamed protein product [*Vitis vinifera*]
9	9	13.9	70	gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	82	−295.3		gi|157353734|[CD] unnamed protein product [*Vitis vinifera*]
10	9	13.9	65	gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	52	−192.2		gi|3329392| class IV chitinase [*Vitis vinifera*
11	9	13.9	27	gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	86	−231		gi|157353734|[CD] unnamed protein product [*Vitis vinifera*]

Table 3. Continued

gel band	HIC fraction	RP-HPLCRT	SDS-PAGE MW[b]	accession no.[c]	protein name [*organism*][d]	total no. of peptides matched[e]	log(e)[f]	protein homologues in X!Tandem[g]	protein homologues in Blast [h]
12	9	14.3	70	gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	43	−150.6		gi|2306811| class IV endochitinase [*Vitis vinifera*]
13	11	19.5	80	gi|33329391|gb|AF532966|-1	*Vitis vinifera* class IV chitinase (Chi4D) mRNA, complete cds	65	−236.2	gi|33329392| log(e) −236.2 class IV chitinase [*Vitis vinifera*]	
14	11	19.5	31	gi|33329391| gb|AF532966|-1	*Vitis vinifera* class IV chitinase (Chi4D) mRNA, complete cds	50	−267.1	gi|33329392| log(e) −267.1 class IV chitinase [*Vitis vinifera*]	
				gi|110384879|gb|EC949346|2	WIN0546.C21_F23 Cab Sauv flower, leaf, and root normalized (WIN05) *Vitis vinifera* cDNA clone WIN0546_F23 3′, mRNA sequence	3	−48.1	gi|147788074| log(e) −48.1 hypothetical protein [*Vitis vinifera*]	
15	11	19.5	29	gi|33329391|gb|AF532966|-1	*Vitis vinifera* class IV chitinase (Chi4D) mRNA, complete cds	38	−196.4	gi|33329392| log(e) −196.4 class IV chitinase [*Vitis vinifera*]	
16	4	9.0	55	gi|33329391|gb|AF532966|-1	*Vitis vinifera* class IV chitinase (Chi4D) mRNA, complete cds	3	−8.4	gi|33329392| log(e) −8.4 class IV chitinase [*Vitis vinifera*]	
17	4	9.0	24	gi|75184387|	ripening-related protein grip22 precursor; class: standard	3	−16.4	gi|7406671| log(e) −16.4 putative ripening-related protein [*Vitis vinifera*]	
18	9	5.5	10	gi|30130330|gb|CB915669|3	VVD101F08_368999, an expressed sequence tag database for abiotic stressed berries of *Vitis vinifera* var. Chardonnay *Vitis vinifera*	7	−44.9	gi|28194084| log(e) − 44.9 lipid transfer protein isoform 1 [*Vitis vinifera*]	
19	11	10.7	25	gi|89242714|	thaumatin-like protein [*Vitis vinifera*]	14	−52.5		
20	13	10.2	23	gi|147785114|	hypothetical protein [*Vitis vinifera*]	6	−22.7	gi|1839046| log(e) −22.7 osmotin-like protein [*Vitis vinifera*].	
21	13	13.3	26	gi|110403092|gb|EC964243|1	WIN087.C21_K19 Cab Sauv seed normalized (WIN08) *Vitis vinifera* cDNA clone WIN087_K19 3′, mRNA sequence	19	−73.6	gi|1839578| log(e) − 60.4 vacuolar invertase 1, GIN1 [*Vitis vinifera*]	
22	13	13.3	22	gi|33329389|gb|AF532965|-1	*Vitis vinifera* thaumatin-like protein (TI3) mRNA, complete cds	9	−42.0	gi|89242714| log(e) −42.0 thaumatin-like protein [*Vitis vinifera*]	
				gi|110359132| gb|EC920118|-2	WIN013.BR_E17 Cab Sauv pericarp non-normalized (WIN01) *Vitis vinifera* cDNA clone WIN013_E17 5-, mRNA sequence	5	−25.7	gi|1839578| log(e) − 25.7 vacuolar invertase 1, GIN1 [*Vitis vinifera*]	

Table 3. Continued

gel band	HIC fraction	RP-HPLCRT	SDS-PAGE MW[b]	accession no.[c]	protein name [*organism*][d]	total no. of peptides matched[e]	log(e)[f]	protein homologues in X!Tandem[g]	protein homologues in Blast [h]
23[i]	9	24.5		gi|110409999|gb|EC975674|2	WIN1028.C21_P01 Muscat Hamburg postveraison pericarp normalized (WIN10) *Vitis vinifera* cDNA clone WIN1028_P01 3′, mRNA sequence	8	−16.8		gi|157353734|[CD] unnamed protein product [*Vitis vinifera*]
24[j]	13	10.9	23	gi|33329389|gb|AF532965|-1	*Vitis vinifera* thaumatin-like protein (TI3) mRNA, complete cds	125	−189.5	gi|33329390| log(e) −189.5 thaumatin-like protein [*Vitis vinifera*]	
				gi|147785114|	hypothetical protein [*Vitis vinifera*]	122	−172		
				gi|110377838|gb|EC942933|-3	WIN0527.C21_E16 Cab Sauv flower, leaf, and root normalized (WIN05) *Vitis vinifera* cDNA clone WIN0527_E16 3-, mRNA sequence	31	−41.6	gi|147854517| log(e) −41.6 hypothetical protein [*Vitis vinifera*]	

[a] The origin of the bands (HIC fraction number and HPLC retention time) and their apparent MW on nonreducing SDS-PAGE are also given. A list of the identified peptides is given in Supporting Information Table S1. [b] Approximate MW by SDS-PAGE under nonreducing conditions. [c] Protein identification number provided by the NCBInr database. [d] Proteins ID identified by using X!Tandem searching in the GPM database. [e] Total number of peptides identified by the X!Tandem program, which matched with the identified protein. [f] Base −10 log of the expectation that the assignment is stochastic. [g] Proteins that also use some subset of the spectra used to match the protein listed on the main ID column. Proteins with high similarity to those identified. [h] X!Tandem identified peptides giving a significant homology when loaded in Blast for alignment. CD, chitinase domain detected in Blast. [i] Sample 23 (RT 24.5 min) was not loaded on the gel but analyzed by nanoLC-MS/MS directly in liquid form because its protein content was too low to become visible in the gel. [j] Sample 24 corresponded to the identified TL protein (gi|33329390) contained in HIC fraction 13 (see **Table 2**).

Putative Thaumatin-like Protein [V. vinifera] and Thaumatin-like Protein [V. vinifera]. The TL proteins are, after the chitinases, the second most prominent grape and wine proteins (*27*, *31*, *37*). This was confirmed in the results shown in **Figure 5** and **Table 3**, in which seven spots were recognized as TL proteins. In particular, three spots (5−7) contained putative TL proteins, whereas four (19, 20, 22, and 24) contained TL proteins. These two TL classes differ in both HPLC retention time (respectively, 9.3 and 10.2−13.3 min) and hydrophobicity characteristics (lower for the putative TL proteins). It is to be noted that the three putative TL protein bands derived all from the same SDS-PAGE lane. Particularly, the HPLC peak (9.3 min) appeared in 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 bands with apparent MW of 40 and 16 kDa on nonreducing SDS-PAGE are likely modified isoforms. Proteolysis can be most likely excluded when the identified peptides are fitted to a linear protein chain of 222 amino acids. Particularly, peptides from the 40, 22, and 16 kDa bands started from positions 33, 25 and 48, respectively, and all finished at amino acid 217. Therefore, these MW incongruences could be attributed to the nonreducing conditions in which the analysis was performed (*38*).

Spots 19, 20, 22, and 24 were all identified as TL proteins, and some differences among isoforms have been highlighted. In particular, a TL protein (gi|89242714) was recovered in bands 19 and 22, whereas the two other bands were identified as a hypothetical protein very similar to an osmotin-like protein (band 20) and a TL protein (gi|33329390, band 24) that was previously identified (see **Figure 3** and **Table 2**). Moreover, bands 22 and 24 showed multiple matches. In particular, band 22 also contained vacuolar invertase, and these two matches resulted from the identification of different sets of peptides, thus indicating the presence of multiple proteins in the same gel band, indirectly confirming the association and the difficulty of separating these two classes of protein encountered in the first part of this paper. On the contrary, band 24 matched two hypothetical proteins through the same set of peptides, indicating that this result was due to homology between the proteins.

The TL proteins identified in bands 19 and 22 were isolated from two different HIC fractions (11 and 13, respectively). This fact could be explained in two ways: (i) the presence of different TL protein isoforms in our samples recognized as the same protein in the database; (ii) a partial modification of the same TL protein that resulted in a changing of properties such as its MW or hydrophobicity. The second hypothesis seems to be supported by data obtained by Pocock and colleagues (*27*), which showed the presence of a main TL protein and a minor TL protein that behaved, by HPLC analysis, similarly to, respectively, proteins from spots 22 and 19 (see Supporting Information Figure S2, fractions 13 and 11, respectively). Accordingly, our experimental data suggest that the second hypothesis is the most probable. In particular, the TL protein could have incurred some modifications, likely proteolysis. This hypothesis is supported by data from the identified peptides, with protein from band 22 recognized only on the basis of peptides from position 84 of the linear protein chain, whereas protein from band 19 started from position 41 of the chain. A certain amount of endogenous proteolytic activity is detectable in musts (*39*), but this activity is not sufficient to degrade the highly resistant PR proteins (*5*). However, these enzymes could be responsible for a partial modification of some peptides, resulting in a slightly different behavior of the protein during the fractionation processes. In fact, the four TL protein bands identified showed similar, but not identical, hydrophobicities. Besides, in HPLC this protein showed up at four RTs, whereas in SDS-PAGE it showed apparent MWs from 22 to 25 kDa. The same reasoning might explain also the appearance of three bands in the same SDS-PAGE lane from only one HPLC peak (spots 5−7) as discussed above.

PR-4 Type Protein [V. vinifera]. This class of protein was eluted in HIC fraction 9 and showed a HPLC RT of 8.1 and 9.3 min and has been identified on three occasions, with only band 4 containing purely PR-4 protein. The three PR-4 matches have two peptides in common. Generally, the PR-4 class of protein is mainly composed of chitin-binding proteins (*40*). These proteins present antifungal activity that is mainly due to their ability to bind fungal cell wall chitin (*41*). The presence of this class of

proteins in grapevine seems to be due to some antifungal mechanism of the plant, although Tattersall and colleagues (*42*) attributed a ripening-related role to this class. PR-4 proteins behaved, in SDS-PAGE, similarly to the putative TL proteins, with the appearance of two bands at different MWs on the same lane, recognized as the same protein (spots 3 and 4). Only two of the three bands identified as PR-4 protein had MWs compatible with the theoretical MW of 15.2 kDa, whereas band 3 had a MW (by SDS-PAGE) more similar to that of a class IV chitinase. Moreover, Van Damme and colleagues (*43*) 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, together with the possible SDS-PAGE bands overlapping, might explain the recognition of band 3 containing both chitinase and PR-4 proteins.

Putative Ripening-Related Protein [V. vinifera].One band (spot 17) was identified as a putative ripening-related protein. This protein had a very low hydrophobicity because it was eluted in HIC fraction 4, before the gradient started. It has a HPLC RT (9.0 min) similar to that of putative TL proteins. The slight difference in predicted p*I* between this protein (4.83) and the thaumatin one (4.94) may be the reason for the slightly different RT in HPLC.

Lipid Transfer Protein Isoform 1 [V. vinifera]. In the literature there are two closely related types of nonspecific (ns) LTPs, types 1 and 2, which differ in protein sequence, molecular weight, and biological properties (*44*). Many nsLTP1 proteins, including those of grapevine origin, have been characterized as allergens in humans (*10*). The LTP identified here (band 18) belongs to isoform 1 and showed very low MW in SDS-PAGE and early RT in HPLC. It is interesting to point out the suitability of the HPLC method, originally designed to achieve a good separation between TL proteins and chitinases, to also isolate the putative allergen LTP, which 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 [V. vinifera = Grape Berries, Sultana, Berries, Peptide, 642 aa]. Some authors (*16*, *28*) highlighted the preferential expression of the GIN1 isogene with respect to the GIN2 in the pericarp, confirming the results described in this paper. After the identification of a vacuolar invertase in HIC fraction 13 with a MW of approximately 66 kDa (see **Figure 3** and **Table 2**), gel bands 21 and 22 were also shown to contain a vacuolar invertase. Both bands derived from the same HPLC peak (RT 13.3 min) but behaved differently in nonreducing SDS-PAGE (26 and 22 kDa, respectively) and, especially, from the theoretical MW of this protein (71 kDa). Okuda and co-workers (*45*), by using 2D-PAGE, noted the presence of invertase fragments in Chardonnay wine with MWs of 39, 38, and 29 kDa, highlighting for the first time the presence of hydrolyzed invertase 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 formerly discussed for the TL proteins. This hypothesis is supported by looking at the distribution of the identified peptides along the linear chain of the protein. Particularly, both bands 21 and 22 were identified on the basis of peptides that matched starting from position 538

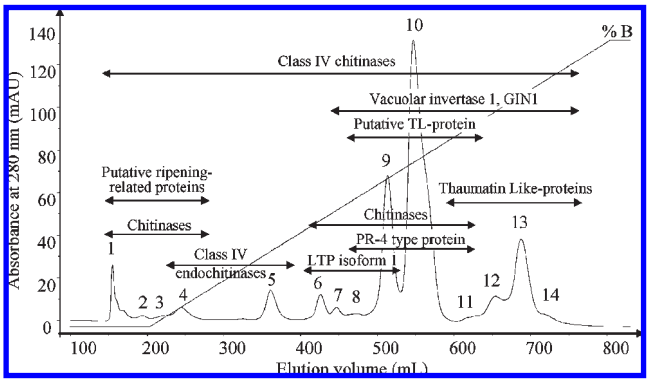

Figure 6. General scheme of the nature and distribution of proteins fractionated during HIC.

of 642 of the sequence. The fragmentation of invertases could also explain the presence of truncated isoforms throughout eight HIC fractions. However, some band overlapping during the SDS-PAGE cannot be excluded.

The results discussed above are graphically summarized in **Figure 6**. When one considers the distribution of proteins throughout the HIC fractionation (**Figure 6**), putative ripening-related proteins appear to be the least hydrophobic because they eluted before the gradient began. Chitinases eluted throughout the gradient. This phenomenon could be ascribed to the presence of several classes of chitinases (*31*) with differing hydrophobicities and to fragmentation of single protein(s) that led to a number of shorter isoforms with modified hydrophobicity.

After the chitinases, the second protein 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 and thus bound to the resin, whereas with the attainment of an ammonium sulfate concentration of approximately 0.8 M in the buffer, a resolubilization of the invertase started and continued until the end of the gradient. The causes of this event need to be investigated. We hypothesize that heterogeneous glycosylation resulting in differing interaction with the resin could be one possible explanation. Invertase fragmentation due to proteolysis could also explain this phenomenon. It is noteworthy that proteins at RP-HPLC RT of 13.3 min were associated with both invertases and TL proteins (see gel bands 21 and 22, **Table 3**). This fact could further explain the difficulty in obtaining a TL protein without some invertase contamination.

Some proteins were eluted in a small chromatographic 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 thought to be a very hydrophobic protein. In fact, LTP is known to have a hydrophobic pocket that endows it with the capacity to bind hydrophobic molecules (*46*). Consequently, this LTP behavior on the HIC resin in this study was likely due to its superficial hydrophobicity more than to its intrinsic characteristics.

In our experimental conditions, a TL protein (gi|33329390, in band 24) was eluted last and thus behaved as the most hydrophobic protein from Semillon grape juice. This fact differentiates this protein class from all others and allowed us to isolate it in relatively high purity after a single HIC step (see **Figures 3** and **4**).

Results previously discussed highlight that HIC can be used for grape protein fractionation and that it is a versatile tool for partial or total protein purification. By coupling this technique

with RP-HPLC analysis, SDS-PAGE, and nanoLC-MS/MS, it was possible to identify a large number of grape proteins and to obtain preliminary results on their hydrophobicity characteristics.

In summary, hydrophobic interaction chromatography combines good preparative fractionation with high protein purity for grape and wine proteins. In particular, a *V. vinifera* TL protein was isolated in a single step with high recovery percentage and >90% purity from both Semillon juice and wine. Moreover, the chromatographic system used has shown its ability to purify more than one protein, especially in wine in which the profile is less complex compared to grape juice, and, for this reason, further studies could utilize this approach to purify other grape and wine protein classes. The application of HIC in wine studies is likely to improve knowledge of grape and wine proteins. Moreover, by combining this technique with other chromatographic methods, a more efficient protein purification method in terms of both quantity and quality can be achieved.

ABBREVIATIONS USED

BSA, bovine serum albumin; Cyt C, horse heart cytochrome C; HIC, hydrophobic interaction chromatography; LTP, lipid transfer protein; MW, molecular weight; p*I*, isoelectric point; PR proteins, pathogenesis-related proteins; RT, retention time; SEC, size exclusion chromatography; TL protein, thaumatin-like protein; VvTL, *Vitis vinifera* thaumatin-like protein.

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Supporting Information Available: Table including the number and sequence of unique peptides identified by the X!Tandem search engine (Table S1) and RP-HPLC chromatograms from all wine (Figure S1) and grape juice (Figure S2) HIC fractions. This material is available free of charge via the Internet at http://pubs.acs.org.

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