JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 11969–11976 11969
DOI:10.1021/jf102603r

Molecular Basis of the Interaction between Proteins of Plant Origin and Proanthocyanidins in a Model Wine System

Tiziana Mariarita Granato,[†] Federico Piano,[†,#] Antonella Nasi,[§] Pasquale Ferranti,[§] Stefania Iametti,[†] and Francesco Bonomi*,[†]

[†]DISMA, University of Milan, Via G. Celoria 2, 20133 Milan, Italy, and [§]DSA, University of Naples 'Federico II', Parco Gussone, Portici, Italy. [#]Present address: DISTAM, University of Milan, Via G. Celoria 2, 20133 Milan, Italy.

Plant proteins are being used as a replacement for animal proteins in wine fining. The surface hydrophobicity of plant proteins in four commercial preparations differing for their origin and processing was assessed by using a fluorescent hydrophobic probe in wine-like media. Displacement of the probe by addition of wine phenolics was measured as a way to compare and predict to some extent the efficiency of these proteins in wine fining. It was found that the binding of polyphenols was much more specific than that of the hydrophobic probe. Further analysis of the polyphenol pattern in protein-treated wine-like solutions pointed out two relevant facts: (1) proteins may interfere with the chemistry of the interactions between polyphenols and other wine components; (2) individual protein preparation having different surface hydrophobicities also have different specificities in binding different polymeric forms of the polyphenols and in their substitution products. These findings are related to the possible carry-over of transition metals and may be worth exploring for custom tailoring the fining process. Whether the practical application of the latter finding will call for production and/or screening of plant-derived proteins with features appropriate to this task remains to be investigated. However, the approaches presented in this study may be used for large-scale screening of protein suitability for fining application under laboratory conditions, providing guidelines for their use in actual winemaking applications.

KEYWORDS: Wine fining; plant proteins; anthocyanidins; protein hydrophobicity; mass spectrometry

INTRODUCTION

Proteins have been used as wine fining agents for a long time. They not only allow clarification of colloidal suspensions but the precipitation of complexes between tannins and proteins in the process known as fining, which softens the gustatory appraisal and can reduce the astringency of otherwise rough wines. Fining also improves wine stability, limiting browning and overoxidation in white wine (*1*, *2*) and stabilizing the color of red wine (*3*), as well as acting on bitterness and roughness in both red and white wines during aging.

A broad range of animal proteins have been used as fining agents, but the recent bovine spongiform encephalopathy pandemic led to the prohibition of the use of bovine plasma and blood cells (EC regulation 2087/97). Winemakers have been encouraged to stop using bovine gelatin as well, and there are also reservations on using egg albumin because of its animal origin (*4*). In this scenario, the use of plant-derived proteins as wine fining agents has become of much interest.

The influence of treatments with proteins, of both animal and vegetable origins, is related to protein−polyphenolic compound associations, in which hydrogen bonds and hydrophobic interactions are responsible for the expected flocculation and clarifying action (*5*−*7*). Improved knowledge of the functional properties of proteins used as fining agents and of the structure of polyphenolic compounds interacting with various classes of clarifying agents is expected to take the whole protein-based fining process beyond the empiricism that has characterized it so far.

The three-dimensional protein structure is dependent on a broad range of factors, which must be taken into account in a synergistic way to explain the functional properties of proteins relevant to the food business (*8*). Among these factors, surface hydrophobicity is known to be significantly related to the functional properties of food and nonfood proteins (*9*). Fluorescent probes are often used to measure the number and relative affinity of hydrophobic groups on the protein surface that are able to bind the probe. One of the most valuable and widely used noncovalent hydrophobicity probes is 1-anilino-8-naphthalenesulfonate (ANS). ANS has been used in studies concerning process-induced modification of isolated food proteins (*10*−*13*) and of complex food systems undergoing processes of various natures (*14*−*18*).

Here we studied the molecular basis of noncovalent interactions between proteins of plant origin and polyphenolic compounds, known for their role in organoleptic as well as stability properties of wines. Surface hydrophobicity of proteins of plant origin was investigated in wine-like model systems by studying changes in the binding properties of ANS, used as extrinsic fluorescent probe. Hydrophobic interactions between phenolic compounds and proteins were evaluated by the study of competition of phenolic compounds with probe for the same binding sites. Polymer chain length and the

*Corresponding author (phone +39-02-50316819; fax +39-02-50316801; e-mail francesco.bonomi@unimi.it).

Published on Web 10/28/2010

Table 1. Surface Hydrophobicity Properties of Proteins in the Various Fining Agents[a]

fining agent	total protein content, mg/g	soluble protein, % of total	F_{max}, fluorescence at saturating probe concn × (mg of protein)$^{-1}$	K_d^{app}, μM	PSH
soy protein isolate	918 ± 12	0.56	1033 ± 16	30.09 ± 1.2	186.73 ± 3.7
pea protein isolate	900 ± 22	0.94	676 ± 32	26.53 ± 0.2	65.39 ± 3.2
lentil flour	315 ± 18	0.24	278 ± 12	24.11 ± 0.7	20.26 ± 1.1
gluten	975 ± 31	<0.10	432 ± 16	33.23 ± 2.2	17.80 ± 2.1

[a] Data and standard deviations are from a minimum of three determinations.

chemical nature of interacting phenols was also addressed by carrying out a qualitative and quantitative characterization of phenolic compounds retained by fining agents by means of mass spectrometry techniques (LC-ESI-MS, MALDI-TOF MS).

MATERIALS AND METHODS

Chemicals and Reagents. Water was purified with a Milli-Q system (Millipore, Bedford, MA). The wine-like model solution used was ethanol/water (10:90 v/v) containing 20 mM tartaric acid and buffered to pH 3.5 with NaOH. Unless otherwise specified, all chemicals were from Sigma Chemical Co. (St. Louis, MO). Oligomeric proanthocyanidin complexes (OPCs) from *Vitis vinifera* seeds were supplied by International Nutrition Co. (INC, Loosdrecht, The Netherlands). The fining agents for experimental activities included commercial protein extracts from soybean and pea, lentil flour, and gluten proteins (all from Prodotti Gianni, Milan, Italy). Chemical data for the various protein preparations are given in **Table 1**.

Probe Binding Studies. Protein surface hydrophobicity was assessed by using ANS as the fluorescent probe. Spectrofluorometric measurements were performed in a Perkin-Elmer Luminescence LS 50 spectrometer using 2.5 nm bandwidths for both excitation and emission. Spectrofluorometric titration of protein samples with negligible volumes of aqueous solutions of the hydrophobic fluorescent marker ANS was performed at 25 °C with magnetic stirring, as done before on protein suspensions (*10*−*12*, *16*). Binding of ANS was monitored at λ_{ex} 390 nm and λ_{em} 460 nm. Multiple additions of the fluorescent probe were done up to saturation with the probe (no increase in fluorescence intensity upon further addition of the probe). The increase in fluorescence intensity (F) upon progressive addition of ANS was analyzed by binding algorithms based on the relationship

$$F = F_{max}[\text{ANS}]/(K_d^{app} + [\text{ANS}])$$

that allowed estimation of the overall binding capacity of the proteins for the probe (given as fluorescence intensity at saturating ANS, F_{max}) and the apparent dissociation constant of the proteins−ANS complex (K_d^{app}). The overall binding capacity (F_{max}) was then corrected for the total protein content of each sample. A protein surface hydrophobicity index (PSH) was calculated as [F_{max} (corrected for the protein content) × $(K_d^{app})^{-1}$] (*15*).

The ability of insoluble proteins to bind ANS was measured by adding an excess of the fluorescent probe ($> 2K_d^{app}$) to a suspension of proteins in a wine-like solution. The suspension was then centrifuged (3000*g*, 10 min, 20 °C). An aliquot of the supernatant was mixed with a detergent solution (aqueous Triton X-100, 2% w/v), which incorporated free ANS and ANS bound to soluble proteins (*19*). The ANS content in the micellar phase was then quantified spectrofluorometrically by adding appropriate amounts of ANS as an internal standard.

Competition studies. Hydrophobic interactions between polyphenol compounds and proteins of plant origin were evaluated by competition studies. Excess ANS ($> 2K_d^{app}$) was added to protein suspensions. The decrease in ANS fluorescence due to probe displacement or by quenching was measured as a function of added polyphenolics [catechin or oligomeric proanthocyanidins, (OPCs)]. Concentration of OPCs was expressed as catechin equivalents. Titration with polyphenolics was continued until no further changes in fluorescence were observed.

In a different approach, the disappearance of ANS binding sites in the insoluble fraction of the various plant protein preparation after interaction with polyphenols was studied. Polyphenols were added to protein suspensions at concentrations corresponding to those at which no more fluorescence changes were observed in the ANS displacement experiments presented above. Excess ANS ($> 2K_d^{app}$ for each individual protein system) was then added, and the amount of ANS remaining in the soluble fraction was measured after centrifugation (3000*g*, 10 min, 20 °C) by the detergent inclusion/internal standard procedure described in the section above.

High-Performance Liquid Chromatography−Electrospray Ionization−Mass Spectrometry (HPLC-ESI-MS). Proteins (200 mg/L) were added to OPC solutions (1 mg/mL) in 20 mL of wine-like buffer. Each sample was mixed for 30 min and centrifuged (3000*g*, 15 min, 20 °C). Both the supernatant and the pellet were analyzed by LC-ESI-MS. Pellets were taken up either in wine-like buffer or in a 2:1 mixture of acetonitrile and 0.1% TFA in water and centrifuged as above before analysis of the supernatant.

LC-ESI-MS was carried on a single-quadrupole instrument (HP1100-MSD, Agilent Technologies, Santa Clara, CA) and by using C18 columns (Vydac, Hesperia, CA; 2.1 × 250 mm). The eluents were 0.1% (v/v) TFA in HPLC-grade water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). OPCs were separated at a constant flow rate of 0.2 mL/min, with a linear gradient of solvent B in the following proportions (v/v): 4 min, 0% B; 4−14 min, 0−18% B; 14−22 min, 18−28% B; 22−24 min, 28% B; 24−26 min, 28−60% B; 26−27 min, 60−80% B; and 27−30 min, 80−100% B. The total run time was 30 min with UV detection at 280 nm. Calibration curves were prepared using flavan-3-ol monomers [(+)-catechin, (−)-epicatechin, and (−)-epigallocatechin-3-*O*-gallate] in the 50−250 mg/L concentration range. Five different concentrations were used for each analyte, and experiments were performed in triplicate.

MALDI-TOF MS. MALDI-TOF spectra were recorded in positive-ion mode, using a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N_2 laser (337 nm). α-Cyano-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) was used as the matrix and prepared by dissolving 5 mg in 1 mL of aqueous 50% acetonitrile (v/v)/0.1% TFA (v/v). The instrument operated with an accelerating voltage of 20 kV. Mass spectrum acquisition was performed in both positive linear and reflectron mode. External mass calibration was performed with peptide standards (Sigma Chemical Co., St. Louis, MO).

RESULTS AND DISCUSSION

Protein Surface Hydrophobicity. Surface hydrophobicity plays an important role in protein functionality. Several studies have reported the use of ANS to characterize the surface hydrophobicity of soluble and insoluble proteins, such as those in cereal-based products (*20*−*22*). Binding parameters for ANS may be inferred directly from titration experiments regardless of the presence of heterogeneous phases (*10*−*12*, *16*) and describe rather accurately the surface properties of proteins (*18*).

Spectrofluorometric titrations with ANS of protein suspensions in wine-like buffer are presented in **Figure 1** and confirm the general applicability of this procedure also to particulate and multiphasic systems. **Figure 1** also shows that the various preparations of plant proteins had evident differences in their overall binding capacity toward the probe. The number of surface sites available for binding of the probe is expressed by F_{max}, the fluorescence at saturating probe concentration corrected for the protein content of individual preparations. As listed in **Table 1**, soybean proteins were characterized by the highest number of binding sites per unit mass protein, followed by pea proteins, gluten, and proteins in lentil flour.

From the titration curves in **Figure 1** it was possible also to calculate the apparent dissociation constants of the protein−ANS complexes (K_d^{app}), which were similar in the various samples (**Table 1**). The ANS binding properties of individual protein

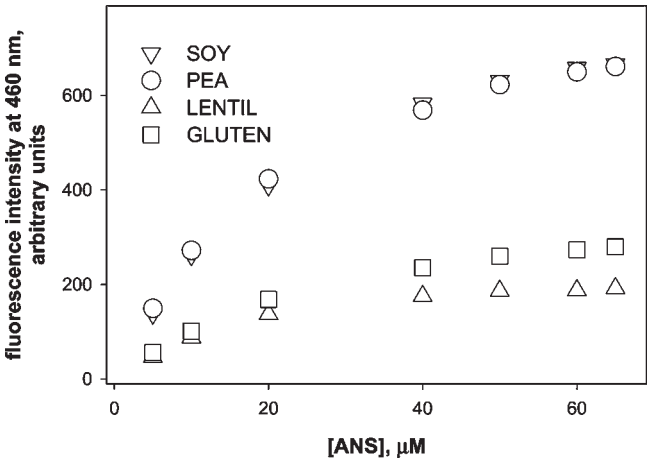

Figure 1. Fluorometric titration with ANS of soybean, pea, lentil flour, and gluten proteins (each at 1 mg of protein/mL in a wine-like model solution). Data are the average of at least triplicate measurements. Symbol size is larger than the standard deviation.

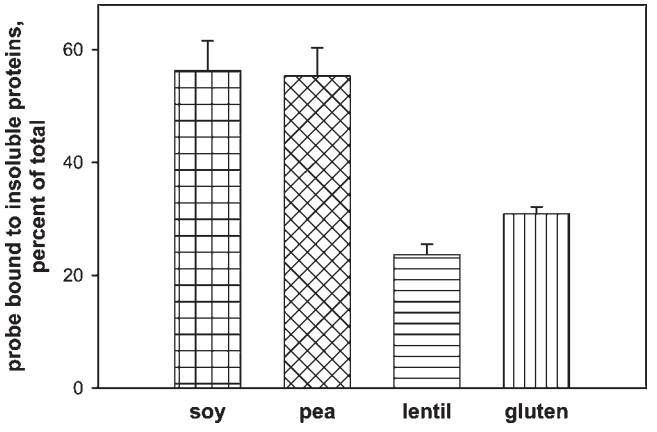

Figure 2. Percent fraction of ANS associated with the insoluble fraction of various proteins (1 mg/mL in a wine-like model solution) after interaction with 60 μM ANS.

preparations may conveniently be expressed, for comparative purposes, by combining the number of sites available for binding of the probe and their average affinity in a single surface hydrophobicity index (PSH = $[F_{max}/\text{prot}]/K_d^{app}$ (*14*, *15*). As summarized in **Table 1**, PSH increased in the order gluten < lentil flour ≪ pea protein isolate < soybean protein isolate.

ANS partition studies were carried out to discriminate between binding of the probe to soluble proteins and binding of the probe to insoluble proteins that are simultaneously present in all of the preparations used here but gluten. Binding of hydrophobes to the insoluble protein fraction is obviously of paramount relevance to the wine fining process. These studies were also meant to set up conditions suitable for carrying out the competition experiments reported in a following section.

A slight excess (~ $2K_d^{app}$) of ANS was added to individual protein suspensions, and the amount of ANS remaining in solution after centrifugation was assessed by a detergent-stripping method (*19*). As shown in **Figure 2**, the insoluble protein fraction in all preparations had a remarkable capability of retaining the probe.

Insoluble proteins in soybean and pea preparations (accounting for 99.44 and 99.06% of total proteins, respectively, in the wine-like buffer used in these studies, see **Table 1**) captured almost 50% of the fluorescent probe initially present, whereas the almost completely insoluble gluten and insoluble proteins in lentil flour (99.76% of the

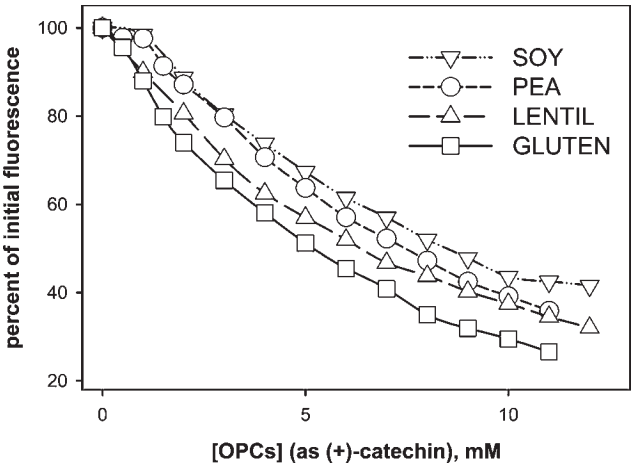

Figure 3. Decrease of ANS fluorescence upon addition of increasing amounts of oligomeric proanthocyanidins (OPCs) to wine-like model solutions containing 60 μM ANS and 1 mg/mL of proteins of various origin. Concentration of OPCs is given as cathechin equivalents. Data are the average of duplicate measurements. Symbol size is larger than the standard deviation.

total proteins) managed to capture about 30 and 20%, respectively, despite the modest overall affinity of these proteins for the probe as assessed by the titration studies shown in **Figure 1**.

Competition Studies. The specificity of the interactions between polyphenols and proteins of plant origin was evaluated by competition studies, in which polyphenols were tested for their ability to displace protein-bound ANS. As shown in **Figure 3**, the addition of increasing amounts of OPCs resulted in a decrease of ANS fluorescence in all cases. The results of similar experiments carried out with catechin (not shown) were overlapping those obtained with OPCs. At the concentrations used here, both catechin and OPCs had little effect on the very low fluorescence of free ANS. Thus, the fluorescence decrease in **Figure 3** stems from detachment of the hydrophobic probe from the protein and seems to confirm that the interactions between proteins and phenolic compounds are governed by hydrophobic forces. Indeed, for both catechin and oligomeric proanthocyanidins, the ability to compete with the probe was highest for those proteins having the lowest ability to bind ANS (see **Figures 1** and **2**) and, thus, the lowest surface hydrophobicity.

The same competition approach was used to assess the amount of ANS remaining bound to the insoluble fraction after incubation of each protein system in the presence of fixed concentrations of ANS (0.1 mM) and oligomeric proanthocyanidins (10 mM as cathechin equivalents). As shown in **Figure 4**, the 100-fold excess of OPCs was unable to prevent binding of ANS to the insoluble proteins in any of the systems. The fluorescence decrease observed in **Figure 3** may be explained as being due to ANS displacement from the soluble fraction of these proteins, but this hypothesis cannot explain what was observed in the case of the totally insoluble gluten. Thus, a more molecular based explanation of the experiments in **Figures 3** and **4** more likely implies that polyphenols bind to proteins "on top" of the bound ANS, quenching its fluorescence and simulating its displacement. Therefore, a more direct approach is required to assess the extent and specificity of the binding of polyphenols to plant-derived proteins and to verify whether the fining process may be finely tuned by an appropriate choice of the involved proteins.

Structural Characterization of Phenolic Compounds before and after Interaction with Proteins Used as Fining Agents. To investigate the molecular basis of tannin−protein associations, OPCs were incubated with each of the various proteins in the same wine-like

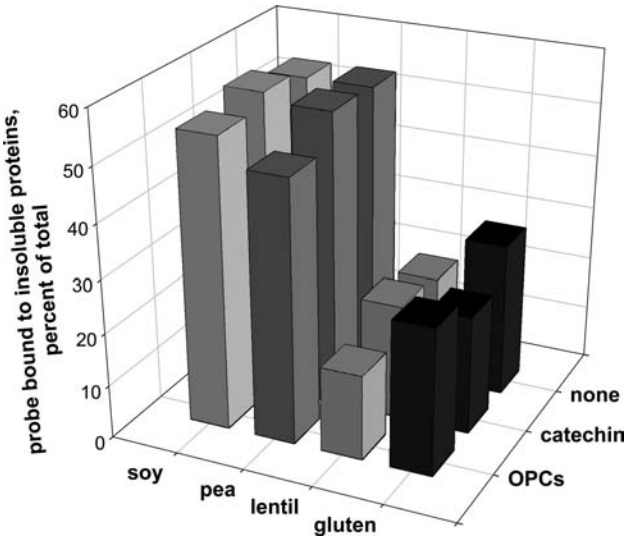

Figure 4. Percent fraction of ANS associated with the insoluble fraction of various proteins (1 mg/mL in a wine-like model solution) after interaction with 60 μM ANS in the absence (no addition) or in the presence of 10 mM catechin and 10 mM OPCs. Concentration of OPCs is given as cathechin equivalents. Data are the average of at least duplicate measurements. In all cases, standard deviation (not given for the sake of clarity) was within 5% of the measured value.

model solution used above. The identification of newly formed compounds and the changes in composition and concentration of OPCs were monitored by HPLC in combination with electrospray mass spectrometry (ESI-MS). To increase the sensitivity of the ESI-MS measurements, the samples were assayed twice, scanning from m/z 100 to 1000 and from m/z 1000 to 2000, respectively. Proanthocyanidin solutions, without addition of fining agents, were used as controls.

The MS total ion chromatogram (TIC) of the positive molecular ions of OPC standard solution (0.1 mM) in the range m/z 100−1000 (not shown) indicated the presence of P1 (m/z 291), P2 (m/z 579), P3 (m/z 867), and some proanthocyanidin gallates (PnGn), including P1G1 (m/z 443), P2G1 (m/z 731), and P2G2 (m/z 883). Polymerized OPCs, in the range m/z 1000 −2000, are predominantly distributed at HPLC retention times of ca. 20−28 min. The mass spectra obtained from the TICs of the extract showed the molecular ion peaks of P4−P6 as well as those of the gallate derivatives P3G1 (m/z 1019), P3G2 (m/z 1172), P4G1 (m/z 1308), P4G2 (m/z 1460), P5G1 (m/z 1595), P5G2 (m/z 1748), and P6G1 (m/z 1884).

More complete data about polymerized tannins were obtained by MALDI-TOF MS analysis in positive-ion linear and reflectron modes. MALDI-TOF is able to measure masses in complex mixtures of low and high molecular weight compounds. In model white wine were detected an oligomeric series of catechin/epicatechin units and their gallic acid ester derivatives (sodium adduct ions M + Na^+), up to the decamer (see **Table 2**). Additionally, masses corresponding to a series of polygalloyl polyflavans were also detected.

Fining model systems were set up by adding each of the fining agents (at the commonly used concentration of 20 g/100 L, corresponding to a protein concentration of 200 mg/L) to OPC solutions in our wine-like buffer, which were stirred for 30 min and centrifuged. The resulting supernatants and the pellets, taken up in a wine-like solution to dissociate weakly bound tannins, were analyzed by LC-ESI-MS. Representative TIC and MS tracings from one of these experiments are shown in **Figure 5**. The TICs of all treated wine-like systems resembled those obtained for standard solutions of OPCs, suggesting an identity of small oligomeric flavan-3ols. This finding was also supported by MALDI-TOF MS results, as summarized in **Table 2**.

The results of MALDI-TOF analysis of fined wine-like systems also suggest that all tested protein fining agents selectively removed polymeric proanthocyanidins, lowering their apparent average degree of polymerization in fined model wine with respect to the original untreated solution of OPCs. These results are in accordance with previous studies, which assume that the largest proanthocyanidin molecules are precipitated first in fining experiments (*23*). This effect could be due to the higher number of phenolic rings present in the more polymerized proanthocyanidins, which increases their hydrophobicity and allows for more effective removal (*24*).

Characterization and estimates of the relative amounts of polyphenols precipitated from wine-like model system were made by LC-ESI-MS analysis of pellets after fining treatment. All of the pellets showed the presence of newly formed products. For instance, we observed the presence of vinylcatechin and vinylepicatechin (m/z 316), eluting later than their unmodified forms (**Figure 6**), and originating by catechin/epicatechin autopolymerization induced by acetaldehyde.

The acetaldehyde present in fining model systems derives from the oxidation of ethanol, either catalyzed by transition metals such as iron and copper (that reportedly are found associated with plant-derived proteins) or through coupled oxidation of phenols (*25*). The reaction starts with the nucleophilic addition of the protonated form of acetaldehyde to the flavanol. The newly formed ethanol adduct, losing a water molecule, is attacked by a second nucleophilic flavanol unit to yield an ethyl-linked flavanol dimer. The ethyl linkages generated by acetaldehyde in the polycondensated tannins are not stable and cleave into vinylflavanol monomers and oligomers (*26*). Compared to direct condensation between flavanols, the rapid polymerization mediated by acetaldehyde gives rise to instability and precipitation (*27*, *28*). This could explain the presence of vinylflavanol products in the pellets precipitated by finings and not in the supernatant of treated samples. In addition to these compounds, various dimeric and oligomeric ethyl-bridged molecules were also detected by MALDI-TOF analysis of pellets. These newly formed species included adducts of trimers and their gallic acid derivatives (m/z 923; m/z 1075.9).

We also carried out a detailed quantitative LC-MS analysis of the flavonoid compounds most important with respect to white wine oxidation (monomeric and dimeric proanthocyanidins) to evaluate which molecules were most easily removed by the various proteins. The browning capacity of white wines depends largely on the nature of polyphenols. Due to their catechol (*o*-diphenol) structure, most of them are rather readily oxidized in winemaking processes, The monomeric catechins and the dimeric procyanidins contribute to the browning more intensely than other phenolics (*29*), and there is strong evidence of epicatechin being the most relevant browning agent among redox-active polyphenols (*30*).

In the OPCs control solution (5 mg/mL) the calculated total concentration of monomeric and dimeric molecules (catechin, epicatechin, monomers gallate, dimers, dimers gallate, dimers digallate) was 2.9 mg/mL. A general decrease in the concentration of all these species, considered as a whole, was observed after treatment with proteins. Lentil flour was the most effective removal agent, giving a 16.4% decrease in OPCs, followed by gluten, soy, and pea proteins, which gave decreases of 12.6, 9.26, and 8.44%, respectively. These differences in clarifying efficiency are likely related to the molecular composition, the biochemical characteristics, and the conformation of proteins relevant to the complex interactions that ultimately lead to flocculation of their

Table 2. Oligomeric Proanthocyanidin Detected by MALDI-TOF MS before and after Fining Wine-like Model Solutions (10 mM Proanthocyanidins) with Soybean, Pea, Lentil Flour, and Gluten Proteins (200 mg/L)[a]

		molecular mass, Da					
				fined wine-like solutions			
	no. of galloyl units	calcd ($M + Na^+$)	original OPCs ($M + Na^+$)	soybean	pea	lentil flour	gluten
dimer	0	601.3	602.5	602.1	602.4	601.8	602.2
	1	753.3	754.3	753.8	754.1	753.4	754.0
	2	905.3	907.7	905.6	905.9	905.2	905.7
trimer	0	889.8	890.9	889.7	889.9	889.5	889.9
	1	1041.9	1042.4	1041.4	1041.6	1040.8	1041.4
	2	1194.0	1194.2	1193.0	1194.5	1194.0	1193.3
	3	1346.1	1347.4	1344.8	1345.6	nd	1346.3
tetramer	0	1178.0	1178.0	1177.2	1177.7	1176.7	1178.2
	1	1330.1	1331.8	1329.0	1329.3	1329.9	1329.6
	2	1482.2	1483.0	1481.2	1483.1	1480.9	1483.0
	3	1634.4	1634.6	nd	1633.4	1636.3	nd
	4	1786.5	1787.9	1785.7	1785.7	1784.5	nd
pentamer	0	1466.3	1466.1	1465.9	1466.4	1465.0	1466.0
	1	1618.4	1618.8	nd	1617.4	1615.3	1617.8
	2	1770.5	1770.8	nd	1771.0	1769.0	1769.1
	3	1922.6	1922.9	1919.0	1920.9	nd	1920.8
	4	2074.7	2074.6	nd	2075.8	2077.9	2074.7
hexamer	0	1754.5	1754.9	1752.7	1752.1	1751.1	1754.5
	1	1906.7	1907.6	1905.0	1905.9	1903.0	1905.9
	2	2058.8	2059.9	2057.4	2057.4	2056.5	2055.8
	3	2210.9	2211.3	nd	2210.0	nd	2209.9
	4	2363.0	2363.6	nd	nd	nd	nd
	5	2515.1	nd	nd	nd	nd	nd
	6	2667.2	nd	nd	nd		nd
heptamer	0	2042.8	2042.3	2042.4	2041.0	2043.3	2042.8
	1	2194.9	2194.7	nd	2192.4	nd	2194.9
	2	2347.0	2347.9	2346.5	2345.0	nd	2344.0
	3	2499.1	2499.4	nd	2495.0	nd	2494.4
	4	2651.2	2650.6	nd	nd	nd	nd
	5	2803.3	2802.5	nd	2805.9	nd	2800.2
	6	2955.4	2956.2	nd	2956.2	nd	2959.1
	7	3107.5	3106.3	3106.7	3104.8	3103.3	nd
octamer	0	2331.1	2330.5	nd	2332.2	2327.8	nd
	1	2483.2	2481.7	nd	2482.1	nd	nd
	2	2635.3	2634.0	2634.4	2632.5	nd	2634.2
	3	2787.4	2786.4	nd	2876.5	2786.4	nd
	4	2939.5	2936.8	nd	2939.5	2941.7	nd
	5	3091.0	3093.6	nd	3088.8	nd	3089.9
	6	3243.0	3241.0	nd	3244.4	nd	nd
	7	3395.0	nd	3394.2	3397.2	nd	nd
nonamer	0	2619.3	2619.1	2619.0	nd	nd	nd
	1	2771.4	2771.1	nd	nd	nd	nd
	2	2923.5	2922.6	nd	nd	nd	2924.8
	3	3075.6	3072.6	nd	nd	nd	nd
	4	3227.7	3228.3	nd	3229.6	nd	3226.7
	5	3379.8	3377.3	nd	nd	3378.6	nd
decamer	0	2907.6	2905.0	nd	nd	nd	2909.3
	1	3059.7	3056.0	nd	3053.8	3059.3	nd
	2	3211.8	3209.5	nd	nd	nd	nd
	3	3363.9	3360.7	nd	nd	nd	nd
	4	3516.0	nd	nd	nd	nd	nd
	5	3668.1	nd	nd	nd	nd	nd

[a] OPCs, oligomeric proanthocyanidins; nd, not detected.

complexes with polyphenols and to clarification of the model wine-like solutions used here.

At first sight the interactions brought forward by the clarifying ability of proteins from the various sources seem to be in disagreement with the surface hydrophobicity data and the competition experiments reported in other sections of this work. On the basis of these latter data, we expected that proteins characterized by the highest surface hydrophobicity should have given the highest

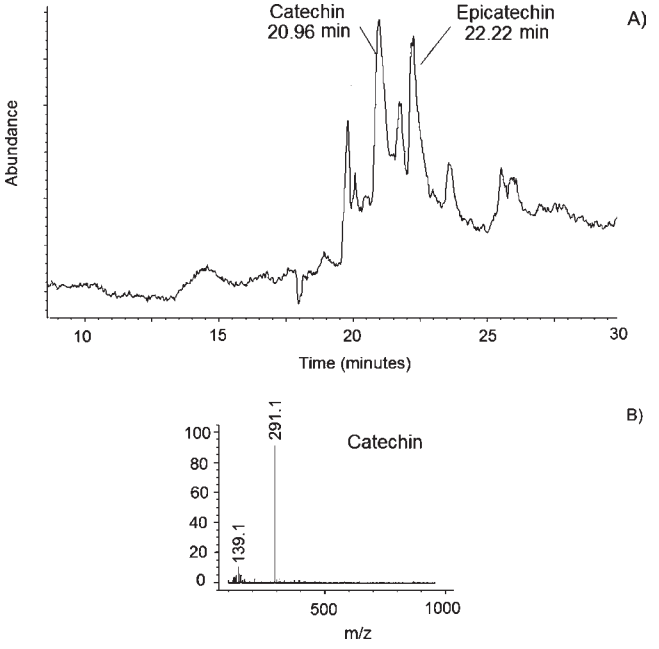

Figure 5. (**A**) Ttotal ion current (TIC) chromatogram obtained in positive ion mode by injection of wine-like model solution after fining with pea protein isolate (1 mg/mL protein, 10 mM proantocyanidins). (**B**) ESI mass spectrum obtained from the TIC chromatogram for 20.92 min elution time, showing the $[M - H]^+$ peaks of catechin (*m/z* 291).

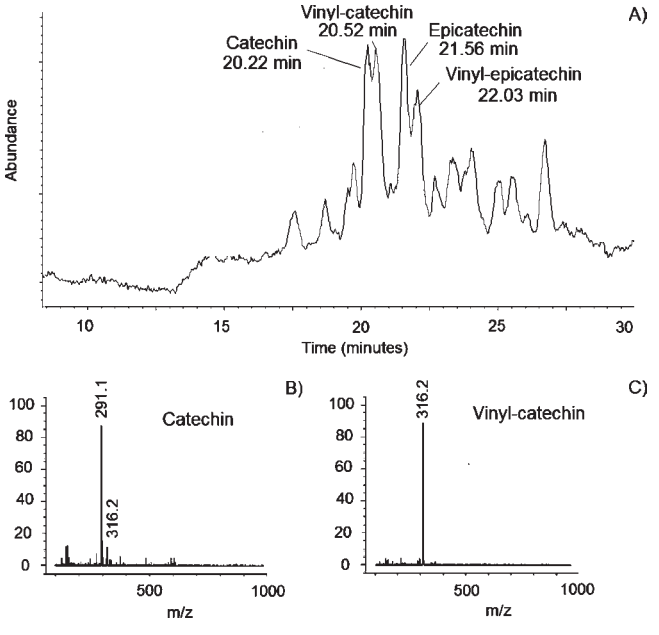

Figure 6. (**A**) Total ion current (TIC) chromatogram obtained in positive ion mode by injection of insoluble fraction of pea protein isolate (1 mg/mL protein, 10 mM proantocyanidins). (**B**, **C**) ESI mass spectra obtained from the TIC chromatogram for 20.22 and 20.52 min elution time, respectively, showing the $[M - H]^+$ peaks of catechin (*m/z* 291) and vinylcatechin (*m/z* 316).

removal of OPC from the wine-like medium, whereas the direct measurements reported above indicate that the actual rank was reversed, at least when OPCs are considered as a whole.

However, if the loss and recovery are analyzed in terms of individual molecules, there is evidence that this discrepancy is more apparent than substantial and that molecular specificity plays a role in governing the interaction between hydrophobic sites on the protein surface and the molecules considered here.

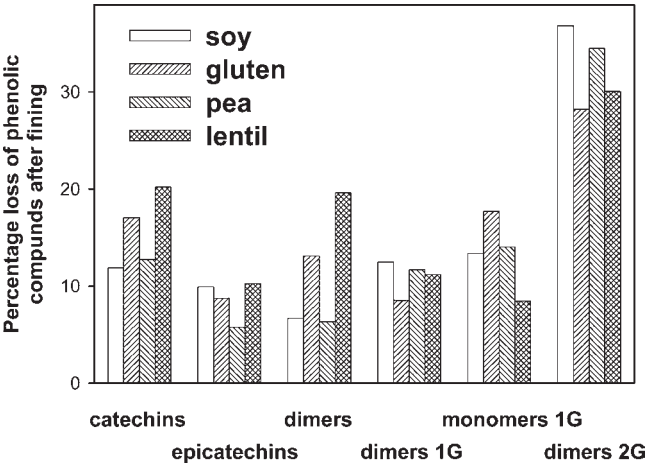

Figure 7. Percentage loss of individual flavan-3-ols species in fined wine-like samples, as assessed by means of LC-ESI-MS analysis.

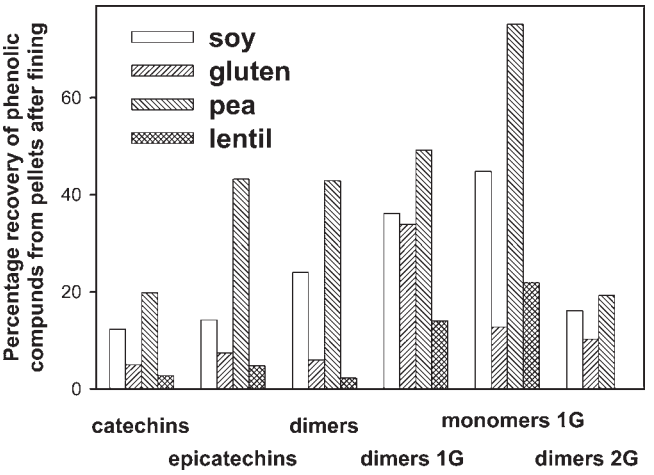

Figure 8. Percentage recovery of phenolic compounds from pellets obtained after fining processes carried out with the various proteins (ratio between the concentration of each molecules in the pellet and their loss in fined wine-like sample).

Figure 7 presents quantitative data as for the removal of individual species after treatment with the various proteins, obtained through HPLC analysis. For instance, the efficiency of the various proteins in removing (+)-catechin, and (−)-epicatechin, which differ only on the spatial position of one OH group with respect to the ring, was remarkably different. In particular, (+)-catechin was more specifically removed than (−)-epicatechin by all of the protein fining agents tested, especially by the lentil flour and the gluten proteins. Moreover, the levels of galloylated proanthocyanidin precipitation appeared to be higher than those of the other phenols for all protein fining agents (from 28% loss with gluten proteins to 36% loss with soy proteins), indicating that the more galloylated proanthocyanidins were removed in a preferential way.

Pellets obtained from the fining processes carried out with the various proteins also were treated with water and with more apolar solvents to assess the nature and intensity of the forces involved in the interaction. Pellets were dissolved in acetonitrile/water 0.1% TFA (2:1) to dissociate soluble and insoluble tannin−protein complexes. The percentage recoveries of phenolic compounds from pellets (ratio between the concentration of each molecules in the pellet and their loss in fined wine-like sample) are compared for each of the tested proteins in **Figure 8**.

Pea and soy proteins were the fining systems in which proanthocyanidins were more easily recovered from pellets. This is in accordance with the evidence gathered from competition studies and confirms that "weak" forces, such as hydrophobic ones, were most relevant to the interactions responsible for precipitation by these fining agents. The interaction between polyphenols and gluten proteins and, above all, lentil flour systems (characterized by higher loss in treated wine-like samples, lower surface hydrophobicity, and low recovery from the pellets) could be instead governed by other types of attractive forces such as hydrogen or covalent bonds (cross-linkages between proteins), which may impair the release of polymerized tannins.

In conclusion, the structural characterization of proteins of plant origin in terms of surface hydrophobicity provided a comparative estimate of the number and of the affinity of binding sites on the surface of the various proteins. The indications provided by these studies were confirmed to a large extent by competition/displacement experiments. However, a straightforward interpretation of the displacement experiments was made difficult by simultaneous binding of the fluorescent probe and of polyphenols. This indicates the existence of multiple binding sites on the protein surface, with a possible different specificity for different molecules, as observed for many other food and nonfood proteins (*31*, *32*). This implies that proteins of different origin may selectively bind peculiar fractions in a complex mixture of polyphenols, as confirmed by LC-MS analysis of the pattern of bound and residual polyphenols in mixtures simulating the actual fining process. This finding is of possible practical interest, in that it paves the way to a selective use of protein agents for "fine tuning" the properties of the finished product with respect to important organoleptic properties and their stability.

However, it was also noted that treatment of simulated wine with the commercial protein preparations used here favors some chemical reactions among some of the polyphenols and other wine components. The significance of these reactions in a real wine (in which they may be affected by other wine components) remains to be evaluated. The possible requirement for plant-protein-based fining agents chosen and/or processed ad hoc for this particular purpose also remains to be evaluated. Should this be appropriate or necessary, the methodologies presented here could be fruitfully exploited to assess whether these materials will be suitable for this particular use, for instance, by testing their surface hydrophobicity properties, prior to resorting to exceedingly laborious, time-consuming, and expensive experimentation in actual winemaking applications.

ABBREVIATIONS USED

ANS, 1-(anilino)naphthalene-8-sulfonate; PSH, protein surface hydrophobicity; F_{max}, maximum fluorescence intensity; K_d^{app}, apparent dissociation constant of the protein−ANS complex; LC-MS, liquid chromatography−mass spectrometry; ESI, electrospray ionization; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TFA, trifluoroacetic acid; TIC, total ion current; OPCs, oligomeric proanthocyanidins complexes; (PnGn), proanthocyanidin gallates.

ACKNOWLEDGMENT

The assistance of Dennis Van der Vlies at INC in preparation of the proanthocyanidins used in this study is gratefully acknowledged.

LITERATURE CITED

(1) Cosme, F.; Richardo-da-Silva, J. M.; Laureano, O. Interactions between protein fining agents and proanthocyanidins in white wine. *Food Chem.* **2008**, *106*, 536−544.

(2) Spagna, G.; Barbagallo, R. N.; Pifferi, P. G. Fining treatements of white wines by means of polymeric adjuvants for their stabilization against browning. *J. Agric. Food Chem.* **2000**, *48*, 4619−4627.

(3) Tschiersch, C.; Nikfardjam, M. P.; Schmidt, O.; Schwack, W. Degree of hydrolysis of some vegetable proteins used as fining agents and its influence on polyphenol removal from red wine. *Eur. Food Res. Technol.* **2010**, *231*, 65−74.

(4) Marchal, R.; Marchal-Delahaut, L.; Lallement, A.; Jeandet, P. Wheat gluten used as a clarifying agent of red wines. *J. Agric. Food Chem.* **2002**, *50*, 177−184.

(5) Versari, A.; Barbanti, D.; Potentini, G.; Parpinello, G. P.; Galassi, S. Preliminary study on the interaction of gelatine-red wine. *Ital. J. Food Sci.* **1999**, *11*, 231−239.

(6) Sarni-Machado, P.; Deleris, A.; Avallone, S.; Cheynier, V.; Moutounet, M. Analysis and characterization of wine condensed tannins precipited by proteins used as fining agent in enology. *Am. J. Enol. Vitic.* **1999**, *50*, 81−86.

(7) Yokotsuka, K.; Singleton, V. L. Interactive precipitation between phenolic fraction and peptides in wine-like model solutions: turbidity, particle size and residual content as influenced by pH, temperature and peptide concentration. *Am. J. Enol. Vitic.* **1995**, *46*, 329−338.

(8) Li-Chan, E. Hydrophobicity in food protein systems. In *Encyclopedia of Food Science and Technology*; Hui, Y. H., Ed.; Wiley: New York, 1991; pp 1429−1439.

(9) Nakai, S.; Li-Chan, E. *Hydrophobic Interactions in Food Systems*; CRC Press: Boca Raton, FL, 1988.

(10) Bonomi, F.; Iametti, S. Real-time monitoring of the surface hydrophobicity changes associated with isothermal treatment of milk and milk protein fractions. *Milchwissenschaft* **1991**, *46*, 71−74.

(11) Iametti, S.; Bonomi, F. Monitoring the surface hydrophobicity of milk proteins: a real-time study on heat induced modifications. *IDF Bull. Spec.* **1993**, *9303*, 111−116.

(12) Cairoli, S.; Iametti, S.; Bonomi, F. Reversible and irreversible modification of β-lactoglobulin upon heating. *J. Protein Chem.* **1994**, *13*, 347−354.

(13) Iametti, S.; Scaglioni, L.; Mazzini, S.; Vecchio, G.; Bonomi, F. Structural features and reversible association of different quaternary structures of β-lactoglobulin. *J. Agric. Food Chem.* **1998**, *46*, 2159−2166.

(14) Bonomi, F.; Iametti, S.; Pagliarini, A.; Peri, C. A spectrofluorimetric approach to the estimation of the surface hydrophobicity modifications in milk proteins upon thermal treatement. *Milchwissenschaft* **1988**, *43*, 281−285.

(15) Pagliarini, A.; Iametti, S.; Peri, C.; Bonomi, F. An analytical approach to the evaluation of heat damage in commercial milks. *J. Dairy Sci.* **1990**, *73*, 41−44.

(16) Iametti, S.; Negri, E.; Bonomi, F.; Giangiacomo, R. The estimation of changes in protein surface hydrophobicity during surface cheese ripening. *Neth. Milk Dairy J.* **1991**, *45*, 183−191.

(17) Iametti, S.; Donnizzelli, E.; Pittia, P.; Rovere, P.; Squarcina, N.; Bonomi, F. Properties of a soluble form of high-pressure treated egg albumen. *J. Agric. Food Chem.* **1999**, *47*, 3611−3616.

(18) Bonomi, F.; Mora, G.; Pagani, M. A.; Iametti, S. Probing structural features of water-insoluble proteins by front-face fluorescence. *Anal. Biochem.* **2004**, *329*, 104−111.

(19) Pagani, M. A.; Iametti, S.; D'Egidio, M. G.; Bonomi, F. The evaluation of protein surface hydrophobicity as a quality index for semolina. In *Plant Proteins from European Crops: Food and Non-Food Applications*; INRA; Nantes, France, 1998; pp 225−228.

(20) Genot, C.; Tonetti, F.; Montenay-Garestier, T.; Drapron, R. Front-face fluorescence applied to structural studies of proteins and lipid−protein interactions of visco-elastic food products. 1. Designing of front-face adaptor and validity of front-face fluorescence measurements. *Sci. Aliment.* **1992**, *12*, 199−212.

(21) Genot, C.; Tonetti, F.; Montenay-Garestier, T.; Drapron, R. Front-face fluorescence applied to structural studies of proteins and lipid−protein interactions of visco-elastic food products. 2. Application to wheat gluten. *Sci. Aliment.* **1992**, *12*, 687−704.

(22) Sironi, E.; Guerrieri, N.; Cerletti, P. Surface properties of gluten investigated by a fluorescence approach. *Cereal Chem.* **2001**, *78*, 476−480.

(23) Ricardo-Da-Silva, J. M.; Cheynier, V.; Souquet, J. M.; Moutounet, M.; Cabanis, J. C.; Bourzeix, M. Interaction of grape seed procyanidins with various proteins in relation to wine fining. *J. Sci. Food Agric*. **1991**, *57*, 111−125.

(24) Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566−5577.

(25) Ross, A. R. S.; Ikonomou, M. G.; Orians, K. J. Characterization of dissolved tannins and their metal-ion complexes by electrospray ionization mass spectrometry. *Anal. Chim. Acta* **2000**, *411*, 91−102.

(26) Fulcrand, H.; Duenas, M.; Salas, E.; Cheynier, V. Phenolic reactions during winemaking and aging. *Am. J. Enol. Vitic*. **2006**, *57*, 289−297.

(27) Es-Safi, N. E.; Cheynier, V.; Moutounet, M. Role of aldehydic derivatives in the condensation of phenolic compounds with emphasis on the sensorial properties of fruit-derived foods. *J. Agric. Food Chem*. **2002**, *50*, 5571−5585.

(28) Es-Safi, N. E.; Le Guerneve, C.; Moutounet, M. Effect of copper on oxidation of (+)-catechin in a model solution system. *Int. J. Food Sci. Technol*. **2003**, *38*, 153−163.

(29) Lee, C. Y.; Jaworski, A. W. Phenolic and browning potential of white grape grown in New York. *Am. J. Enol. Vitic*. **1988**, *39*, 337−340.

(30) Sioumis, N.; Kallithraka, S.; Makris, D. P.; Kefalas, P. Kinetics of browning onset in white wines: influence of principal redox-active polyphenols and impact on the reducing capacity. *Food Chem*. **2006**, *94* (1), 98−104.

(31) Lozinsky, E.; Iametti, S; Barbiroli, A.; Likhtenshtein, G. I.; Kálai, T.; Hideg, K.; Bonomi, F. Structural features of transiently modified β-lactoglobulin relevant to the stable binding of large hydrophobic molecules. *Protein J*. **2006**, *25*, 1−15.

(32) Barbiroli, A.; Beringhelli, T.; Bonomi, F.; Donghi, D.; Ferranti, P.; Galliano, M.; Iametti, S.; Maggioni, D.; Rasmussen, P.; Scanu, S.; Vilardo, M. C. Bovine β-lactoglobulin acts as an acid-resistant drug carrier by exploiting its diverse binding regions. *Biol. Chem*. **2010**, *391*, 21−32.

Received for review July 7, 2010. Revised manuscript received October 4, 2010. Accepted October 11, 2010. Work supported in part by PRIN2008 "Evoluzione di composti solforati varietali nella trasformazione enologica".