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REVIEWS

Effects of Protein−Polyphenol Interactions on Beverage Haze, Stabilization, and Analysis

Karl J. Siebert[†]

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

The haze-forming activity of a polypeptide depends greatly on its proline content. Haze-forming polyphenols have at least two binding groups, each of which has at least two hydroxy groups on an aromatic ring. The protein/polyphenol ratio has a strong influence on the amount of haze formed; the largest amount occurs when the numbers of polyphenol binding ends and protein binding sites are nearly equal. This has important consequences for turbidimetric methods used to measure haze-active proteins and polyphenols in beverages. The ratio also influences the effectiveness of a number of stabilization procedures.

Keywords: *Light scattering; analysis; adsorbents; stabilization*

INTRODUCTION

Clear beverages are generally intended to remain clear until they are purchased and consumed. Hazes or sediments may result from microbial growth; from the formation of starch, pentosan, or oxalate crystals in beer (Gjertsen, 1972; Hudson, 1981; Rudin, 1977); from deposits of starch, tartrates, or pectins in fruit juices and wine (Fogarty and Ward, 1972; Van Buren, 1983; Heatherbell, 1976a; Van Buren, 1984); or from solid material such as fining agents, adsorbents, or filter aids that are not completely removed (Glenister, 1974). The most frequent cause of haze in beer, wine, and clear fruit juices, however, results from protein−polyphenol interaction (Goertges, 1982; Heatherbell, 1976b; Hough et al., 1982), and these clear beverages are typically stabilized to delay the onset of protein−polyphenol haze formation.

[†] Telephone (315) 787-2299; fax (315) 787-2284; e-mail kjs3@cornell.edu

Although certain aspects of what makes a protein or a polyphenol haze-active (HA) have been known for some time (Asano et al., 1982; Moll, 1987), the recent discovery of the importance of the ratio of the two in a beverage on the amount of haze formed (Siebert et al., 1996c) has led to better understanding of some of the analytical methods used to measure the HA species and the mechanisms of stabilization methods. This review is intended to describe these aspects of beverage haze formation.

DISCUSSION

Composition of Beverage Haze. Much work has been done over many years to characterize HA materials in beverages [see the thorough review by Moll (1987)]. Mostly this has been approached by collecting haze or sediment material by centrifugation or by ammonium sulfate precipitation. The collected material has often been subjected to chemical analysis, amino acid analysis, chromatography, or electrophoresis. It has long been

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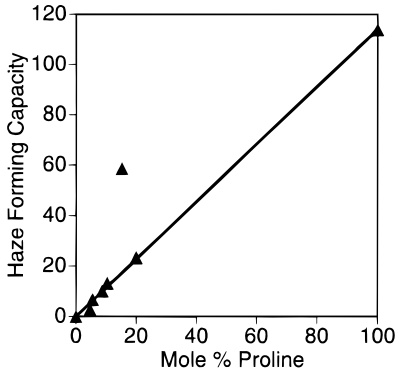

Figure 1. Relationship between the mole percent of proline in synthetic polypeptides and in natural proteins and haze formed in a model system with catechin at 100 °C. Data are taken from Asano et al. (1982).

known, for example, that haze material collected from beer usually contains a substantial amount of carbohydrate, often exceeding the amount of protein, and only a small amount of polyphenol (Belleau and Dadic, 1981; Dadic and Belleau, 1980; Siebert et al., 1981). The carbohydrate, however, appears to be passively coaggregated with the other materials, as its concentration in the haze tends to increase with time and because no carbohydrate-removal treatment is needed to confer stability on these beverages. Rather, reducing the level of protein or polyphenol is sufficient.

Nature of Proteins That Are Haze-Active. It was shown long ago that the barley prolamins, a class of alcohol soluble, proline-rich proteins called the hordeins, were associated with polyphenols in malt (Pollock et al., 1959). Later work, which included amino acid analysis, demonstrated that beer HA proteins were derived from the barley hordeins (Asano et al., 1982). The HA proteins isolated from beer were of several different molecular weights (Asano et al., 1982). This indicates either that several of the known barley hordeins (Shewry, 1993) are involved or that one or more of these are degraded to different degrees by the extensive proteolysis that occurs during mashing.

Of particular interest is the finding that, in a buffer model system with catechin, the amount of haze developed appeared to be essentially linearly related to the mole percent of proline in a polypeptide (Asano et al., 1982) (see Figure 1). A number of amino acid homopolymers that did not contain proline were shown to have no haze-forming activity. This was confirmed with some additional homopolymers, and it was demonstrated that not even the closely related polyhydroxyproline formed measurable haze (Siebert et al., 1996c). This is consistent with results from a number of studies of the binding of polyphenols to proteins using other methods (Hagerman and Butler, 1981; Oh et al., 1980). Model system studies with a number of synthetic proline-containing peptides, polyproline, and two sequential copolymers $(gly\text{-}pro\text{-}ala)_n$ and $(pro\text{-}pro\text{-}gly)_5$, showed that all of these were bound by a number of dimeric and trimeric proanthocyanidins (Outtrup et al., 1987).

Although the origin and nature of the beer HA protein are well established, the situation is less clear in fruit juices and wine. Many of the investigations of fruit juice or wine HA proteins have, as in beer, found the activity distributed over a range of molecular weights and P_i values (Beveridge and Tait, 1993; Hsu, 1986; Hsu et al., 1987; Dawes et al., 1994; Waters et al., 1991, 1992, 1996). Although there is no stage of fruit juice processing during which significant proteolysis is thought to occur, it is possible that some of the enzyme preparations added to liquefy fruit before pressing may contain proteases as well as their stated carbohydrate-hydrolyzing activities. In some cases amino acid analyses of isolated protein fractions have been carried out. Unfortunately, in many of these studies the amino acid analysis procedure used did not determine proline, which we can now see is a serious shortcoming.

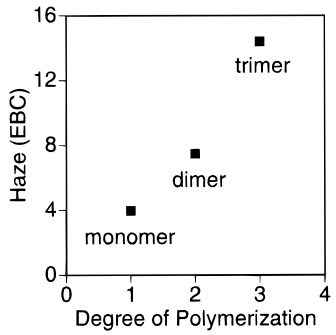

Figure 2. Effect of degree of polymerization on haze formed when 60 mg/L of the indicated (+)-catechin compound was added to beer and assessed by the alcohol chill test. Reprinted with permission from Mulkay and Jerumanis (1983). Copyright 1983 Cerevisia.

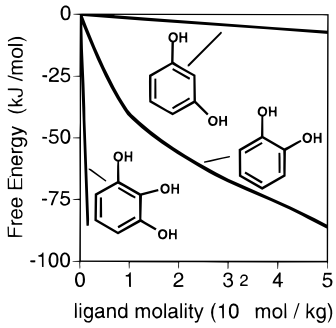

Figure 3. Energy released when polyphenols bound to bovine serum albumin at pH 6.5. Data adapted from McManus et al. (1985).

In one study of haze material isolated from apple juice, proline comprised 5−16% of the amino acids in the haze proteinaceous material (Johnson et al., 1968). This suggests that, although grains and fruits are very different, the HA proteins in both are likely rich in proline.

Nature of Polyphenols That Are Haze-Active. Research has shown that simple phenols and most polyphenol monomers, when combined with beer HA proteins in model systems, produced no haze (Eastmond and Gardner, 1974; Asano et al., 1984). A small amount of haze was produced with epicatechin and catechin. Dimers and higher polymers of the proanthocyanidins have haze-forming activity that increases with the degree of polymerization (see Figure 2) (Mulkay and Jerumanis, 1983).

An interesting study was carried out in which the energy released when each of several polyphenols bound to bovine serum albumin (BSA) was measured (McManus et al., 1985) (see Figure 3). *m*-Diphenol, in which the OH groups were separated, released only a small amount of energy upon binding to the protein, whereas *o*-diphenol, in which the OH groups were adjacent on the ring, bound considerably more strongly. The vicinal triphenol (1,2,3-triphenol) bound much more strongly yet. These results were obtained at pH 6.5, and

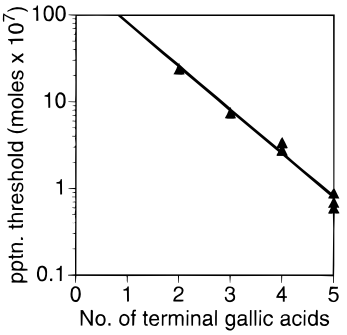

Figure 4. Relationship between β-glucosidase precipitating activity and the number of terminal gallic acid moieties in a gallotannin. Data adapted from Haslam (1974).

results at pH 2.2 were substantially different. The binding strength of these compounds at the pH of the beverages of interest (generally near pH 3 for grape juice and wine and close to pH 4 for apple juice and beer) is not known, although it has been shown that pH exerts a strong influence on the amount of haze formed from the same concentrations of HA protein and HA polyphenol (see later).

Tannic acid is a member of the gallotannins, a family of compounds that has some number of gallic acid (GA) moieties connected to a glucose molecule by ester linkages. Gallotannins with a variety of configurations are produced naturally by different organisms (Haslam, 1974). In some of these there are chains in which two or more gallic acids are connected to each other by an ester linkage and then connected to the glucose. This reduces from three to two the number of available OH groups on the gallic acid moieties in the interior of the chains and likely hinders access to them as well. Haslam and co-workers obtained a number of gallotannins of known structure and determined their activities in precipitating β-glucosidase. Their published data were examined, and a striking ($r^2 = 0.980$) relationship between the logarithm of the concentration of gallotannin that just began precipitating the enzyme and the number of terminal (end of chain) gallic acid groups in the gallotannin molecule was found (see Figure 4). The cube root of the precipitation concentration versus the number of terminal GAs gave an equally strong relationship, indicating that the phenomenon is probably related to volume. This is not surprising, as molecules of roughly the same size can only bridge so far, regardless of the number of points of attachment. Presumably the strength of the attachment between two proteins can be increased by making multiple connections (Baxter et al., 1997). It appears unlikely from steric considerations that one polyphenol molecule would be able to join three protein molecules.

The naturally occurring HA polyphenols in beer are members of the proanthocyanidins. These are monomers, dimers, trimers, and higher polymers of catechin, epicatechin, and gallocatechin (see Figure 5). Of these, it has been shown that the concentrations of the two most prominent dimeric proanthocyanidins in beer, procyanidin B3 (catechin−catechin) and prodelphinidin B3 (gallocatechin−catechin) (see Figure 6), are closely related to haze formation (McMurrough et al., 1992). The rate of haze formation was shown to be very well explained ($r = 0.965$) by the product of the sensitive proteins (measured by tannic acid induction of haze) and the sum of the dimeric proanthocyanidins measured by HPLC. If the same binding strength relationships

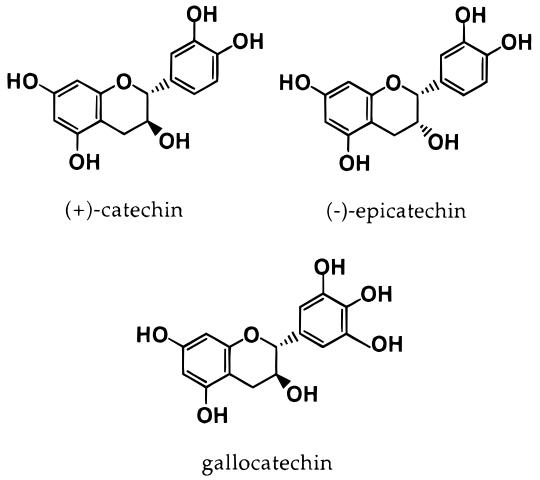

Figure 5. Structures of the proanthocyanidin monomers typically found in beer.

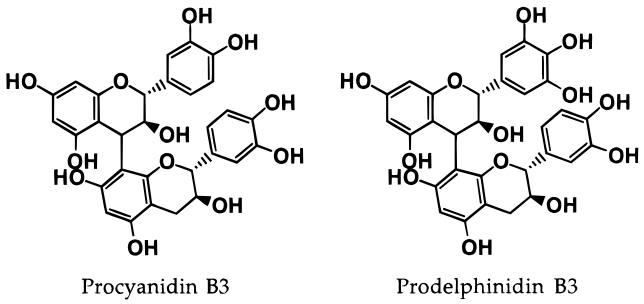

Figure 6. Structures of procyanidin B3 and prodelphinidin B3, the prominent proanthocyanidin dimers in beer.

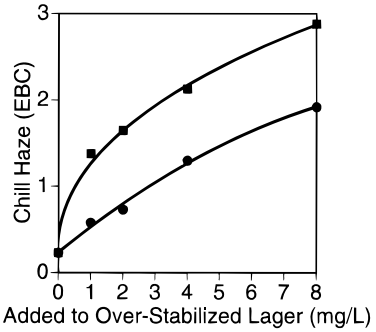

Figure 7. Effects of the addition of prodelphinidin B3 (■) and procyanidin B3 (●) on haze developed in beer at 60 °C. Data are adapted from McMurrough et al. (1996).

described in Figure 3 hold, catechin and epicatechin should each have one medium-strength binding site and one weak binding site. Gallocatechin should have one strong and one weak binding site. Procyanidin B3 (catechin−catechin) should have two medium-strength binding sites and two weak ones, whereas prodelphinidin B3 (gallocatechin−catechin) should have one strong binding site, one medium-strength site, and two weak ones. There is, in fact, evidence that prodelphinidin B3 has greater haze-forming activity than procyanidin B3 (McMurrough et al., 1996; Mulkay and Jerumanis, 1983) (see Figure 7). The degree of proanthocyanidin polymerization was found to exert a stronger effect on haze formation than the number of OH groups on a ring (see Figure 8) (Mulkay and Jerumanis, 1983). The McMurrough model of haze formation considered only proanthocyanidin dimers, yet successfully described haze formation behavior; this agrees with reports that

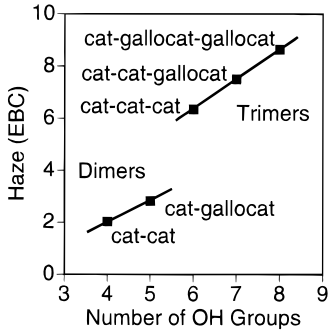

Figure 8. Comparison of the effects of degree of polymerization and number of hydroxyl groups on haze when 60 mg/L of the indicated compound was added to beer and assessed by the alcohol chill test. Reprinted with permission from Mulkay and Jerumanis (1983). Copyright 1983 Cerevisia.

the amounts of trimers and higher degree of polymerization compounds actually present in beer are quite small (Jerumanis, 1979; McMurrough and Baert, 1994; Ohtsu and Hashimoto, 1982), probably as a result of limited extraction from malt and losses during processing. The predominant proanthocyanidin in apple juice is procyanidin B2 (epicatechin−epicatechin), whereas the major dimers in pear juice are procyanidin B1 (epicatechin−catechin) and B2 (Spanos and Wrolstad, 1992). Grape juice contains procyanidins B1, B2, B3, and B4 (catechin−epicatechin).

Interactions between Haze-Active Proteins and Polyphenols. Considerable work has been carried out on the nature of protein−polyphenol interaction. It is well-known that at least the initial reaction is not covalent bonding, because most haze caused by chilling partially or totally dissolves when a hazy beverage is warmed. This phenomenon has frequently been attributed to protein−polyphenol complexes held together by some combination of hydrogen and/or hydrophobic bonding (Asano et al., 1982; Hagerman and Butler, 1981; Oh et al., 1980). Ionic bonding is definitely not involved. This was demonstrated by showing that salt did not interfere with haze formation (Asano et al., 1982) or cause freshly formed haze to dissolve (Siebert et al., 1996c), whereas a nonpolar solvent (dioxane) and a hydrogen bond acceptor (dimethyl formamide) both prevented haze formation (Asano et al., 1982) and dissolved freshly formed haze (Siebert et al., 1996c). Recent work has reported that interactions between polyphenols and proline-containing peptides may involve formation of π-bonded complexes in which the rings of the two compounds overlap (Baxter et al., 1997; Bianco et al., 1997).

When an HA protein (e.g., gelatin or gliadin) and an HA polyphenol (e.g., tannic acid) were combined in various proportions in a buffer model system, a pattern was seen in the results (Siebert et al., 1996a,c) (see Figure 9). As the protein concentration was held constant and polyphenol increased, the haze rose to a maximum but then declined at higher polyphenol levels. Similarly, when polyphenol concentration was held constant and protein increased, the observed haze first rose to a maximum and then declined at higher protein levels. A model that explains this behavior (Figure 10) was proposed (Siebert et al., 1996c). If an HA protein is conceptualized as having a fixed number of sites to which a polyphenol can bind (presumably the proline residues) and an HA polyphenol is thought of as having two (or more) ends that can bind to HA protein, then

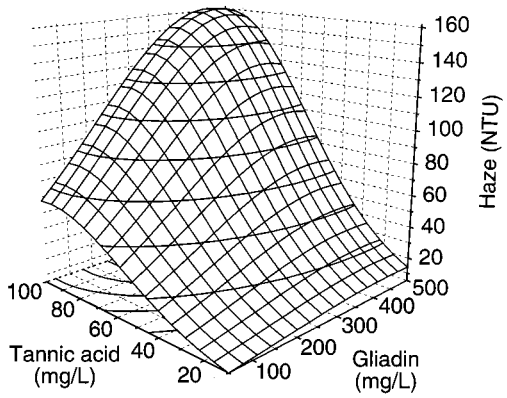

Figure 9. Effects of the concentrations of gliadin and tannic acid on predicted haze at 6% (v/v) alcohol and pH 3.7. Reprinted with permission from Siebert et al. (1996a). Copyright 1996 American Chemical Society.

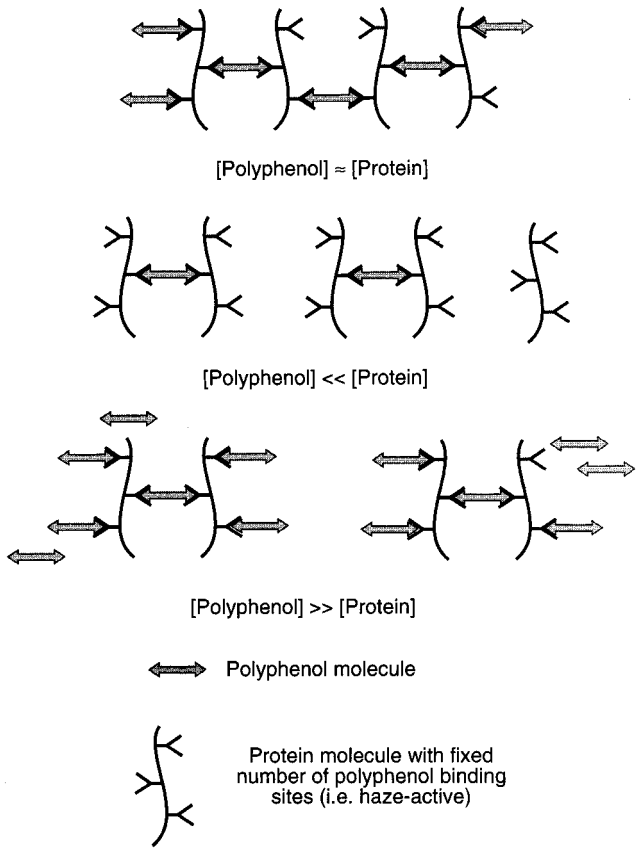

Figure 10. Conceptual mechanism of protein−polyphenol interaction. Reprinted with permission from Siebert et al. (1996c). Copyright 1996 American Chemical Society.

the situation where the total concentration of polyphenol ends is roughly equal to the number of binding sites in the protein will result in a large network, corresponding to large colloidal particles and maximum light scattering. In a situation such as beer, in which there is a large excess of HA protein to HA polyphenol (Siebert et al., 1996a), each HA polyphenol molecule should be able to find binding sites in two proteins to attach to. However, it is unlikely that there will be sufficient additional polyphenol molecules to bridge many of these "sandwiches" or "protein dimers" together. The result is smaller particles and less haze. In beverages such as apple juice, where there is a large excess of HA polyphenol to HA protein (Siebert et al., 1996a), nearly all of the sites in the proteins would be occupied. That would

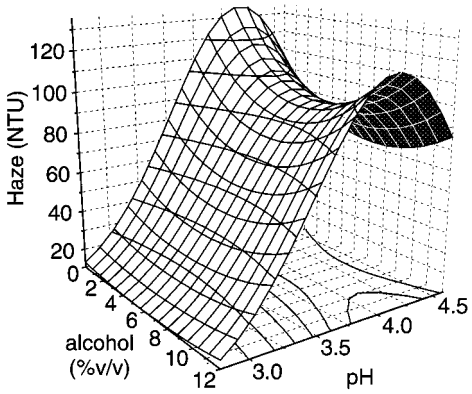

Figure 11. Effects of alcohol and pH on haze predicted by the response surface model at 275 mg/L gliadin and 55 mg/L tannic acid. Reprinted with permission from Siebert et al. (1996a). Copyright 1996 American Chemical Society.

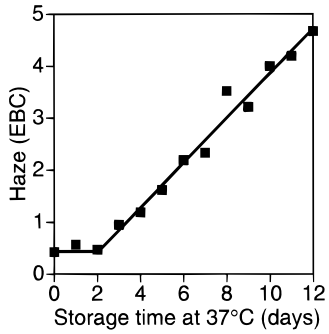

Figure 12. Time course of haze formation for lager beer [adapted from McMurrough et al. (1992)].

make it difficult for a polyphenol attached at one end to find an available site on another protein to bridge to. The result would again be smaller particles and less light scattering.

The effects of pH and ethanol concentration on haze formation were studied in a model system (Siebert et al., 1996a). It was seen that pH had a striking effect (Figure 11); approximately 7 times as much haze was produced with the same amounts of protein and polyphenol near pH 4.0−4.2 than at pH 3.0. At pH values >4.2, the haze declined. This effect may be due to the increasing charge on the protein at pH values above and below its isoelectric point; because the interaction appears to be nonionic, greater charge may result in repulsion of protein molecules from one another. Ethanol had no effect on the haze near the pH of grape juice and wine, but at the pH of beer and apple juice, resulted in first a modest decline in haze, followed by an increase at higher concentrations. Because we know that a nonpolar solvent can prevent haze formation (Asano et al., 1982), it seems possible that a semipolar solvent (ethanol) may interfere with haze formation to some extent. At higher levels ethanol may cause protein precipitation.

Pattern of Haze Development. It has long been known that the pattern of haze formation in packaged beer has two stages (Gardner and McGuinness, 1977; McMurrough et al., 1992) (see Figure 12). At first, no change in haze is observed. After some time, the haze begins to increase linearly. There are two possible explanations for this behavior. Changes must occur during the initial, flat part of the curve. Perhaps small protein−polyphenol complexes are formed that are initially soluble and so do not scatter light. Alternatively, some other chemical reaction may have to take place before haze formation begins in earnest. Two possible mechanisms were described in the review by Gardner and McGuinness (1977) (see Figure 13). In the one that has most often been proposed (left side of Figure 13), the reaction is polymerization of simpler polyphenols to the higher molecular weight compounds that are known to be more haze-active. The other mechanism is "activation" of existing polyphenol compounds that then react with proteins to develop haze (right side of Figure 13). There is some evidence that the mechanism is not the former, because radiolabeled epicatechin did not polymerize in beer to form dimers or trimers (McGuinness et al., 1975a) and it was incorporated into haze only to a small extent, whereas labeled dimeric catechin was readily incorporated into beer haze. Some caution should be exercised in drawing conclusions here due to the recent finding of substantially larger amounts of haze formed with catechin than epicatechin under some circumstances (Siebert and Lynn, 1998). One study found, however, that dimeric proanthocyanidins actually depolymerized in wort and beer (Derdelinckx and Jerumanis, 1987). It has been shown that beers with relatively high levels of dimeric catechin can have fairly long shelf lives, provided that package oxygen content is low. On the other hand, beers with low dimeric catechin levels can be fairly stable even with high oxygen levels (McGuinness et al., 1975b).

Although the protein/polyphenol ratios in most fruit-based beverages are very different from those in beer, the patterns of haze formation in Concord grape juice and cranberry juice cocktail prepared on the pilot scale, packaged, and stored at 37 °C were remarkably similar

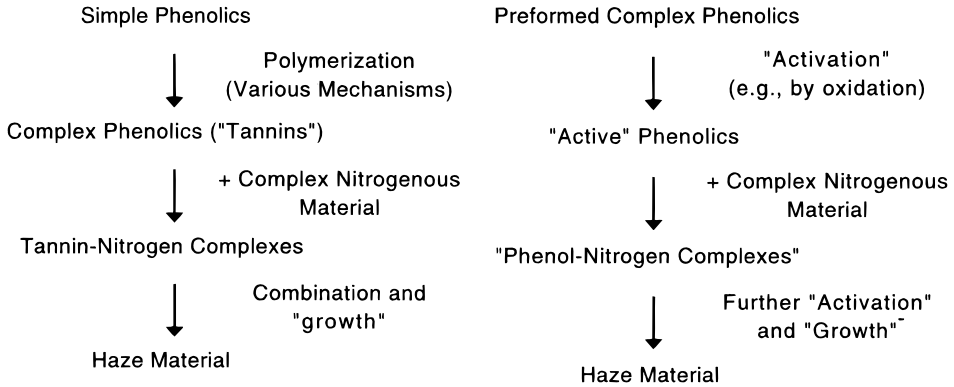

Figure 13. Possible mechanisms accounting for the observed pattern of haze development in beer. Concept is taken from Gardner and McGuinness (1977).

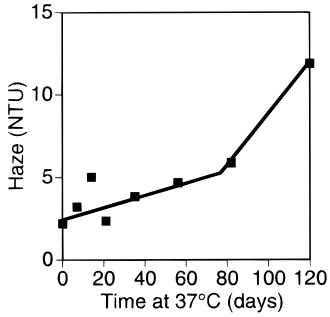

Figure 14. Time course of haze formation at 37 °C in Concord grape juice prepared and packaged on the pilot scale. Packages were attemperated to 25 °C and opened just prior to the measurement of haze in nephelos turbidity units.

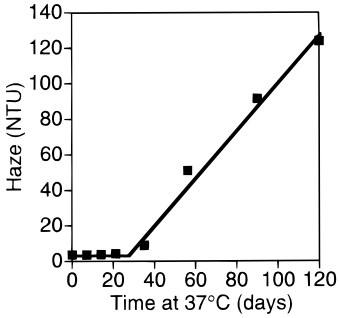

Figure 15. Time course of haze formation at 37 °C in cranberry juice cocktail prepared and packaged on the pilot scale. Packages were attemperated to 25 °C and opened just prior to the measurement of haze in nephelos turbidity units.

(see Figures 14 and 15). This suggests that a mechanism similar to that in beer occurs in these cases. An experiment to test the oxidation hypothesis was carried out with commercial, bottled apple juice (see Figure 16). Bottles were divided into four groups. Those in the first (control) were not opened. Bottles in the other three groups were opened in a laminar flow hood (to avoid microbial contamination) to admit air to the presumably oxygen-depleted headspace. Sulfite was added to the bottles in one of these groups, and ascorbate was added to another. The packages were then reclosed, and all four groups were stored while haze development was monitored. The control followed the same haze development pattern seen previously, with a flat phase followed by a linear increase. The samples to which air but no antioxidant had been added exhibited a shorter flat phase and a shallower slope once the haze increase started. The bottles with both added air and antioxidants behaved very similarly to the control. Obviousy, oxygen had an effect on the haze development pattern. This is consistent with reports of the effect of anaerobic processing on haze stability in apple juice (Wall et al., 1996).

Situation in Various Beverages. It has been demonstrated that beer is high in HA protein and low in HA polyphenols, whereas apple juice is just the opposite (Siebert et al., 1996a). Grape juices are low in HA protein, but much more variable in HA polyphenol; both white and red juices were found to range from low to quite high concentrations (Siebert et al., 1996a). White wines made from *vinifera* grapes were uniformly very low in HA polyphenols, whereas wines made from some American varieties were distinctly higher, and those of hybrids tended to be intermediate (Siebert et al., 1996b). All of the white wines examined had low levels of HA protein. Red wines generally had high levels of HA polyphenols (considerably higher than the white wines). The HA protein levels in red wines made from both *vinifera* and American grapes tended to be quite low but reached moderate levels in wines made with hybrid grapes.

Other Phenomena Involving Protein−Polyphenol Interaction. Protein−polyphenol interaction is important not just in beverage haze. Dietary tannins (defined as water soluble plant phenolic materials with molecular weight ≥500 Da and the ability to precipitate gelatin and other proteins from aqueous solution) have an antinutritional effect (Mehansho et al., 1987; Baxter et al., 1997). Tannins depress the growth rate of rodents and chicks and decrease protein utilization in humans. Hamsters are particularly sensitive to tannins, which can be lethal to them in as little as 3 days. According to

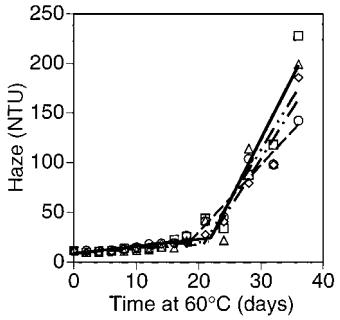

Figure 16. Time course of haze development in commercial, bottled apple juice stored at 60 °C: control (□), with air (○), with air plus sulfite (△) to achieve 58 mg/L SO_2, and with air plus ascorbate (◇) to achieve 200 mg/L in the package.

Mehansho et al., the mechanism of the antinutritional effect is unclear. The response of most mammals to a dietary tannin challenge is to produce higher levels of a class of salivary proteins known as the proline-rich proteins (PRPs). These can contain up to 45 mol % proline. They can be induced by a dietary tannin challenge to levels as high as 70% of total salivary protein and appear to be the body's "first line of defense". Perhaps the PRPs compete efficiently for the dietary polyphenols, thus improving the nutritional availability of less proline-rich dietary proteins.

Astringent taste perception has been associated with interactions between polyphenols in food and salivary PRPs (Luck et al., 1994; Baxter et al., 1997). One hypothesis is that small (presumably colloidal sized) particles are formed in the mouth; these are then perceived by tactile sensation, probably by the trigeminal nerve. Another possibility is that interaction of PRPs with tannins results in a loss of lubricity normally provided by the salivary proteins.

Mechanisms of Stabilization. A number of approaches are employed for stabilizing beverages. The usage patterns make a great deal of sense in view of the mechanism in Figure 10. In beer it is desired to remove HA material while preserving the foam-active protein. The traditional method is to put the fermented beer into a tank and hold it just above freezing to induce haze formation and natural sedimentation of HA material. The disadvantage of this method is that it occupies tanks for a long time (months), which few breweries can afford. Fining with gelatin, isinglass, or tannic acid is often employed to hasten the process. This is followed by a cold, sharp filtration, usually through diatomaceous earth, to remove the particulate matter. An approach that was widely used in North America about 25 years ago was the addition of a small amount of proteolytic

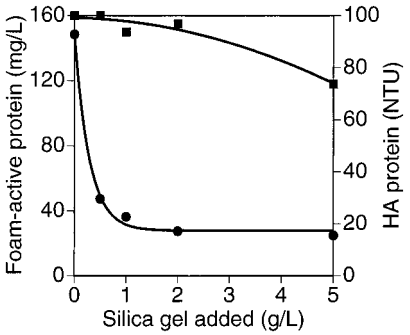

Figure 17. Effects on beer foam active (■) and haze-active (●) protein of treatment with silica hydrogel. Reprinted with permission from Siebert and Lynn (1997c). Copyright 1997 American Society of Brewing Chemists.

enzyme (most frequently papain) to attack the HA protein and delay the onset of haze formation (de Clerck, 1969). This was quite effective, but unfortunately it also degraded foam-active protein and required additional treatment to compensate for this. The treatments most often used for beer stabilization today are fining agents or adsorbents that remove either proteins or polyphenols.

Bentonite functions very nonspecifically and removes foam-active as well as HA protein from beer with similar efficiencies (Siebert and Lynn, 1997c). Silica gels (hydrogels or xerogels) are much more specific and remove HA protein with virtually no effect on foam-active protein unless very high treatment levels (much higher than commercial practice) are applied (see Figure 17). This specificity has been shown to result because silica gel binds to the same sites in the HA proteins (proline residues) as do HA polyphenols (see Figure 18) (Siebert and Lynn, 1997c). In beverages that are polyphenol-rich, silica gels do not work as well as in beer because most of the proline sites in the proteins (where the adsorbent attaches) are already occupied by polyphenols (Siebert and Lynn, 1997b).

Polyvinylpolypyrrolidone (PVPP) adsorbs HA polyphenols. PVPP has considerable resemblance to polyproline; both have five-member, saturated, nitrogen-containing rings, amide bonds, and no other functional groups. Recent results have suggested that PVPP and HA proteins bind to HA polyphenols in a similar manner (see Figure 19) (Siebert and Lynn, 1998). PVPP is more effective in polyphenol-rich beverages (Siebert and Lynn, 1997b) than it is in beer (Siebert and Lynn, 1997c), where it appears that most of the HA polyphenols are bound to HA protein at both ends and thus unavailable to the adsorbent.

Bentonite is often used to stabilize fruit juices and wine because it works well in those applications. It effectively and indiscriminately removes protein. Ultrafiltration, which removes all proteins larger than the membrane molecular weight cutoff, is also commonly used to stabilize fruit juices but would be unsuitable for beer as it, like bentonite, would take out foam-active as well as HA protein.

Impacts on Analytical Procedures. Only a small proportion of the total protein in beverages is involved in haze formation (Siebert and Lynn, 1997a), and its composition is likely to be unusual (presumably high in proline in all cases and, at least in the case of beer, high in glutamine as well). As a consequence, many of the assumptions of common methods of protein analysis may not be fulfilled.

Coomassie blue dye binding is commonly used to determine proteins in solution (Bradford, 1976) or to detect proteins on electrophoresis gels. In the case of beer it is known that the Bradford method provides only about one-seventh the average response to beer protein as to the bovine serum albumin typically used for calibration (Hii and Herwig, 1982). This was explained by Siebert and Knudson (1989), who compared data from the response of Coomassie blue to amino acid homopolymers (Compton and Jones, 1985) with the amino acid composition results for barley hordein (Asano et al., 1982). Coomassie blue response is highly biased toward homopolymers of the basic and aromatic amino acids, particularly arginine, and gives little, if any, response to other homopolymers (Compton and Jones, 1985). Hordein is dominated by proline (≈20 mol %) and glutamine (≈30 mol %), homopolymers of which do not produce any Coomassie blue response (Siebert and Lynn, 1997c), and it is poor in the amino acids that produce a significant response. As a result, hordein produces even less response to Coomassie blue than the average beer protein, which is already quite poor. Wine HA protein may be similar, as it has been reported that it is 50−80% underrepresented by Coomassie blue (Waters et al., 1991).

Determination of protein by measuring absorption at 280 nm assumes a typical content of aromatic amino acids and is subject to interferences from many other compounds that absorb in that part of the UV spectrum; it is reportedly satisfactory for studying wine HA protein (Somers and Ziemelis, 1973).

The Kjeldahl method assumes all of the nitrogen present is contained in proteins that have a typical amount of nitrogen (i.e., which comprise amino acids of average molecular weight ~150 Da per nitrogen) and is relatively insensitive. In the case of hordein, the average molecular weight per nitrogen is low because of the high content of glutamine (with two nitrogens) and proline (which has a relatively low molecular weight). Much of the nitrogen in beer and fruit juices is in the form of free amino acids.

The bichinchonic acid method appears to be less biased against HA protein than Coomassie blue, but, although it gives a linear response to peptides composed of primary amines, it gives a weak and curvilinear response to polyproline (Siebert and Lynn, 1997c) and also to homopolymers of other secondary amines such as hydroxyproline and sarcosine; the extent to which this may influence results for proline-rich proteins is not known.

Turbidimetric methods, such as the "sensitive proteins" assay (Thompson and Forward, 1969), have the advantage that they respond specifically to just the portion of the total protein able to react with an HA polyphenol to develop haze. However, the response depends on the proportion of HA protein to HA polyphenol during haze development, so endogenous polyphenol will influence results. It is possible, however, to remove the endogenous polyphenol before haze induction, which improves the inherent bias of the procedure (Siebert and Lynn, 1997a). Another approach is the turbidimetric titration developed by Chapon and implemented in the Tannometer, a commercial instrument designed for beverage analysis (Chapon, 1993). This records the haze while a titrant such as tannic acid is gradually added; it can give a good indication of the amount of HA protein even in beverages that contain a fair amount of endog-

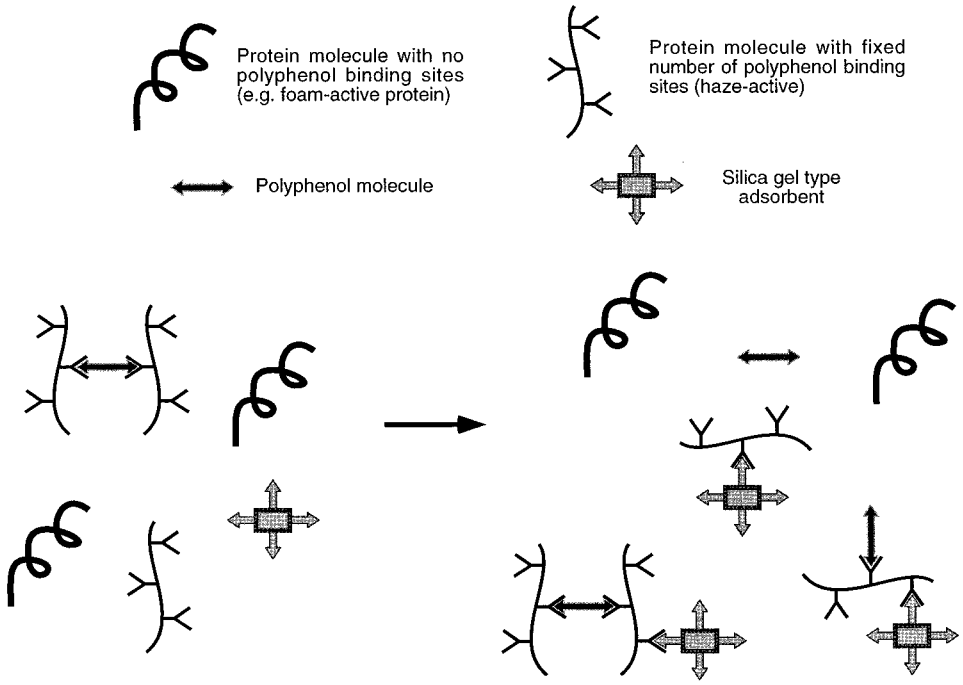

Figure 18. Mechanism of silica gel adsorption of beer HA protein. Silica gel binds to the proline residues in proteins, which are also the sites for polyphenol attachment. As a result, silica gel is specific for HA protein. Reprinted with permission from Siebert and Lynn (1997c). Copyright 1997 American Society of Brewing Chemists.

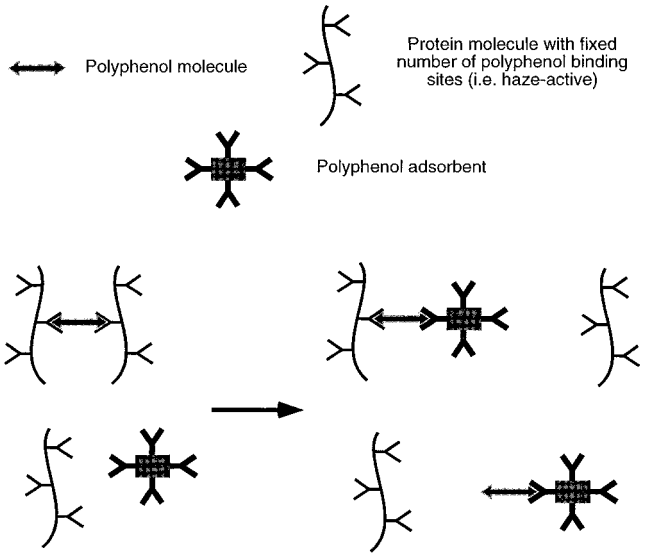

Figure 19. Possible mechanism of PVPP adsorption of beer HA polyphenol. PVPP binds to the same part of the polyphenol molecule that attaches to HA protein. As a result, PVPP is specific for HA polyphenol. Reprinted with permission from Siebert and Lynn (1997c). Copyright 1997 American Society of Brewing Chemists.

enous HA polyphenol. In low HA protein beverages (e.g., stabilized, commercial, bottled apple juice) the amount of HA protein present is so little that adding tannic acid provokes almost no response (Siebert et al., 1996a). It is possible to get a slightly larger response from unstabilized juice (e.g., mechanically clarified cider).

In analogy to the sensitive proteins method, an approach to measure HA polyphenol was developed, based on the addition of an HA peptide to a sample, followed by haze development and a turbidimetric measurement (Siebert et al., 1996a). Gelatin provoked considerable response in apple juice, but almost none in beer (Siebert et al., 1996a). Polyproline was more effective, presumably because it was able to better compete against the beer HA protein for HA polyphenol (Siebert and Lynn, 1997c). Polyproline was also found to be superior to gelatin for determining wine and apple juice HA polyphenol (Siebert et al., 1996b; Siebert and Lynn, 1997a), giving a more linear response. It is likely this occurs because the synthetic polyproline contains a range of molecular weights that tends to offset the normal tendency toward a curved response.

CONCLUSIONS

HA proteins contain proline, and their haze-forming activity is mainly related to the mole percent of proline in the protein. The binding sites of haze-forming polyphenols require at least two and preferably three hydroxy groups on an aromatic ring; vicinal arrangements are more haze-active. The degree of polymerization of polyphenols has a stronger effect on polyphenol binding than does the number of OH groups on an aromatic ring. The proportion of protein to polyphenol exerts a very strong influence on the amount of haze formed; the largest amount of haze is observed when the numbers of polyphenol binding ends and protein binding sites are nearly equal. Higher ratios of HA protein/HA polyphenol, such as are found in beer, result in less haze formation, as do higher proportions of polyphenol, such as are seen in apple juice. This effect of proportionality and the unusual composition of HA proteins have important consequences for analytical methods used to measure HA protein and HA polyphenol in beverages. The protein/polyphenol proportion also influences the effectiveness of some stabilization procedures. Silica gels are very effective in beer, but much less effective in apple juice. PVPP works very effectively in apple juice, but less so in beer. This behavior can be explained by mechanisms that account for adsorbent action. Bentonite works equally well in both polyphenol-rich and polyphenol-poor beverages.

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