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Electrophoretic Method for the Identification of a Haze-Active Protein in Grape Seeds

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A simple, fast, and more selective approach is presented in this study for the identification of haze-active proteins. Grape seed proteins were unfolded by 1% SDS and then interacted with different amounts of tannin at 4 °C, followed by gel electrophoresis. It was found that the intensity of the band at 45 kDa was decreased as tannins increased. The amino acid composition of this isolated 45-kDa protein was higher in proline (9.49%) than the average proline content of total grape seed proteins (4.85%). To verify the selectivity of the proposed method, a globular protein (bovine serum albumin, BSA) and a proline-rich protein (gelatin) were selected and used in the model system. As expected, gelatin was removed as it reacted with the increasing added tannins, whereas BSA did not. These results showed that it is possible to identify haze-active proteins by modulating the accessibility of protein to tannins, suggesting this new method can be used by the beverage industry to trouble-shoot haze problems and for quality control.

KEYWORDS: Gel electrophoresis; grape seed protein; protein−polyphenol interaction

INTRODUCTION

Polyphenols, otherwise known as tannins, are secondary metabolites that are widespread in the plant kingdom. They possess an abundance of phenolic groups and, on the basis of their chemical structures, are usually classified as either condensed or hydrolyzable tannins. Condensed tannins are polymers of flavonoid units, and hydrolyzable tannins are esters, usually containing D-glucose, gallic acid, and its derivatives (*1*). Tannins have a significant affinity for extended proteins and peptides that contain a high proportion of proline residues (*2*, *3*). This feature is the origin of their harsh astringent taste and the cause of haze formation in beer, wine, and fruit juices (*4*). Studies on animals have demonstrated a number of detrimental biological effects caused by tannins such as antinutritional effects, and these manifest as a decrease in both growth rate and body weight gain (*5*, *6*), as an interference with iron absorption (*7*), as an interference with pancreatic digestion (*8*), and as an inhibition of digestive enzymes (*9*), as well as the formation of insoluble complexes with dietary proteins. Esophageal cancer and systemic toxic effects (*10*) have also been reported. The ability of polyphenols to serve as antioxidants has been addressed recently (*11*−*13*), and the optimal daily intake for humans has been estimated to be ∼1 g of tannin (*14*).

One of the defensive mechanisms developed to reduce the adverse effects of tannins on mammals is the production of tannin-binding proteins. Saliva from many species contained a family of proline-rich proteins (PRPs) (*6*), which represented up to 70% of salivary proteins in human parotid saliva and which bind more strongly to polyphenols than to other salivary proteins (*15*). It has been suggested that PRPs contain 28−40% proline residues, which are grouped with glutamine and glycine residues and are arranged into 5−15 almost identical repeats, accounting for 70−80% of the total amino acid residues in PRPs (*16*). Acidic, basic, and glycosylated PRPs have also been described (*15*), and the main function of basic PRPs appears to be to bind dietary polyphenols, thereby neutralizing the harmful actions of tannins (*17*).

The complexing of tannin to PRPs is principally a hydrophobic stacking of the polyphenol ring against the pyrrolidine ring face containing the Cα proton. Secondary hydrogen-bonding effects stabilize the complex (*18*). The binding occurs primarily at proline residues together with the preceding amide bond and amino acid and particularly favors the first proline residue of a Pro-Pro sequence (*3*). This may be due to the X-Pro sequence, in which proline imposes a β-sheet conformation on the preceding residue (*19*). Two-dimensional ^{1}H NMR studies indicate that human salivary proteins with multiple tandem repeats (SPPGKPQGPPQQ) demonstrate a much stronger interaction than a single repeat sequence (*20*). The increased affinity cannot be exclusively attributed to the longer protein containing more binding sites, but must also involve synergistic effects related to folding and "wrapping around" the tannin by the longer protein. These result in an increase in association by cooperative intramolecular interactions (*20*, *21*). NMR studies also reveal that the larger and more complex polyphenols with a smaller dissociation constant (K_d) interact more strongly with the PRP fragments. Procyanidin B-2 (condensed tannin) has the strongest binding affinity to a PRP fragment compared to trigalloylglucose (hydrolyzable tannin), proanthocyanidin mono-

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mer [(−) epicatechin], and propyl gallate (*3*). As the number of galloyl groups increases, the binding affinity of the hydrolyzable tannins strengthens (*3*). This effect can be ascribed to the increase in hydrophobicity of the molecules with more galloyl groups and a sterically restricted structure that preferentially forms secondary binding interactions (*3*, *22*). Proline residues in the PRP fragment not only act as binding sites but also maintain the peptide in an extended form (*23*) and maximize the availability of binding surfaces (*24*). The rigidity of the proline-rich sequence is favorable for tannin binding; the nonproline residues then can encompass and make multiple contacts with bound polyphenol molecules.

A group of histidine-rich salivary proteins are also important in tannin binding (*22*). In contrast to the large molecular sized proline-rich proteins, these proteins have low molecular weights (histatin 5, 3037 Da) and contain high levels of basic amino acids. In a similar way to proline-rich proteins, the histatins characteristically have a random coil structure, and this makes the basic amino acids accessible to interaction with tannin aromatic rings (*25*).

The formation of soluble or insoluble complexes caused by protein−polyphenol interaction (haze) poses a problem in the food industry. Visible haze formation can limit the shelf life of products that the consumer expects to be clear. These include beer, wine, fruit juices, coffees, and teas (*4*, *26*). Not all proteins are equally involved in forming hazes, and proteins rich in proline residues have been suggested as being responsible for haze formation in beverages. The haze-forming proteins in beer are derived from the hordeins and are relatively rich in proline (*27*); it has also been reported that hydrolysates of protein from apple juice sediments contain 5−16 mol % proline (*28*). Addition of adsorbents such as bentonite, silica gel, polyvinylpolypyrrolidone, or gelatin to stabilize a beverage by the removal of either polyphenols or proteins has been investigated (*29*, *30*). The efficacy of the adsorbents varies with the type of beverage (*29*). One disadvantage of adsorbent treatment is the concomitant removal of flavor compounds (*31*). A method for the removal of proteins important in the formation of hazes has been developed (*32*, *33*). It was suggested that haze formation should be forced by heating to 80 °C, followed by cooling to 4 °C for a number of hours (*33*). Although this approach was able to remove hazes, it failed to selectively identify the key haze-active proteins. This was because of the resultant greater protein unfolding and exposure of internal amino acids that complex with polyphenols. The purpose of this study was to develop a method to identify haze-active proteins by modulating the accessibility of binding sites with 1% SDS to tannins. To our knowledge, this is the first time SDS has been used in studying the haze-active protein. Grape seed is selected as target sample in this study because seed proteins, presented in juices due to the breakage of seeds during processing, may have an effect similar to that of barley in beer on haze formation.

MATERIALS AND METHODS

Preparation of Grape Seed Proteins. Grape (var. Black Queen, a hybrid of *Vitis vinifera* and *Vitis labruscana*) seed protein was chosen as target protein in this study. It was extracted by homogenizing 200 g of grape seeds in 1 L of 50 mM Tris-HCl buffer (pH 6.8) containing 10 mM β-mercaptoethanol, 2.0 mM EDTA, 20 mg/L phenylmethanesulfonyl fluoride (PMSF), 1% Triton X-100 (or 1% SDS), and 5% polyvinylpolypyrrolidone (PVPP) (*4*). The homogenate was centrifuged for 30 min at 3000*g* at 4 °C. The supernatant was concentrated with a 10 kDa cutoff membrane (Millipore, Billerica, MA).

Purification of Grape Seed Proteins. The concentrated solution was applied to an Amersham Bioscience CM Sepharose fast flow (Uppsala, Sweden) column (1.5 cm × 50 cm), previously equilibrated with 50 mM Tris-HCl buffer (pH 6.8) containing 10 mM β-mercaptoethanol, 2.0 mM EDTA, and 20 mg/L PMSF. Protein was eluted with the help of a gradient of sodium chloride ranging from 0 to 1 mol L^{-1} in the same buffer. The flow rate was 30 mL h^{-1}, and 2 mL fractions were collected. Fractions eluted at a salt concentration of 0.34 mol L^{-1} showing absorbance at 280 nm were pooled and again concentrated using a Microcon centrifugal filter device with a 10 kDa cutoff membrane. The concentrated sample containing the 45-kDa protein was then applied to a Biogel-P polyacrylamide gel (Bio-Rad, Hercules, CA) column (1.5 cm × 50 cm), previously equilibrated with 50 mM phosphate buffer (pH 6.8). All protein purification was carried out on a BioLogic LP system (Bio-Rad, Hercules, CA) at 4 °C. Elution was performed at a flow rate of 30 mL h^{-1} with a fractionator, and 2-mL fractions were collected. The fractions from the gel filtration with a molecular weight ranging from 29 to 66 kDa (Sigma protein molecular weight marker was used) were pooled and concentrated in a Microcon centrifugal filter device with a 10 kDa cutoff membrane (Millipore) for further studies. Protein concentration determination was carried out by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) and was calibrated by bovine serum albumin (BSA).

Haze-Active Protein Determination for Grape Seed Proteins and Model System. Extracted grape seed proteins containing detergents were mixed with 0, 2, or 4 g/L tannins (Sigma, St. Louis, MO) in 50 mM Tris-HCl buffer (pH 6.8) at 4 °C for 30 min. After centrifugation at 3000*g*, supernatants were analyzed by SDS-PAGE. Gel image was analyzed by ImageMaster VDS-CL (Amersham Biosciences, Uppsala, Sweden). For the verification model, BSA and gelatin from Sigma were selected. Protein solutions, with or without 1% SDS, were mixed with different levels of tannins (0, 4, 5, 6, and 7 g/L) in 50 mM Tris-HCl buffer (pH 6.8) at 4 °C for 30 min. Gel electrophoresis was performed after supernatants were centrifuged at 3000*g* for 30 min.

Electrophoresis. To determine the molecular weight of the purified protein, SDS-PAGE with 15% acrylamide gels containing 0.2% bisacrylamide was used. The buffer system was the same as that used by Laemmli (*34*). The electrophoresis conditions were 80 V for 30 min followed by 150 V for 2 h. Proteins were fixed in the gel with 45% methanol and 1% of acetic acid for 20 min and were visualized with 0.1% (w/v) Coomassie Blue G-250 in 17% (w/v) $(NH_4)_2SO_4$, 34% (v/v) methanol, and 0.3% (v/v) phosphoric acid overnight. The p*I* of the purified protein was determined by two-dimensional electrophoresis according to the manufacturer's instructions provided by Amersham Biosciences. A pH 3−10 strip was used for the first dimension, and a 15% SDS-PAGE gel was used for the second dimension. Gel was stained with Coomassie Blue (*35*, *36*). The gel image was captured and analyzed by a cooled digital CCD camera system, ImageMaster VDS-CL (Amersham Biosciences).

Amino Acid Analysis. To investigate the amino acid composition of the extracted protein sample, it was hydrolyzed in 6 M HCl at 110 °C for 16 h under argon. Amino acid analysis was then performed on a Beckman 6300 high-performance amino acid analyzer according to the method of Spackman et al. (*37*).

RESULTS AND DISCUSSION

The formation of protein−polyphenol complexes is largely based on the structure of the proteins (*3*, *22*). Extended or random coil proteins such as proline-rich proteins and histatin with open structures allow hydrophobic amino acid residues to readily interact with the aromatic rings on tannins. Hydrogen bonding helps to stabilize the complexes and may also contribute to protein−tannin attraction. To locate proteins with a high tendency toward tannin binding, we searched for proteins with a loose, extended structure such as proteins high in proline or histidine residues. Efforts have been made to identify such proline-rich proteins, employing isotope-labeled [^{3}H]proline (*35*, *38*) or construction of a cDNA library for cloning of carrot extensin (*39*). These approaches have merits as they allow a precise study of the target proteins. Heat treatment is commonly used to investigate proteins involved in haze formation in

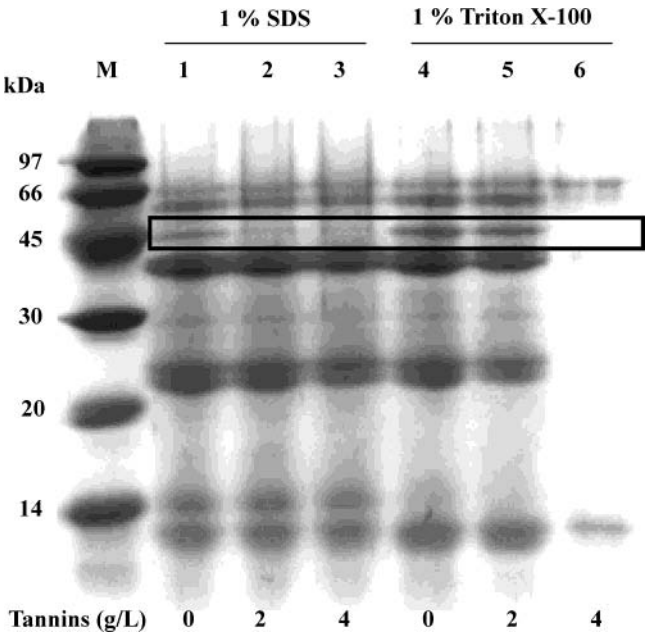

Figure 1. Comparison of the band intensity of grape seed proteins at 1% SDS or 1% Triton X-100 with different levels of tannins: (lanes 1–3) with 1% SDS at tannin levels of 0, 2, and 4 g/L; (lanes 4–6) with 1% Triton X-100 at tannin levels of 0, 2, and 4 g/L. Proteins (2 mg/mL) were incubated with 1% SDS and then reacted with 0, 2, or 4 g/L tannins for 30 min at 4 °C. After centrifugation at 3000*g*, the supernatant was collected for SDS-PAGE.

beverages such as beer, fruit juice, or wine (*33*). However, it is time-consuming (80 °C heating for 6 h, then 4 °C cooling for 12 h), and nonspecific reaction may occur due to the unselective nature of the heat-forced complex formation between tannins and unfolded hydrophobic amino acid residues.

The nature of haze-active proteins in fruit juices and wine has been studied. Some of their characteristics have also been described (*32*, *33*, *40*), but no clear identification has been made. Beer haze-active proteins are high in proline and originate from barley hordein (*27*). Grape seeds, rich in tannins, may contribute to haze formation in juices or wine if structurally loose or extended proteins are also present. Castriotta and Canella (*41*) reported the amino acid composition of total grape seed proteins (**Table 1**); this had a high percentage of glutamate (23%) and a fairly low level of proline (5%).

Table 1. Amino Acid Composition of Grape Seed Proteins and Isolated 45-kDa Protein (Grams per 100 g of Protein)

amino acid	45-kDa protein	total protein	total protein[a]
Lys	4.21	2.41	3.14
Met	3.52	1.25	1.40
Cys	1.74	0.91	(Met + Cys)
Phe	3.29	3.64	4.59
Tyr	2.87	2.90	2.27
Ile	4.19	4.72	4.98
Leu	8.63	7.33	8.16
Thr	5.34	3.56	4.09
Val	6.87	6.94	5.54
His	2.11	2.43	2.27
Arg	4.26	6.51	6.89
Gly	9.21	11.39	7.90
Ser	5.65	6.01	4.99
Ala	7.81	6.49	5.34
Asp	9.49	8.16	9.14
Glu	9.38	20.49	23.74
Pro	9.49	4.85	5.67

[a] Castriotta and Canella (*41*).

Grape seed proteins mixed with 1% SDS were combined with different amounts of tannins (0, 2, and 4 g/L). After separation by SDS-PAGE (**Figure 1**), the intensity of a protein band at a molecular weight of ~45 kDa was noticeably decreased by an increase in tannins. The results of image analysis (**Figure 1**) indicated that prior to the addition of tannins, the band intensity of a protein at 74 kDa was similar to that for the 45-kDa protein. However, after treatment with tannins, the band intensity of the 45-kDa protein was decreased to 55% at the 2 g/L tannin level and to 50% at the 4 g/L tannin level. Notable intensity changes for other major bands (62, 40, and 22 kDa) were not observed (**Figure 1**). Increasing SDS concentration (**Figure 2**) led to much less removal of the 45-kDa protein and slightly reduced removal of other proteins, so it reduced selectivity. It was of interest to specifically purify this particular protein for further characterization. The extracted protein was purified by gel

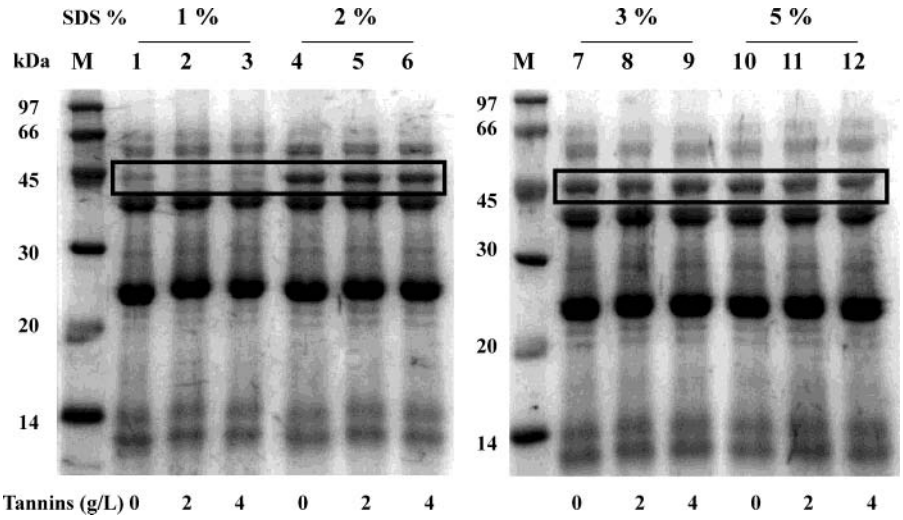

Figure 2. Comparison of band intensity of grape seed proteins with different amounts of SDS (1, 2, 3, and 5%) at various tannin concentrations (0, 2, and 4 g/L): (lane 1) 1% SDS + 0 g/L tannins; (lane 2) 1% SDS + 2 g/L tannins; (lane 3) 1% SDS + 4 g/L tannins; (lane 4) 2% SDS + 0 g/L tannins; (lane 5) 2% SDS + 2 g/L tannins; (lane 6) 2% SDS + 4 g/L tannins; (lane 7) 3% SDS + 0 g/L tannins; (lane 8) 3% SDS + 2 g/L tannins; (lane 9) 3% SDS + 4 g/L tannins; (lane 10) 5% SDS + 0 g/L tannins; (lane 11) 5% SDS + 2 g/L tannins; (lane 12) 5% SDS + 4 g/L tannins. Proteins (2 mg/mL) were incubated with 1% SDS and then reacted with 0, 2, or 4 g/L tannins for 30 min at 4 °C. After centrifugation at 3000*g*, the supernatant was collected for SDS-PAGE.

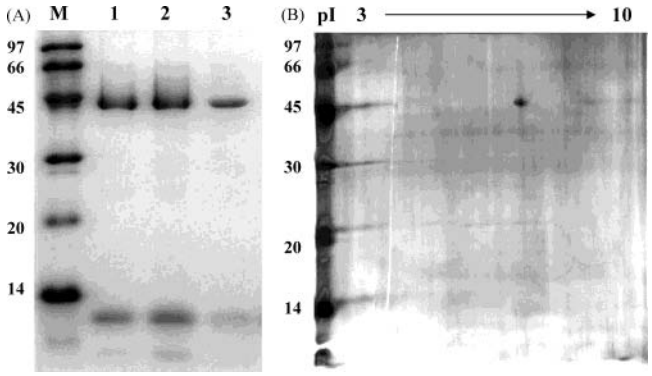

Figure 3. Isolation of the 45-kDa protein (**A**) and its p*I* value from two-dimensional electrophoresis (**B**); (lanes 1 and 2) CM ion exchange chromatography; (lane 3) after gel filtration chromatography. M, protein marker.

filtration and ion exchange chromatography (**Figure 3A**); its p*I* value of 7.2 was determined by two-dimensional electrophoresis (**Figure 3B**). Amino acid analysis indicated that this 45-kDa protein had a higher proportion of proline residues (9.49%) than the proline content (4.85%) in total protein (**Table 1**). It has been demonstrated that basic and aromatic amino acids were also involved in polyphenol binding (*18*, *22*). The contents of basic and aromatic amino acids in the 45-kDa protein were 10.58 and 6.16%, respectively.

A globular protein (BSA) and a proline-rich protein (gelatin) were chosen to verify the new method. Different tannin levels (4, 5, 6, and 7 g/L) were mixed with BSA or gelatin with or without 1% SDS (**Figure 4**). The band intensity of BSA under investigation was not markedly affected by an increase of tannins at the 1% SDS level; however, the BSA disappeared at higher levels of tannins without SDS (**Figure 4A**). In contrast, the band intensity of gelatin decreased with an increase in tannins both with and without 1% SDS (**Figure 4B**). This pattern shown on the gel might be due to the protein structure and its amino acid composition. Most of the prolines (the major polyphenol binding sites) in BSA (5%) were in the interior of a compact, globular protein and thus sterically impeded from participating in specific interactions with polyphenols. The tannin/BSA association probably involved surface-exposed aromatic residues (*3*, *23*). While mixing with SDS, BSA was strongly bound and denatured by the detergent, leading to the unavailability of polyphenol binding sites for the subsequently added tannins.

Gelatin contains a high proportion of hydroxyproline (13.3%) and proline (15.5%) and has long been suggested for use as a clarification agent in the wine industry. The extended protein exposed binding sites to tannins at both natural and denatured forms. SDS shielded the binding sites on gelatin, causing the impediment of interaction. However, as shown in **Figure 4B**, with an increase of tannins, the gradually reduced band intensities were observed. It was likely that the remaining polyphenol binding sites on gelatin had more opportunities to interact and form complexes.

SDS in this study not only facilitated protein dissolution but also played an important role in distinguishing haze-active proteins by modulating the interactions between polyphenol binding sites and tannins. Without the interaction with SDS, the band intensities of grape seed proteins were all nonselectively reduced at higher tannin levels (data not shown). Once the anionic detergent was added, haze-active protein was easily identified.

A nonionic surfactant, Triton X-100, is usually used to refold protein following SDS-PAGE and has also been used to displace

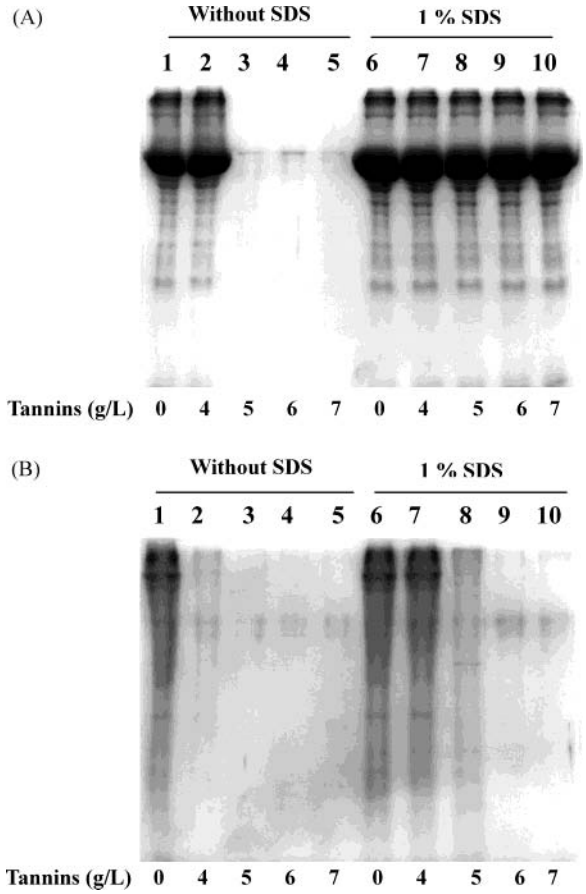

Figure 4. Comparison of the band intensity of (**A**) bovine serum albumin and (**B**) gelatin with different levels of tannins and SDS content: (lanes 1–5) without SDS; (lanes 6–10) with 1% SDSl; (lane 1) protein + 0 g/L tannins; (lane 2) protein + 4 g/L tannins; (lane 3) protein + 5 g/L tannins; (lane 4) protein + 6 g/L tannins; (lane 5) protein + 7 g/L tannins; (lane 6) protein + 0 g/L tannins; (lane 7) protein + 4 g/L tannins; (lane 8) protein + 5 g/L tannins; (lane 9) protein + 6 g/L tannins; (lane 10) protein + 7 g/L tannins. Proteins (4 mg/mL) were incubated with or without 1% SDS and then reacted with 0, 4, 5, 6, or 7 g/L tannins for 30 min at 4 °C. After centrifugation at 3000*g*, the supernatant was collected for SDS-PAGE.

protein bound SDS by forming a stable complex with protein (*42*). This detergent, however, exhibited a lower effect on the reduction of band intensities of gelatin and BSA with the increase of tannins (**Figure 5**). This could be attributed to the folding effect of the surfactant. As the concentration of Triton X-100 increases, the interaction of proteins−polyphenols may be hampered.

In summary, we have demonstrated the feasibility of a new method for the identification of proteins involved in haze formation, which is an important issue in the beverage industry. The proposed method is simple, fast, and able to selectively identify proteins with a high tendency to react with tannins. It is based on the hydrophobic interaction between structurally extended or loose proteins and the aromatic rings of the polyphenols. Proteins with higher proline, basic, and aromatic amino acid residues were found to react strongly with tannin and formed complexes. Because grape seed proteins were denatured and exposed polyphenol binding sites to tannins by 1% SDS, it was then possible to differentiate haze-active proteins. A specific haze-active protein with a molecular weight of 45 kDa and a p*I* value of 7.2 was isolated. Amino acid analysis indicated that this isolated protein was high in proline. The application of such a method for the identification of haze-

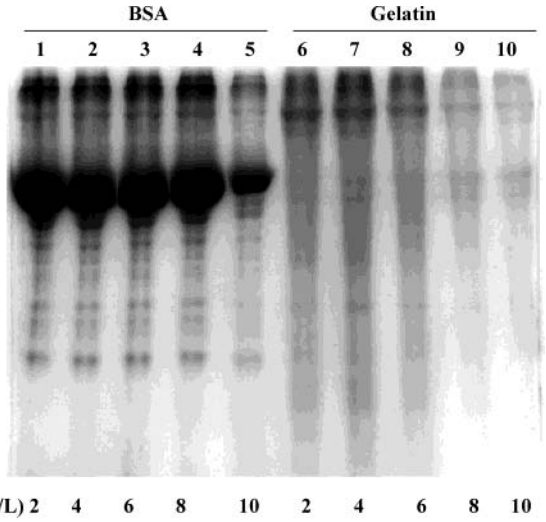

Figure 5. Comparison of the band intensity of bovine serum albumin and gelatin at 1% Triton X-100 with several levels of tannins: (lanes 1–5) BSA; (lanes 6–10) gelatin; (lanes 1 and 6) 2 g/L tannins; (lanes 2 and 7) 4 g/L tannins; (lanes 3 and 8) 6 g/L tannins; (lanes 4 and 9) 8 g/L tannins; (lanes 5 and 10) 10 g/L tannins. The proteins (4 mg/mL) were incubated with 1% Triton X-100 and then reacted with 2, 4, 6, 8, or 10 g/L tannins for 30 min at 4 °C. After centrifugation at 3000*g*, the supernatant was collected for SDS-PAGE.

active protein could open new opportunities to distinguish haze-active proteins and also allow the design of an immunoassay for the detection of haze-active protein in beverages. The precise amount of adsorbents used to stabilize beverages and prolong their shelf life can also be tuned using this method by avoiding the removal of flavor compounds. The sensory quality of beverages can therefore be better controlled to meet the expectation of consumers.

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