Nature of Polyphenol–Protein Interactions

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Proteins and polyphenols were combined in model systems, and the resulting hazes were measured by light scattering. The amount of haze formed depends both on the concentrations of protein and polyphenol and on their ratio. A conceptual model in which a protein molecule has a fixed number of polyphenol binding sites explains the observed behavior and has implications for turbidimetric methods for estimating haze-active protein and haze-active polyphenol in beverages. The ranking of haze-forming activity of the test polypeptides was different with tannic acid than with catechin; this indicates differences in binding site availability, bridging ability, or specificity for the two polyphenols. More haze was observed when model systems were heated, suggesting that polyphenol binding sites are exposed when protein hydrogen bonds are broken. Freshly formed haze dissolved when dimethylformamide or dioxane was added; this may be useful for recovering compounds from isolated hazes for analysis.

Keywords: *Haze-active protein; haze-active polyphenol; beverages; model systems; gelatin; tannic acid*

INTRODUCTION

Proteins and polyphenolic compounds can combine to form soluble complexes; these can grow to colloidal size, at which they scatter light, and larger still, which can lead to sediment formation. Visible haze formation can limit the shelf life of products the consumer expects to be clear including beer (Gramshaw, 1970; Rudin, 1977; Whitear, 1974), wine (Heatherbell, 1976; Goertges, 1982), fruit juices (Heatherbell, 1976; Van Buren, 1972), and coffees and teas (Seshadri and Dhanaraj, 1988). The same phenomenon can contribute to the fouling of process equipment surfaces with deposits that are difficult to remove by in-place cleaning. Improved understanding of the proteins and polyphenols that form haze and of the nature of their interactions should lead to better measurement and stabilization procedures and possibly better cleaning methods.

At least initially, the protein–polyphenol complexes are held together by weak associations and haze can be dispelled by warming; in brewing this is commonly referred to as "reversible haze" or "chill haze" (Chapon, 1968). The mechanism appears to be a noncovalent interaction in which protein molecules are held together by polyphenolic compounds acting as bridges.

Not all proteins are equally involved in forming hazes, and some efforts to characterize the haze-active protein fraction in beer have been made. Asano and co-workers showed that the haze-forming proteins in beer are derived from the hordeins (barley prolamins) and are relatively rich in proline (Asano et al., 1982). In their model system, peptides that contained proline formed haze roughly in proportion to the mole percentage of proline in the peptide. Polypeptides that contained little or no proline produced little or no haze. These authors also showed that haze formation was inhibited by the presence of a hydrogen bond acceptor or a nonpolar solvent, but not salt, and concluded that the complexes are held together by some combination of hydrogen and/or hydrophobic bonding. Outtrup and co-workers also studied the involvement of proline-containing peptides in haze formation and found greater activity with higher proline contents (Outtrup et al., 1987).

Efforts have also been made to characterize the haze-active polyphenolic compounds in beer. Proanthocyanidins (dimers and trimers of catechin, epicatechin, and gallocatechin) were shown to be active in forming haze with peptides in model systems (Outtrup et al., 1987). The concentrations of the proanthocyanidin dimers of catechin and/or epicatechin were the beer phenolic compounds found to be most closely related to the rate of haze formation (McMurrough et al., 1992).

Some evidence for preferential involvement of particular protein and polyphenol fractions in fruit juice hazes has been reported. Stabilization of grape juice and wine required removal of 12 600–30 000 Da fractions of pI 4.1–5.8, at least some of which were glycoproteins (Hsu et al., 1987, 1989). Hydrolysates of protein from apple juice sediments contained from 5 to 16 mol % proline (Johnson et al., 1968); this is a much higher proline percentage than found in most proteins. Condensed tannins have been implicated in haze formation in apple juice (Wakayama and Lee, 1987; Johnson et al., 1968). It is instructive to study the general nature of protein–polyphenol interactions that lead to haze development in beverages.

MATERIALS AND METHODS

Chemicals. Proteins, homopolymeric amino acids, amino acid copolymers, and catechin were purchased from Sigma Chemical Co. (St. Louis, MO). Tannic acid was purchased from Baker (FCC 0380-04) or Mallinckrodt (1764). Gelatin (G-0510 calfskin, type IV, 60 bloom or bovine, type B, 75 bloom) was also obtained from Sigma. Dimethylformamide (DMF) and dioxane were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Stock Solutions. Sodium or potassium phosphate buffer, 0.02 M (pH 4.02 or pH 4.2), was prepared fresh daily in HPLC grade (deionized, distilled, and filtered) water.

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In several experiments gelatin was weighed and added to each test container as the dry powder. In the other cases gelatin was dissolved in hot HPLC grade water. Ethanol (20% of the final stock solution volume) was then added, and the solution was brought to final volume with HPLC grade water.

Tannic acid stock solution was prepared by dissolving tannic acid in ethanol (20% of the final stock solution volume) and making to volume with HPLC grade water.

Measurements. Light scattering measurements were carried out with one of two ratio turbidimeters. The early measurements were made with a Hach Model 18900 instrument (Hach Co., Loveland, CO) using 24 mm diameter cuvettes; this instrument has a measuring range of 0−200 Nephelos turbidity units (NTU). The remaining measurements were made with a Hach Model 2100AN ratio turbidimeter. This instrument measures over the range of 0−10 000 NTU. Two cuvette sizes (24 or 13 mm diameter) were used in different experiments.

Model Systems. In the model system, a peptide (gelatin, gliadin, lysozyme, papain, or a synthetic polypeptide) and a polyphenolic compound (catechin or tannic acid) were combined in 0.02 M [pH 4.2 (or in one experiment pH 4.02)] phosphate buffer in a beaker. The mixture was held in a water bath at 25, 80, or 100 °C for 30 min. The 80 and 100 °C treated samples were then placed in a 25 °C bath for attemperation before haze measurement. Haze determinations were carried out with a Hach ratio turbidimeter using either 13 or 24 mm diameter cuvettes. Smaller incubation volumes (typically 20 mL in a 50 mL beaker) and the 13 mm cuvettes were used for all experiments with synthetic peptides. Larger volumes (typically 100 mL in a 250 mL beaker) and the 24 mm cuvettes were used for experiments in which gelatin or gliadin was the only peptide.

Haze Dissolution Experiment. Amounts of gliadin and catechin calculated to achieve 500 and 750 mg/L, respectively, in the final mixture were combined in 0.02 M sodium phosphate buffer (pH 4.2). The samples were heated for 30 min at 80 °C and then cooled to 25 °C. One sample was a control. To three samples in each of four sets of samples were added, respectively, three different volumes (amounting to 5%, 10%, or 25% of the final volume) of test material (water, salt expressed as a 20% solution, DMF, or dioxane). The samples were mixed and held for 30 min at 25 °C, and the haze of each was measured.

RESULTS AND DISCUSSION

Nature of Protein−Polyphenol Complex Formation. Preliminary work in which peptides and polyphenols were combined in a model system of pH 4.02 potassium phosphate buffer suggested that the amount of haze formed was curvilinearly related to both protein and polyphenol concentration. This was studied in some detail by combining gelatin, a known haze-active protein, and tannic acid, a known haze-active polyphenol, in various proportions. The results are shown in Figure 1. Note that this experiment was conducted with the Model 18900 turbidimeter that has an upper measuring limit of 200 NTU. Some of the highest values were off-scale and could have been significantly higher than 200 NTU. As polyphenol concentration increased at a fixed level of protein, the observed haze at first rose, then reached a plateau, and in most cases declined. Similar behavior was seen at fixed polyphenol levels as the protein concentration increased. The concentrations of protein and polyphenol and their ratio all have strong effects on the amount of haze produced.

A model that could account for the results seen in Figure 1 is depicted in Figure 2. If each polyphenol molecule is viewed as having a fixed number of binding ends (shown here as two) and each protein is viewed as having a fixed number of polyphenol binding sites (shown here as three), then the situation in which the

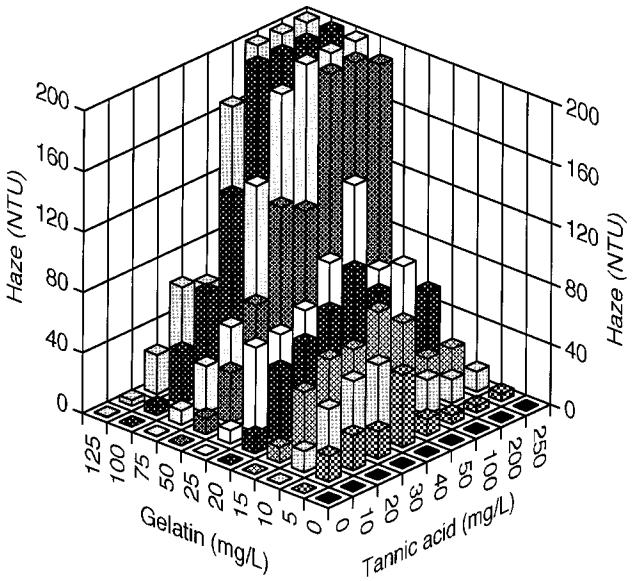

Figure 1. Haze produced (NTU) when various concentrations of gelatin and tannic acid in 0.02 M (pH 4.02) potassium phosphate buffer were held for 30 min at 25 °C. Hazes over 200 NTU were off-scale on the turbidimeter used for this experiment.

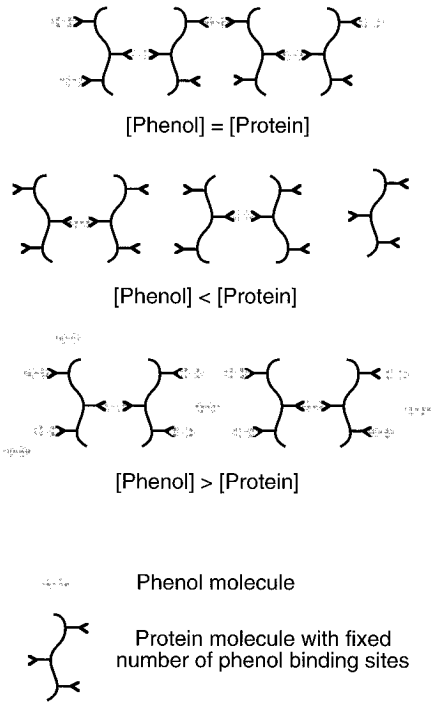

Figure 2. Model for protein−polyphenol interaction that explains the results observed in Figure 1. Polyphenols are depic-ted as having two ends that can bind to protein. Proteins are depicted as having a fixed number of polyphenol binding sites.

number of polyphenol ends equals the number of protein binding sites should produce the largest network, resulting in the largest particles and the greatest amount of light scattering. With a large excess of protein relative to polyphenol, each polyphenol molecule should be able to bridge between two protein molecules, but it would be unlikely that these proteins would be further bridged to others. This would result mainly in protein dimers, smaller aggregates, and less light scattering. With excess polyphenol relative to protein, all of the protein binding sites would be occupied, but the likelihood that bridging would occur would be low

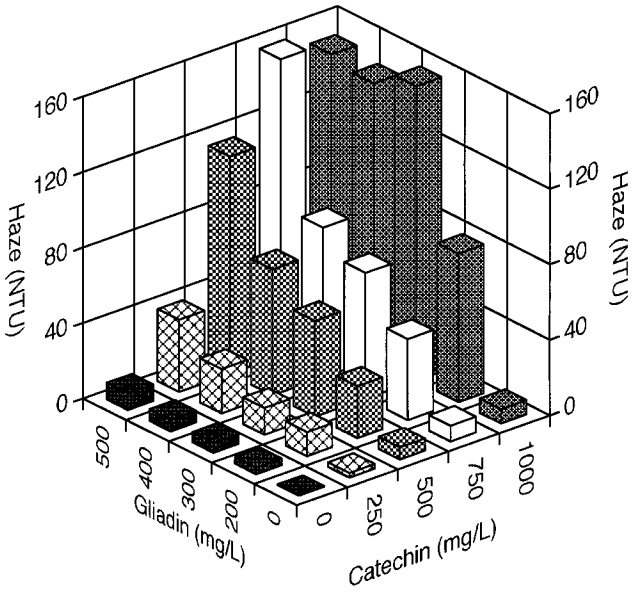

Figure 3. Haze produced (NTU) when various concentrations of gliadin and catechin were combined in 0.02 M (pH 4.2) potassium phosphate buffer and heated for 30 min at 80 °C.

because each free polyphenol end would have a small chance of finding a free binding site on a protein molecule. This, too, would result in small aggregates and less light scattering. Such a model would predict a ridge of increasing haze as the protein and polyphenol concentrations are simultaneously increased (along the diagonal), and this was seen in Figure 1. The model is also consistent with results of previous studies that found the ratio of tannic acid to gelatin measured in precipitates increased significantly, but not proportionately, as tannic acid increased (Van Buren and Robinson, 1969; Calderon et al., 1968). The amount of light scattered is a function of the particle size; a nonlinear response between scattering and particle concentration would result if the average particle size varies within a set of samples (Thorne, 1963). It is very likely that this occurs within the range of conditions shown here.

Gelatin and tannic acid are well-known for their strong activity in forming hazes (Van Buren, 1978; Bernstein, 1981; Heatherbell, 1984; Makkar et al., 1988); it is possible, however, that neither may be typical of the natural proteins or polyphenols found in beverages. A model system experiment was carried out with gliadin (a wheat prolamin rich in proline) and catechin (a polyphenolic compound naturally present in both beer and fruit juices), and this showed a pattern similar to that seen with gelatin and tannic acid (see Figure 3). A plateau was reached when either protein or polyphenol concentration increased while the other was held constant. The decline in haze at higher concentrations was not so evident as it was with gelatin and tannic acid. It may be that the solubility limits for gliadin and catechin make observation of this part of the relationship difficult.

Nature of the Polypeptides That Form Haze. The mole percent of proline in a protein or polypeptide was shown to be essentially linearly related with the ability of that protein to form haze with 400 mg/L catechin (Asano et al., 1982). There was, however, a substantial gap of proline content (between about 20% proline in barley hordein and 100% proline in polyproline) in the polypeptides for which results were reported. Gliadin, which contains approximately 15% proline, formed significantly more haze than expected from the relationship between proline content and haze-forming activity seen with the other peptides. Asano and coworkers also showed that a number of homopolymeric amino acids (polyglutamic acid, polylysine, and polyserine) formed no observable haze under their conditions. The results in Figure 1 indicate that measurements performed at a single point of polyphenol and protein concentration can give a misleading impression of the haze-forming activity elsewhere. Experiments with some of the materials used by Asano et al. were repeated using a range of protein concentrations rather than a single point and somewhat different conditions (600 rather than 400 mg/L catechin and 80 °C rather than 100 °C heating).

Asano and co-workers concluded that hydrogen and/or hydrophobic bonding is involved in the protein−polyphenol interaction. Hydroxyproline is very similar in structure to proline but would be expected to be more active in hydrogen bonding. Hydroxyproline is found in few natural proteins; however, gelatin, which has strong haze-forming activity, contains about 12−14% hydroxyproline and a similar amount of proline. The interaction of polyhydroxyproline with polyphenols was previously examined with somewhat contradictory results. Polyhydroxyproline was shown not to inhibit binding of proanthocyanidin to bovine serum albumin under conditions at which polyproline was strongly inhibitory (Hagerman and Butler, 1981). The authors interpreted this and other results as evidence of strong involvement of hydrogen bonding in protein−polyphenol interaction. When polyhydroxyproline was combined with tannins and light absorbance was measured to assess haze formation, however, absorbance increased almost as much as when polyproline was added (Oh et al., 1980). The levels of polypeptide and tannin used in this experiment were quite high, on the order of 1000 mg/L each, and may have exceeded solubility limits. Oh et al. also reported that increasing salt concentration and temperature resulted in greater haze formation and that detergent addition dissolved haze; they concluded that protein−polyphenol interactions are mainly due to hydrophobic bonding. It was therefore of interest to compare the haze-forming activities of polyhydroxyproline, polyproline, gelatin, and gliadin using light scattering and a range of peptide concentrations.

This comparison was carried out both with catechin at 80 °C and with tannic acid at 25 °C (Figure 4). In both cases polyhydroxyproline produced no haze at any concentration. Gelatin produced a peak of haze at low protein concentration with tannic acid but produced virtually no haze with catechin. Polyproline and gliadin produced more haze than the other polypeptides with both polyphenolic compounds. However, gliadin produced the most haze with tannic acid, while polyproline produced the most with catechin. There are two possible explanations for these differences. The first is simply that the differences between the polyphenolic compounds, such as the much larger size of tannic acid, its aromaticity, and its higher density of hydroxy groups compared with catechin, may be responsible. Alternatively, the different temperatures used (80 vs 25 °C) may have contributed either kinetically or possibly by breaking hydrogen bonds and unraveling the protein to a greater extent. Since hydrophobic groups tend to be concentrated in the interior of water-soluble proteins and one of the more likely mechanisms for the protein−polyphenol interaction is hydrophobic bonding, it is likely that more hydrophobic (polyphenol binding) sites

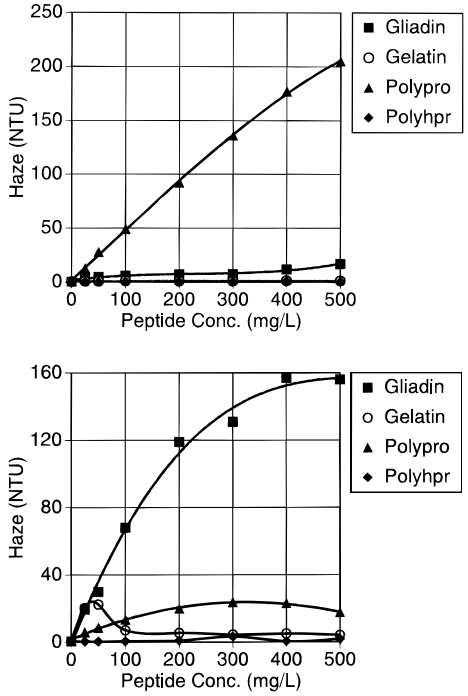

Figure 4. Hazes produced when various polypeptides were combined in 0.02 M (pH 4.2) sodium phosphate buffer (top) with 600 mg/L catechin and held for 30 min at 80 °C or (bottom) with 50 mg/L tannic acid and held for 30 min at 25 °C. Polypro, polyproline; Polyhpr, polyhydroxyproline.

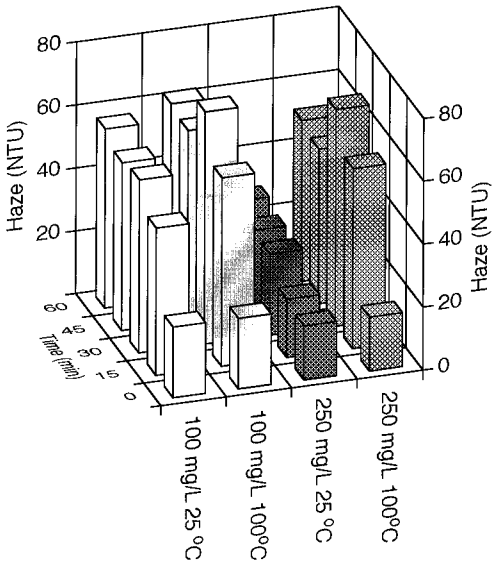

Figure 5. Hazes produced when 100 or 250 mg/L gliadin was combined with 50 mg/L tannic acid in 0.02 M (pH 4.2) phosphate buffer and held for various times at 25 or 100 °C.

could be exposed by heating. Heating has been shown to increase the hydrophobicity of ovalbumin and lysozyme (Kato et al., 1986). A time trial was carried out at two gliadin concentrations at both 25 and 100 °C (see Figure 5). In all cases it appears that a plateau was reached by 30 min and little, if any, additional haze was formed at longer times; this indicates that the difference between the two temperatures is not due to a kinetic effect (when the same ultimate haze level would be reached after a longer time at the lower temperature). The amount of haze formed at 100 °C was greater, particularly with the higher protein concentration. It is notable that with 250 mg/L protein at 25 °C the haze was lower than with 100 mg/L; this apparently coincides with an excess of protein relative to tannic acid.

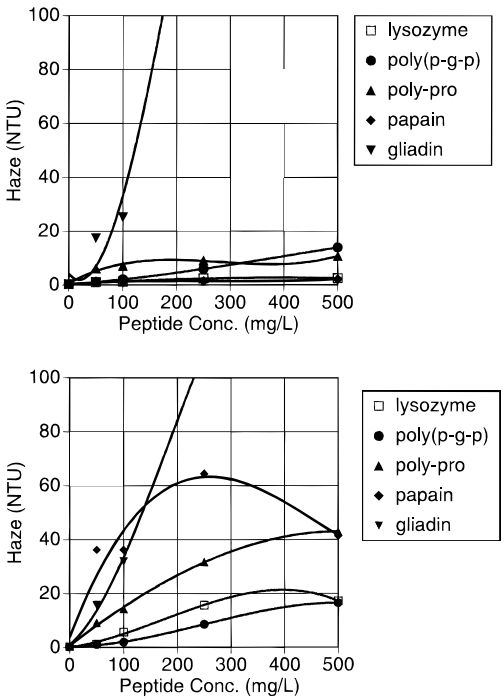

Figure 6. Hazes produced when proline-containing synthetic polypeptides and native proteins were combined in 0.02 M (pH 4.2) phosphate buffer with 50 mg/L tannic acid and (top) held for 30 min at 25 °C or (bottom) held for 30 min at 80 °C. Poly-(p-g-p), proline:glycine (2:1) copolymer; polypro, polyproline.

However, heating the 250 mg/L gliadin sample led to a large increase in haze. This result would be expected if more effective polyphenol binding sites are exposed by heating and is consistent with a greater role for hydrophobic rather than hydrogen bonding.

The reaction with other peptides was examined with tannic acid at 25 and 80 °C (see Figure 6). Here two synthetic polymers, polyproline and a mixed copolymer of proline and glycine in a 2:1 molar ratio, neither of which has a native form, were compared with three proteins. Lysozyme, papain, and gliadin contain about 1%, 5%, and 15% proline, respectively (Asano et al., 1982). The synthetic peptides produced slightly higher amounts of haze at the higher temperature than they exhibited at 25 °C; these compounds have no particular secondary structure and, since they have fairly uniform composition throughout, should exhibit similar properties no matter which way the molecule is folded or unfolded. Lysozyme and papain produced almost no haze at the lower temperature but substantially more at the higher temperature, at which the three native proteins produced haze in rough proportion to their proline content. It appears that the native proteins tend to produce significantly more haze when heat is applied. This is consistent with the hypothesis that heating exposes more polyphenol binding sites and with the model of Figure 2.

Another test of the model can be made by comparing the results obtained with equal weight percent amounts of peptides of the same composition but different molecular weights; these should produce different results because the number of polypeptide molecules changes as the size range is changed at constant concentration (w/v). It is possible to purchase homopolymeric proline in three size ranges. These were obtained and added to tannic acid at 25 and 80 °C. The results are shown in Figure 7. Very little haze was formed at the lower temperature, and the results tended to be variable. At

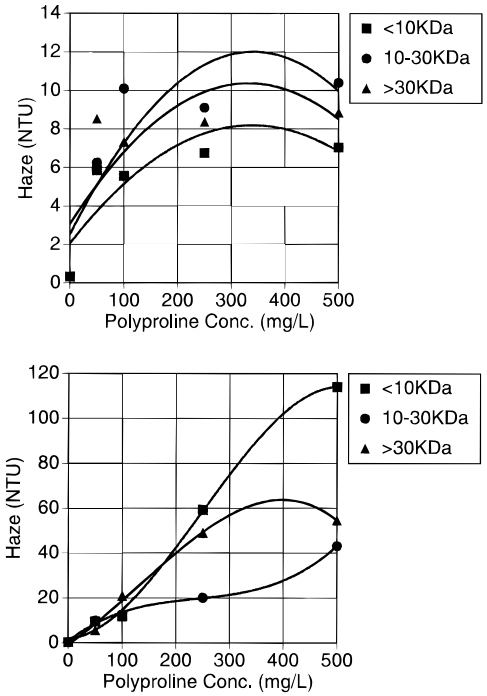

Figure 7. Hazes produced when three different molecular size ranges of polyproline were combined in pH 4.2 phosphate buffer with 50 mg/L tannic acid and held (top) for 30 min at 25 °C or (bottom) for 30 min at 80 °C.

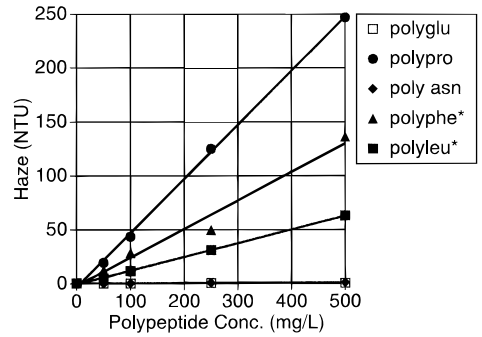

Figure 8. Hazes produced when homopolymeric peptides with various functional groups were combined with 600 mg/L catechin in pH 4.2 phosphate buffer and held for 30 min at 80 °C. Polyglu, polyglutamic acid; polypro, polyproline; polyasn, polyasparagine; polyphe, polyphenylalanine; polyleu, polyleucine. *insoluble; the same haze values were obtained with or without catechin.

80 °C there were clear differences among the three materials and it appears that the peak responses occur at different concentrations. This result is consistent with the model of Figure 2.

A number of homopolymeric amino acids, some of which were examined at a single point by Asano et al., were combined with catechin (see Figure 8). Polyglutamic acid (an acidic amino acid) and polyasparagine (an amide) formed no measurable haze at any concentration tested. Polyphenylalanine and polyleucine (both nonpolar) were also examined; these substances did not dissolve under the experimental conditions and produced the same amount of light scattering whether catechin was present or absent. As a result, it is not known if these two compounds interact with polyphenols to form haze.

Proline is apparently required for peptide haze-forming activity (all polypeptides examined that lacked proline failed to produce haze with polyphenols, and essentially all polypeptides that contained proline formed haze to some degree, at least if heated). Polysarcosine was shown to compete for binding to proanthocyanidins nearly as effectively as polyproline (Hagerman and Butler, 1981), but sarcosine is not normally found in beverage protein. It is likely that proline is a necessary component of the polyphenol binding site. It appears virtually certain that the other amino acids in a polypeptide also exert an influence on haze-forming activity, as it is not entirely explained by the proline content. Some amino acids affect protein folding (and presumably physical access to binding sites). Others may facilitate or inhibit binding if they are near a binding site. Hydroxyproline, for example, which in homopolymer form produced no haze, is well-known to interrupt formation of helical structures in proteins; it is prominent in gelatin, and it may function there to create large regions of the molecule with fairly accessible (and presumably proline-containing) polyphenol binding sites. If the binding mechanism is largely hydrophobic binding, nonpolar amino acids in a peptide could hinder haze formation if they promote folding of hydrophobic regions of the molecule into the interior in a way that renders them inaccessible. Alternatively, they might promote haze formation if they act cooperatively with a nearby proline to enhance the activity of the binding site. Other amino acids in a peptide may exert an influence on binding sterically or electrostatically.

Nature of the Polyphenols That Form Haze. As is evident from Figure 4, rather different polyphenolic compounds (catechin and tannic acid) are capable of forming haze and produce not only different amounts of haze but quite different rankings with the same set of polypeptides. It is likely that the accessibility of binding sites plays a role in the differences. Larger molecules, such as are thought to predominate in most tannic acid preparations (typically represented as one glucose molecule with either three or nine gallic acid moieties attached), can bridge greater distances and may be able to simultaneously attach at more sites, but they may also encounter more steric hindrance than smaller molecules such as catechin. Other differences include the density of hydroxy groups (higher in tannic acid) and the aromatic nature of gallic acid.

Catechin was shown to exhibit haze-forming activity in a model system similar to that of isolated beer polyphenols (Asano et al., 1982). There is evidence that the polyphenols most involved in haze formation in beer are the proanthocyanidin dimers of catechin and/or epicatechin (McMurrough et al., 1992). These would, naturally, resemble catechin more than tannic acid, although the molecules are roughly twice as large as catechin. Similar procyanidins are prominent in fruit juices (Lea, 1984; Spanos and Wrolstad, 1990; Oszmianski and Sozynski, 1986), and the oxidation of procyanidins has been associated with haze formation in juices (Lea, 1984; Wilson and Burns, 1983).

Nature of the Protein−Polyphenol Interaction. When salt, DMF, or dioxane was added to solutions containing polypeptides and catechin, formation of haze was inhibited by DMF (a hydrogen bond acceptor) and dioxane (a nonpolar solvent) and essentially unaffected by salt (Asano et al., 1982). Since the initial protein−polyphenol interaction is thought to be reversible, it should be possible to dissolve already-formed haze with DMF and dioxane. This was tested by combining gliadin and catechin in buffer and holding for 30 min at 80 °C to develop haze. Various amounts of salt

Table 1. Haze (NTU) Observed after Catechin and Gliadin Were Combined in 0.02 M (pH 4.2) Sodium Phosphate Buffer, Held at 80 °C for 30 min To Develop Haze, and the Indicated Amounts of Water, DMF, Dioxane, and Salt Were Added

% of final volume added	water	dioxane	DMF	NaCl (expressed as a 20% solution)
0	220	220	220	220
5	173	30	17	297
10	182	10	12	281
25	166	9	8	416

solution, DMF, and dioxane were then added and the hazes measured. The results are shown in Table 1. Water aliquots added in the same proportions caused a small decline in haze, presumably because of dilution. Relatively small amounts of DMF and dioxane essentially dissolved all of the haze. Salt addition resulted in higher hazes, probably due to salting out of proteins; there certainly was no evidence of dissolution. These observations may be useful in dissolving collected haze from beverages for analysis. The haze-depressing effect seen here with a nonpolar solvent may also occur in beverages that contain ethanol, a somewhat less polar solvent than water.

Conclusions. As reported by Asano and co-workers, peptides and proteins that contain proline formed haze when combined with polyphenolic compounds, while synthetic polypeptides and proteins lacking proline did not. Polypeptides with higher percentages of proline tend to form more haze. The amount of haze formed depends both on the concentrations of protein and polyphenol and on their ratio. A model in which a haze-active protein has a fixed number of polyphenol binding sites at a given temperature accounts for this behavior. This model has ramifications for methods of assessing haze-active proteins or haze-active polyphenols based on generating and measuring haze because of the endogenous proteins and polyphenols present in most beverage samples. The lack of haze formation with polyhydroxyproline and the higher amounts of haze observed upon heating proteins at higher temperatures suggest that hydrogen bonding is not as important in the interaction between proteins and polyphenols as is hydrophobic bonding. Haze, once formed, can be dissolved by either DMF or dioxane.

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