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Characterization of Haze-Active Proteins in Apple Juice

LI-CHEN WU† AND KARL J. SIEBERT*

Department of Food Science and Technology, Cornell University, Geneva, New York 14456

The nature of the haze-active protein (HAP) in apple juice was investigated. Heat treatment removed protein indiscriminately while polyvinylpolypyrrolidone (PVPP) treatment was fairly specific for proteins of 15 and 28 kDa. Presumably, the PVPP bound to polyphenols, which in turn were complexed with protein. Three candidate apple HAPs were isolated. Two were extracted from juice with acetone and fractionated by hydrophobic interaction chromatography and solid phase extraction with C18 (HAP I) or SAX (HAP II) material. Hydroxyproline-rich protein was extracted from apple tissue (HAP III). The order of haze formation with tannic acid was gliadin > HAP III > HAP II > HAP I > bovine serum albumin, which shows increasing haze formation with increasing proline content. The sizes of HAP I, II, and III were 28, 15, and 12 kDa; the first two corresponded to the sizes of proteins removed by PVPP treatment and are involved in juice haze formation.

KEYWORDS: Beverage; heat treatment; PVPP; turbidity; proline-rich protein

INTRODUCTION

Protein−polyphenol interaction is the most frequent cause of haze formation in beer, wine, and clear fruit juices (*1*−*3*). There is little information in the literature regarding the apple juice proteins that are involved in haze formation. Some efforts have been made to characterize haze-active (HA) proteins in apple juice by comparing the juice stability, protein content, and protein band patterns after treatment with adsorbents or fining agents, such as bentonite, silica sol, or gelatin (*4*), or after oxidation (*5*). These studies concluded that proteins that contribute to instability and haze formation in apple juice prepared from Granny Smith apples were in the 21−31 kDa range while those from McIntosh, Golden Delicious, and Jonagold had a molecular size of 32 kDa.

A forcing method in which samples are heated at 80 °C for 6 h and then held at 4 °C for 16 h to develop a haze (*6*) has been widely used for testing protein instability in wine. In one study, the grape proteins involved in heat-induced haze were characterized as low in molecular mass (12.6 and 20−30 kDa) and low in pI (4.1−5.8) (*7*). In another, two protein bands in Muscat of Alexandria wine, of 24 and 32 kDa, were associated with heat haze formation (*8*). The same forcing method was applied to apple juice, and the results indicated that proteins of less than 30 kDa were involved in haze formation (*4*).

The effect of heat on turbidity in a model system was previously investigated (*9*). In that case, turbidity increased when a protein−polyphenol solution was heated at 80 °C for 30 min. It was suggested that the increase in turbidity might be due to either a conformational change in the protein, exposing more sites to which polyphenols can bind, or heat-induced changes in the polyphenols, such as epimerization, or both.

Although bentonite, a nonspecific protein adsorbent, is commonly used in fruit juice stabilization, it binds nonselectively (*10*). In beer, for example, bentonite removes both foam- and haze-active proteins (HAP). Consequently, if apple juice aliquots were treated with increasing amounts of bentonite and then subjected to SDS−PAGE, there would be no assurance that a decrease in the intensity of a protein band would be indicative of a HAP.

Silica gel has been shown to specifically attach to the polyphenol binding sites of HAPs (*11*). However, because apple juice is rich in polyphenols (*12*), most of the binding sites, particularly in most HAPs, would be occupied by polyphenols and thus unavailable to attach to silica gel (*10*). This explains the observed limited effectiveness of silica gel in removing HA protein from apple juice.

Polyvinylpolypyrrolidone (PVPP) has long been used in the beverage industry as a polyphenol adsorbent (*10*, *13*). While it is known to be effective and fairly specific for polyphenols (*11*), it has also been shown to remove some HA protein from apple juice (*10*). This is thought to result from removal of soluble or insoluble protein−polyphenol complexes from the juice. Presumably, the PVPP binds to polyphenol that is in turn attached to protein. Because it is the ability of a protein to bind to polyphenols that makes it HA (*9*, *14*), this fraction is of considerable interest in studies of haze formation. The use of PVPP to study HAPs in apple juice was previously attempted, by comparing high-performance liquid chromatography (HPLC) patterns (using 278 nm detection) before and after PVPP treatment (*15*). While diminution in size of a few peaks was seen, the changes were generally small. It was concluded that either the HAPs in apple juice comprise only a small percentage of the total protein or they may not be detected well at 278 nm.

* To whom correspondence should be addressed. Tel.: 315 787-2299. Fax: 315 787-2284. E-mail: kjs3@cornell.edu.
† Current address: Department of Applied Chemistry, National Chi-Nan University, 1 University Road, Puli, Nantou, 545 Taiwan.

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Proteins involved in tannin−protein complexes have been shown to be proline-rich and/or quite hydrophobic. Butler (*14*) reported that there was a significant difference between proteins high in proline, such as gelatin and salivary proline-rich proteins, and proteins lower in proline, such as bovine serum albumin (BSA) and lysozyme, in their relative affinity for sorghum tannin. Proteins purified from sorghum tannins were quite hydrophobic and bound noncovalently to tannins (*16*). Proline-rich proteins have been widely reported as HAPs (*3*, *17*); proline residues in a protein appear not only to serve as binding sites for polyphenols but also to keep the peptide extended and maximize the available binding surface (*18*). Hydroxyproline-rich proteins are also possibly involved in haze formation, although polyhydroxyproline did not form complexes with catechin or tannic acid (*9*). Hydroxyproline-rich proteins were found to be involved in plant cell wall lignification (*19*) by providing sites for selective complexation with phenolic precursors (*20*). Hydroxyproline residues in a protein likely also promote a loose configuration that favors accessibility to binding sites.

Polyphenol binding to protein is clearly not covalent and appears to be mainly hydrophobic in nature, possibly with some contribution from hydrogen bonding (*3*, *20*−*22*). The interaction of polyphenols with proline-rich proteins was inhibited if dioxane (*9*, *23*) or dimethylformamide was added (*9*, *23*) and enhanced by salt (*9*).

Isolation of apple HAPs has proven difficult because they are low in concentration and associated with tannins. The isolation of tannin-associated proteins in sorghum was achieved by liquid−liquid separation (*16*). An alternative approach is sorbent extraction (*24*). This method shortened the analysis time by approximately 50% and reduced to one-tenth the volume of sample required for the analysis (*25*). This technique has been applied in isolation of polyphenolic compounds from wine (*26*), various foods (*27*), and grapes (*28*).

The objectives of this research were first to study the HAPs in apple juice by applying heat and PVPP treatments. Then, candidate apple HAPs; apple salt-extractable, hydroxyproline-rich proteins; and apple juice hydrophobic proteins were to be isolated and characterized, and their haze-forming activities were tested.

MATERIALS AND METHODS

Preparation of Juices. Fuji, Jonagold, Cortland, and Mutsu apples were harvested in the 1998 season from the New York State Agricultural Experiment Station Orchard at Geneva. Apples were stored at 0 °C and used in juice production within 2−3 months of harvest. Juice was prepared from each single cultivar. Apples were chopped, and juice was expressed using a hydraulic press (Loomis Engineering & Manufacturing Co., Caldwell, NJ). The juice was clarified by centrifugation (20 min at 12 200*g*) at 4 °C and then filtered through glass fiber filter circles (Fisher, Fairlawn, NJ) under vacuum. The juice was then filtered through Whatman 2V, followed by Whatman No. 5 filter paper (*15*).

Tannic Acid Induction of Haze. Hazes were produced by adding 0, 1.25, or 2.5 g/L tannic acid (Mallinckrodt Chemical) to juices of each apple cultivar (*15*). The samples were incubated for 30 min in a water bath at 25 °C. The haze intensity was then measured by turbidimetry.

Assessment of Total Polyphenol Content of Juice. Total phenols were determined with the Folin−Ciocalteu assay (*29*). Juices were centrifuged at 10 000*g* for 10 min at 4 °C before analysis (*30*). Centrifuged juice (0.25 mL) was mixed with 15 mL of distilled water and 1.25 mL of Folin−Ciocalteu reagent (Sigma, St. Louis, MO). A reagent blank was prepared with distilled water. The assay mixtures were allowed to stand for at least 5 min, and 3.75 mL of 20% Na_2CO_3 solution was then added. The solutions were brought to volume (25 mL) with distilled water and mixed thoroughly. After incubation in a water bath at 75 °C for 2 h, the absorbance at 765 nm was measured using a Hitachi U-2000 Spectrophotometer (Hitachi, Tokyo, Japan). The total phenol content of apple juice was calculated from a calibration curve prepared using gallic acid (Sigma).

Assessment of Total Protein Content of Juice. Total protein content was measured as previously described (*5*). Filtered apple juice (1 mL) was concentrated to dryness with a centrifugal ultrafiltration device (5000 molecular weight cutoff (MWCO) membrane, Millipore Corp., Bedford, MA) at 2000*g* and 4 °C. The retentate was dissolved in 0.2 mL of 0.1 N NaOH, and the protein content of the juice was determined by Coomassie blue dye binding (*31*). Resuspended samples (10 μL) were mixed with 200 μL of 5-fold diluted Coomassie blue dye concentrate (Bio-Rad, Hercules, CA). The mixture was allowed to stand for 15 min at room temperature, and then, its absorbance at 595 nm was measured. The total protein content of the juice was calculated from a calibration curve prepared with BSA.

Heat Treatment. The pH of portions of the juice was adjusted with 2 N HCl or 2 N NaOH to achieve pH values of 3.0 and 5.0 at 25 °C. The control and adjusted pH juices were held at 80 °C for 0.5, 2, 4, or 6 h and then cooled at 4 °C for 16 h. The haze formed was measured by turbidimetry.

PVPP Treatment. Prewashed PVPP (*15*) was added to freshly made apple juice at rates of 0, 1, 3, or 5 g/L. The mixtures were stirred for 30 min at room temperature and then filtered through Whatman 2V filter paper.

Turbidimetry. Haze determinations were made with a Hach model 2100AN ratio turbidimeter (Hach Company, Loveland, CO) using 24 mm diameter cuvettes. The instrument was calibrated with formazin standards every 3 months and checked daily with Hach GELEX Secondary Standards. Results were expressed in nephelos turbidity units (NTU).

Isolation of Potential HAPs from Apple Juice. All procedures were performed at 4 °C unless otherwise stated. Cold (−26 °C) acetone (Fisher, Fair Lawn, NJ) was added to freshly made Jonagold apple juice to achieve a final concentration of 80%. The mixture was centrifuged at 15 000*g* for 15 min. The supernatant was collected and concentrated by evaporation in the hood overnight. The concentrated samples were then dialyzed against 0.05 M sodium phosphate buffer (pH 7.5) with 10 kDa MWCO tubing for 24 h at 4 °C. After four changes of the dialysis buffer, samples were concentrated with Aquacide II (Calbiochem, La Jolla, CA). Proteins in the concentrated solution were then loaded onto a 1.5 cm × 30 cm Phenyl Sepharose CL-4B column (Sigma) equilibrated with 1.7 M $(NH_4)_2SO_4$ in 0.01 M sodium phosphate buffer (pH 6.5). Proteins were eluted with a linear gradient decreasing in salt concentration from 1.7 M $(NH_4)_2SO_4$ to distilled, deionized water. The flow rate was 0.5 mL/min, and 5 mL fractions were collected. The absorbances at 220 (A_{220}) and 280 nm (A_{280}) were measured with a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan). Fractions with significant absorbance were examined further.

Solid Phase Extraction. Absorbing fractions that eluted from the Phenyl Sepharose CL-4B column were combined and loaded onto nonpolar adsorbent, C18 (Varian, Harbor City, CA; 2 g sorbent in 12 mL tube), or strong anion exchange adsorbent, SAX (Varian; 2 g sorbent in 12 mL tube) cartridges, to remove polyphenols. Cartridges were preconditioned with methanol and water as described by the manufacturer. Proteins were eluted from the cartridges with water, collected, and concentrated by freeze-drying (Virtis, Gardiner, NY).

Isolation of Hydroxyproline-Rich Proteins from Apples. Salt-extractable, hydroxyproline-rich proteins were isolated by the method of Kleis-San Francisco and Tierney (*32*). Jonagold apples (500 g) were blended with 0.05 M potassium phosphate buffer (pH 5.0), 10% (w/v) poly(vinylpyrrolidone) (Sigma), 5 mM dithioerythritol (Sigma), 0.5% (w/v) ascorbic acid (Fisher), and 4 mM $Na_2S_2O_5$ (Aldrich, Milwaukee, WI) in a blender (Waring, New Hartford, CT). After centrifugation at 5000*g* for 10 min, the pellet was collected and extracted with 500 mL of 0.5% Igepal (Sigma) and 2 mM $Na_2S_2O_5$ for 1 h. The solution was centrifuged at 5000*g* for 15 min, and the pellet was then mixed with 2 mM $Na_2S_2O_5$, 0.5% (w/v) ascorbic acid, and 0.1% (v/v) β-mercaptoethanol to remove the nonionic detergent, Igepal. After three washes,

the pellet was extracted with 200 mM $CaCl_2$ (Fisher) and 4 mM $Na_2S_2O_5$ overnight at 4 °C. The extract was centrifuged at 2000*g* for 15 min. Trichloroacetic acid (TCA) (Sigma) was added to the supernatant to achieve a final concentration of 10%. The mixture was held at 0 °C overnight. The mixture was then centrifuged at 24 000*g* for 15 min, and the supernatant was dialyzed using 10 kDa MWCO tubing (Pierce, Rockford, IL). Then, samples were concentrated with a 10 kDa MWCO microconcentrator (Millipore).

Amino Acid Analysis. Amino acid composition analysis was carried out by the Cornell Biotechnology Resource Center. Proteins were hydrolyzed with 6 N HCl. The amino acids were derivatized with phenyl isothiocyanate, and the resulting phenylthiocarbamyl amino acids were separated by reversed-phase HPLC.

Protein Assays. Protein concentrations were measured by Coomassie Brilliant Blue (CBB) dye binding (*31*) as described for apple juice above, from 280 nm absorbance by calculation of molar absorptivity from amino acid composition (*33*) and by the bicinchoninic acid (BCA) protein assay (*34*).

Protein molar absorptivity at 280 nm, ϵ_M, was calculated from the results of amino acid analysis (*33*):

$$\epsilon_M = 5690n_W + 1289n_Y + 120n_C$$

where n_W, n_Y, and n_C are the numbers of moles of tryptophan, tyrosine, and cysteine in a protein, respectively. The protein concentration was then estimated from the 280 nm absorbance, A_{280}:

$$\text{protein concentration (mol/L)} = A_{280}/\epsilon_M$$

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). The filtered juice (20 mL) was freeze-dried and then resuspended with 2 mL of 1% SDS (*35*). The resuspended samples (10× original concentration) and broad range molecular weight standards (Bio-Rad) were applied to SDS−PAGE (*36*), using a precast Tris-HCl 4−20% acrylamide graduated gel (Bio-Rad). The protein bands in the gel were detected by silver staining (Bio-Rad).

SDS−PAGE of the candidate HAP isolates was carried out with Tris-HCl Ready Gels (Bio-Rad) with either 12% resolving gel with 2.6% cross-linker or a 4−20% gradient of resolving gel with 2.6% cross-linker, depending on the size of the protein. Low, high, or broad range standards (Bio-Rad) were used as appropriate. The staining procedure was as described for the juice.

Haze-Forming Activity Test. Haze-forming activity was measured according to Siebert et al. (*9*). Aqueous solutions of proteins were mixed with 150 mg/L tannic acid (Mallinckrodt, St. Louis, MO) in 0.02 M (pH 4.2) sodium phosphate buffer to achieve final protein concentrations of 0, 20, 40, 60, 80, or 100 mg/L. A blank for each concentration was prepared by the addition of water rather than protein solution. The mixtures were allowed to stand at 25 °C for 30 min. The haze that formed was measured by turbidimetry. Results were subjected to linear regression analysis.

RESULTS AND DISCUSSION

Induction of Haze Formation in Apple Juice. Protein−polyphenol interaction is a major factor in haze formation in beverages (*12*). In apple juice, the concentration of HA proteins is quite low and the polyphenol content is large relative to the amount of protein. The HA protein contents of juices made from different apple cultivars have been shown to vary (*37*). Juices from four apple cultivars (Fuji, Jonagold, Cortland, and Mutsu) were prepared and compared (**Table 1**). The juice made from Jonagold apples produced the greatest increase in haze upon addition of tannic acid, indicating that it was richer in HAP than the other varieties examined. Consequently, this cultivar was selected for further work.

Heat Treatment. The natural pH of the Jonagold apple juice was 3.5, and portions of the juice were adjusted to pH 3 and pH 5. **Figure 1** shows the effects of heat on turbidity development in the juice at the three pH values. The turbidity increased with longer heating times. The highest turbidity was observed at pH 3.0, and the pattern was very similar to the result with the pH 3.5 juice. The apple juice with the highest pH showed a much smaller increase in turbidity. These results appear somewhat different from those reported in a gliadin−tannic acid model system (*12*). In that case, the greatest amount of haze formation was observed near pH 4.2. At both lower pH (near 3) and higher pH (around 4.5), haze formation was considerably less. The apple juice HAP could behave differently than gliadin.

Table 1. Haze (NTU) Developed upon Addition of Tannin Acid to Clarified Apple Juice at 25 °C

	tannic acid added (g/L)[a]		
cultivar	0	1.25	2.50
Fuji	38.6 ± 1.3	43.3 ± 0.6 (+12%)	45.4 ± 1.4 (+18%)
Jonagold	40.7 ± 1.3	47.6 ± 0.7 (+17%)	49.9 ± 1.1 (+23%)
Cortland	36.7 ± 1.1	40.8 ± 0.6 (+11%)	44.3 ± 1.4 (+21%)
Mutsu	39.6 ± 1.3	43.2 ± 1.2 (+9%)	46.8 ± 0.4 (+18%)

[a] Mean of three analyses ± SD (% change from untreated juice).

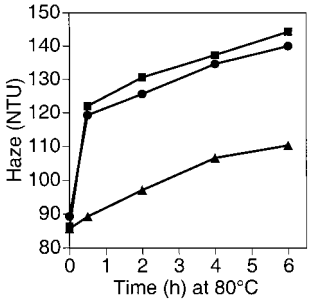

Figure 1. Haze formation in Jonagold apple juice at pH 3.0 (■), 3.5 (●), and 5.0 (▲) subjected to heat treatments (80 °C for the indicated time followed by 16 h at 4 °C).

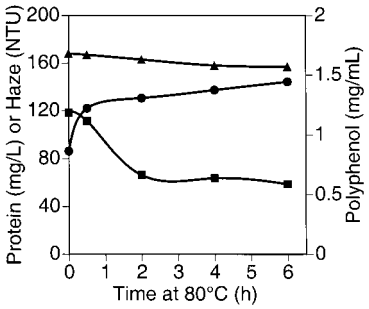

Figure 2. Profiles of total protein (■), total polyphenol (▲), and haze (●) in pH 3.0 Jonagold apple juice held at 80 °C for the indicated time followed by 16 h at 4 °C.

The levels of total protein and total polyphenol remaining in solution after heat treatment and haze removal were measured (**Figures 2−4**). Although the haze intensities observed were quite different, the quantities of total protein and total polyphenol measured at each time point were similar at the three pH values. The total protein content at each pH studied was reduced by slightly more than 50% (**Figures 2−4**) by the longest heat treatment. This finding was similar to results from heat-treated wine, where over 60% of the Voit-N content remained in solution (*39*). Another study reported that about 50% of wine proteins were removed by this process (*7*). In contrast, heat treatment at 80 °C for 6 h reportedly coagulated and removed all of the soluble protein in tested Australian wines (*6*).

Heat treatment reportedly denatures wine proteins, which can cause non-HAPs to become HA by opening their structure and

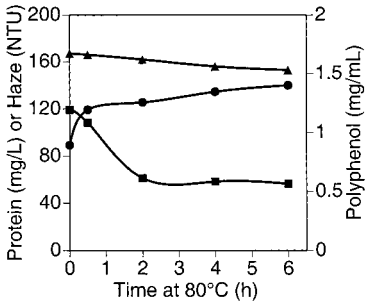

Figure 3. Profiles of total protein (■), total polyphenol (▲), and haze (●) in pH 3.5 Jonagold apple juice held at 80 °C for the indicated time followed by 16 h at 4 °C.

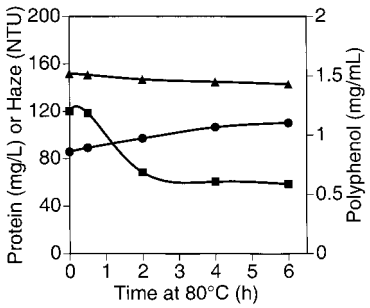

Figure 4. Profiles of total protein (■), total polyphenol (▲), and haze (●) in pH 5.0 Jonagold apple juice held at 80 °C for the indicated time followed by 16 h at 4 °C.

exposing polyphenol binding sites (*39*), or may cause the destruction of heat sensitive proteins (*7*). Because heat treatment removes proteins indiscriminately, it is not an appropriate approach for the study of juice HAPs.

There is a possible danger in using CBB dye binding in a study of HAP. Coomassie blue is highly biased toward the basic and aromatic amino acids, particularly arginine, and gives little, if any, response to peptides that lack these (*40*). It has been shown that CBB produces little response to the grain HAPs (gliadin and hordein) because they are poor in the basic and aromatic amino acids (*3*). Hii and Herwig reported that Coomassie blue only provided one-seventh the average response to beer protein as to the BSA typically used for calibration (*41*). Siebert and Knudson reasoned that this occurred because the major protein in beer (also the HAP) is very poor in the basic and aromatic amino acids (*42*). Wine HAPs have been reported to be 50−80% underrepresented by CBB (*24*). While the nature of the apple HAPs is as yet unclear, it is certainly possible that they too may be underestimated by CBB dye binding.

PVPP Treatment. Apple juice was treated with PVPP at levels ranging from 0 to 5 g/L. The higher end of this range is an excessive treatment level, well beyond the range normally employed commercially (*10*). Reported dosages for stabilization were 0.5 g/L for beer (*43*) and 1.08 g/L for grape juices (*44*). PVPP treatment removed a significant amount of total polyphenol from the juice (**Figure 5**), reaching 64.3% reduction at the highest treatment level (5 g/L). A similar result was reported in a study where the same PVPP dosage rate removed virtually all of the HA polyphenol from apple juice (*10*). In contrast, the total protein content decreased only about 18% with 5 g/L of PVPP (**Figure 5**). This decrease is presumably due to the removal of HA protein complexed with HA polyphenols (*10*).

Subjecting apple juice to SDS−PAGE revealed major protein bands at 15, 28, and 32 kDa. When aliquots of the juice were treated with different levels of PVPP, the intensities of the bands at 15 and 28 kDa were reduced with increased treatment with

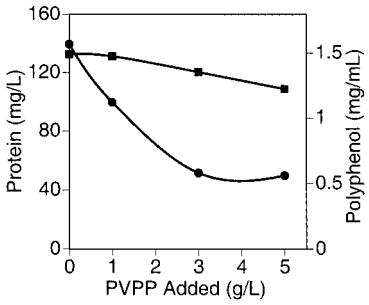

Figure 5. Total protein (■) and total polyphenol (●) contents of Jonagold apple juice after treatment with the indicated amounts of PVPP.

PVPP, suggesting that these two proteins may be HA. The band at 32 kDa was not significantly reduced, indicating that this protein is not as sensitive to polyphenol binding as those of 15 and 28 kDa. There may be some other HAPs present at low concentration in the juice that were not detected.

Isolation of Potential Apple HAPs. The main considerations in seeking HAPs are to isolate proteins that bind readily to polyphenols and to separate them from polysaccharides and polyphenols. It is possible that nonHAPs may share similar molecular weights with HAPs. It is not practical to isolate all of the proteins in apples and then to compare their haze-forming activities or amino acid compositions. Consequently, a selective method to isolate potential HAPs was used. Sorghum tannin-associated proteins isolated in a previous study were characterized as either rich in proline content or high in hydrophobicity (*16*). In another study, Wilson successfully extracted phenolic compounds from apple juice with acetone (*45*). It is very likely that tannin-associated proteins were also simultaneously extracted.

Solid phase extraction with C18 and SAX cartridges has previously been used to separate polyphenols from proteins (*46*). C18 sorbents are designed for retaining nonpolar compounds (*46*) and have been used in isolating wine heat-unstable proteins (*8*) and separating polyphenols (*25*, *47*). However, C18 is generally regarded as a rather nonselective sorbent; it retains almost any organic molecule from aqueous matrixes (*46*). As a result, some tannin-associated proteins in apple juice could be retained by the cartridge due to the interaction between tannic acid and C18 sorbent. SAX, a quaternary amine anion-exchange sorbent, was chosen as another approach to separate polyphenols and polyphenol-associated proteins. The SAX sorbent is positively charged at neutral pH, and anions are retained by the exchanger. Because phenolic acids (hydroxybenzoic acid types) are ionized at pH 7 (*48*), it is feasible to use an anion-exchange sorbent to retain them and elute tannin-associated proteins with water. Proteins containing amino acids with acidic side chains (aspartic and glutamic acids) would also bind to the anion exchange material. Condensed polyphenols, such as flavonoids, are eluted with the proteins since their pK_a values are in the range of 9−10 and consequently they are not ionized at the pH of juice.

The acetone precipitated juice fraction was subjected to hydrophobic interaction chromatography (HIC) (**Figure 6**). The HIC elution profile showed that the absorbance peaks at 280 and 220 nm occurred at low salt concentration, indicating that the corresponding compounds were hydrophobic (*49*). The peak height measured at 280 nm (due mainly to tryptophan and tyrosine) was lower than that at 220 nm (from the peptide bond). The fractions corresponding to the UV absorbing peak were pooled and split in two. One portion was subjected to sorbent extraction with C18, resulting in a potential HAP fraction; this

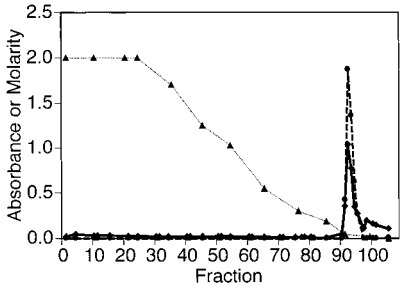

Figure 6. Elution profiles of acetone soluble apple juice proteins from phenyl–sepharose CL-4B. Ammonium sulfate, M (▲); absorbance at 220 nm (●); and absorbance at 280 nm (■).

Table 2. Amino Acid Composition of Apple Protein Isolates

	mole %		
amino acid	HAP I	HAP II	HAP III
Asx[b]	13.4	9.7	8.4
Glx[c]	ND[a]	11.4	10.4
Ser	19.7	8.4	10.0
Gly	23.6	13.8	10.0
His	ND	1.7	2.1
Arg	4.1	5.2	3.3
Thr	ND	5.7	8.3
Ala	6.7	10.2	8.7
Pro	1.8	4.2	5.0
Tyr	2.5	3.0	2.6
Val	1.3	5.9	4.5
Met	2.3	1.9	1.6
Cys	4.6	ND	2.7
Ile	3.4	4.3	3.0
Leu	13.7	6.9	5.5
Phe	ND	3.1	2.3
Trp	ND	ND	ND
Lys	3.1	4.0	4.5
Hyp	ND	0.7	7.5

[a] Not detected. [b] Asx = asparagine and aspartic acid. [c] Glx = glutamine and glutamic acid.

was designated HAP I. The other portion was isolated with the SAX sorbent and designated HAP II.

HAPs are typically proline-rich (*3*, *23*). Some plant cell wall proteins are also rich in proline; these are glycoproteins that are also hydroxyproline-rich (*50*). They are found in many plant tissues, including carrot tubers (*51*), sexual tissues of flowers (*52*), and tomato cell suspension cultures (*53*). It was of interest to investigate such proteins as possible HAPs in apple juice.

Proline/hydroxyproline-rich proteins are soluble in 10% TCA (*54*). Blended apple tissues were extracted with detergent and calcium chloride and followed by a precipitation with 10% TCA. The supernatant of the mixture was collected, dialyzed, and concentrated; it was designated potential HAP III.

Protein Assay and SDS−PAGE. SDS−PAGE of the three isolates revealed that the molecular masses of HAP I, II, and III were 28, 15, and 12 kDa, respectively. These proteins are similar in size to the 12 kDa and 20−30 kDa heat-unstable proteins of grapes (*7*) and the 21−31 kDa HAPs in Granny Smith apples (*4*). Even at high concentrations, these proteins did not stain well with Coomassie blue, so silver stain was used to visualize them.

Amino Acid Analysis. The possible HAP isolates were subjected to amino acid analysis (**Table 2**). They were low in aromatic amino acids, had moderate proline content (1.8−5.0 mol %), and were high in glycine (10.0−23.6 mol %) and serine (8.4−19.7 mol %). Similar results were seen in wine heat-unstable proteins, which were high in serine, glycine, and aspartic acid; proline content was not measured in this study (*8*). HAP II and III contained 10.4−11.4 mol % of glutamine/glutamic acid residues, but these were not detected in HAP I. The high content of serine may be indicative of oligosaccharide linkage points in these proteins. The high content of glycine may be indicative of random coil structures (*55*), which are more likely to react with polyphenols (*56*).

Table 3. Relative Responses of the Bradford (Coomassie Blue) Dye-Binding Assay, the BCA Assay, and the Calculated Concentration Based on 280 nm Absorbance and Amino Acid Analysis for the Apple Protein Isolates and BSA

	protein concn (g/L)		
	Bradford[a]	BCA[a]	amino acid composition[b]
HAP I	0.46 ± 0.03	0.84 ± 0.04	1.0
HAP II	0.13 ± 0.05	0.82 ± 0.06	1.0
HAP III	0.25 ± 0.04	0.77 ± 0.08	1.0
BSA	1.10 ± 0.03	1.15 ± 0.02	1.0

[a] Mean of three analyses ± SD. [b] The known molar absorptivity contributions of tyrosine, tryptophan, and cysteine and the measured 280 nm absorbance of the protein were used to calculate protein concentration from results of the amino acid analyses.

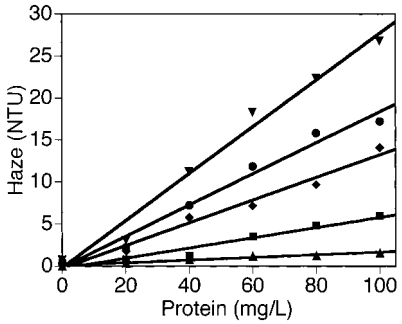

Figure 7. Hazes produced when various proteins were combined with 150 mg/L tannic acid in pH 4.2 sodium phosphate buffer and held for 30 min at 25 °C. HAP I (■), HAP II (♦), HAP III (●), BSA (▲), and gliadin (▼).

Protein Determinations. Several protein determination methods were applied to the isolates (**Table 3**). The Coomassie blue dye binding assay considerably underestimated all of the isolated proteins, indicating that it is unsuitable for quantification of apple HAPs just as it was unsuitable for beer and wine HAPs. The BCA protein assay appeared more suitable for the analysis of apple HAPs (**Table 3**). The BCA response to isolated proteins gave similar results to the estimates based on calculation from 280 nm absorbance and amino acid analysis results. These results agree with the statement that the BCA method is less biased against HAPs than Coomassie blue (*3*).

Haze-Forming Activity Test. The amount of haze formed when various amounts of each of the isolated proteins, gliadin, or BSA were combined with tannic acid is shown in **Figure 7**. The haze-forming activity rankings were gliadin > HAP III > HAP II > HAP I > BSA. For the HAPs and gliadin, this ranking was in the same order as the proline content of the protein (gliadin contains 15%). The BSA haze-forming response was lower than expected based on its proline content (4.6%).

Many reports have indicated that proline residues are the binding sites where polyphenols attach, and proline/hydroxyproline residues keep the protein structure extended, leading to the maximum exposure of binding sites to polyphenols (*3*, *20*). HAP II contained fewer proline and hydroxyproline residues than HAP III but more glycine and alanine residues. The

hydrophobic residues in HAP II, such as alanine, proline, valine, leucine, and phenylalanine may have formed a hydrophobic "pocket", which could sequester polyphenols (*57*); however, it appears that the content of proline was much more influential in polyphenol binding than general nonpolar character. This phenomenon was also seen in HAP I, where the proline content was even less, resulting in less haze formation.

The strongest haze-forming activity was demonstrated by HAP III, which had a total hydroxyproline plus proline content of 12.4 mol % (**Table 3**). In a previous study, the proline content of apple juice haze proteinaceous material was found to range from 4.6 to 16% and was high in Glu, Gly, and Ala (*58*). The fact that the HAP I and II molecular masses correspond to the sizes of the proteins reduced by PVPP treatment is significant and suggests that these play a role in haze formation in apple juice. HAP III was an even stronger haze former, but it is unclear the extent to which it is normally found in juice.

ABBREVIATIONS USED

BCA, bicinchoninic acid; CBB, Coomassie brilliant blue; HA, haze-active; HAP, haze-active protein; HPLC, high-performance liquid chromatography; MWCO, molecular weight cutoff; NTU, nephelos turbidity units; PVPP, polyvinylpolypyrrolidone; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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