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Haze Formation in Model Beer Systems

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The interaction of a haze-active protein (gliadin) and a haze-active polyphenol (tannic acid) was studied in a model beer system in order to investigate the principle mechanisms of haze formation at low temperatures. Low concentrations (g/L) of tannic acid, high concentrations of gliadin, and comparatively high temperatures lead to maximum haze values. When considered on a molar basis, the greatest haze levels are displayed at an approximate 1:1 equivalence of polyphenol and protein. The greater part of haze formation was completed within 0.5 h, irrespective of the concentration of gliadin, the concentration of tannic acid, and the temperature of the model solution.

KEYWORDS: Model; haze; turbidity; polyphenol; protein; beer

INTRODUCTION

Most beers worldwide are served "bright", i.e., without the presence of a haze or sediment. In the absence of precautions, various materials can emerge from solution as turbidity (reviewed in ref *1*). The brewer seeks to avoid this by reducing the levels of one or more of these components. The entire malting and brewing processes can be viewed holistically as being, in part, exercises in eliminating haze-forming materials, of which perhaps the most significant are polypeptides derived from the hordein fraction of barley proteins (*2*) and polyphenols derived from barley and hops (*3*). Among the key stages is the cold conditioning of newly fermented beer. Recently, it was confirmed that it is especially important that beer is taken to as low a temperature as possible (short of freezing the product) in order to maximize the insolubilization processes and that beer held at somewhat warmer temperatures does not develop the same degree of turbidity even after relatively prolonged storage (*4*).

In a separate but related study, now reported, we have substituted beer per se with a model system comprised of gliadin as a source of haze-sensitive protein and tannic acid as a source of polyphenol, the two being combined in various proportions at an alcohol content and pH typical of many beers. The aim was to shed further light on the insolubilization process and assess the impact of varying the proportions of the main substances contributing to haze formation. Gliadin is a wheat-derived protein and, as such, is not found in beers other than those derived from that cereal. It is, though, a protein that is analogous to hordein and quite similar in amino acid composition (*5*); in particular, it is rich in proline and glutamine (*6*). However, the equivalent protein from barley (hordein) is not economically available in sufficient quantities and gliadin has been used previously as a substitute for fundamental studies of haze formation (*7*−*12*). Equally, tannic acid is not a natural component of beer (other than as a precipitant deliberately added downstream), but its molecular characteristics are not vastly different from those of the polyphenols native to beer, and again, it has been featured prominently in prior studies (*7*−*12*).

MATERIALS AND METHODS

Model System. Acetate buffer [0.01 M, pH 4.5, containing 5% (v/v) ethanol] was prepared with high-performance liquid chromatography (HPLC) grade (deionized, distilled, and filtered) water. The buffer was filtered through a 0.22 μm membrane filter and degassed through the application of a vacuum with simultaneous agitation. Tannic acid ($C_{76}H_{52}O_{46}$, reagent ACS, powder, MW 1701.22, no. 419995000) was purchased from ACROS Organics (New Jersey). The tannic acid stock solutions were prepared fresh daily at concentrations of 2440, 4880, and 7320 mg/L by dissolving tannic acid in acetate buffer. Gliadin (crude, from wheat gluten, no. 9007-90-3) was obtained from Sigma Aldrich Chemie GmbH (Steinheim, Germany). As gliadin did not dissolve completely in the acetate buffer containing 5% (v/v) ethanol, a standard solution of gliadin was prepared by adding 500 mg of gliadin to 1 L of buffer. This dispersion was stirred for 30 min at room temperature followed by filtration over a fluted filter to remove undissolved gliadin particles. The protein content was estimated by a UV absorption method (*13*). The values generated by spectrophotometric methods for assessing protein were inherently dependent upon the amino acid composition of the polypeptides; the amino acids affording the most absorbance were tyrosine, tryptophan, and cysteine (*14*). Gliadin contains a preponderance of glutamine and proline and therefore may be expected to display somewhat lower absorbance values. The relationship between "apparent protein" as detected by the u.v. procedure and that quantified gravimetrically was therefore elucidated. Gliadin (200 mg; 14.8% nitrogen as is when determined by total combustion) was dried to constant weight. A 100 mg amount of the dried gliadin was stirred into 200 mL of the acetate buffer and equilibrated for 30 min at 20 °C, before filtering through a dried and weighed filter paper. The contents of the paper were washed with deionized water, and the paper was dried to constant weight at 60 °C before reweighing to determine the quantity of residual gliadin. The difference between this weight and that of the original gliadin introduced into the experiment represents that which is dissolved, and this amounted to 0.4 mg/mL. The protein concentration of the solution made

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Table 1. Molar Concentrations of Tannic Acid and Gliadin Employed in This Study

mg/L	μM	mg/L	μM
	tannic acid		
5	3.0	40	23.5
10	5.9	80	47.0
20	11.8	120	70.5
	gliadin		
78	2.1	234	6.4
156	4.3	312	8.5

was determined to be 0.26 mg/mL according to the u.v. procedure at a suitable dilution. Accordingly, all protein values determined spectrophotometrically were scaled up by a factor of 1.56 to arrive at the "true" dissolved protein concentration. On this basis, the stock gliadin solution was diluted to the target concentrations of 312, 234, 156, and 78 mg/L with acetate buffer. Assuming a molecular weight of 1701 for tannic acid (http://www.jtbaker.com/msds/englishhtml/t0065.htm; date accessed 08.25.2005, page published 02.11.2004) and one of 36500 for gliadin (*15*), we can suppose that the approximate concentrations of the two entities used in this study are as shown in **Table 1**.

Aging Regimens. A 30 mL amount of gliadin solution was transferred to 50 mL disposable centrifuge tubes with screw caps, and 0.5 mL of the tannic acid stock solution was added in order to achieve the final tannic acid concentrations of 40, 80, and 120 mg/L. All defined combinations of gliadin and tannic acid concentrations were combined. The samples were subjected to temperatures of −2.5, 0, or +5 °C for periods of 0.5, 1, and 3 h. In a separate set of experiments with newly prepared materials, the range of tannic acid concentrations employed was 0−40 mg/L, with incubation at 5 °C for 3 h and gliadin concentrations of 312, 234, 156, and 78 mg/L. All experiments were performed in duplicate.

Measurement of Haze. All turbidity measurements were performed in 24 mm diameter cuvettes using a model 2100AN Turbidimeter (Hach Co., Loveland, CO) in the ratio mode using a tungsten light source and U.S. Environmental Protection Agency method 180.1 filter. Results were expressed in nephelos turbidity units (NTU). Prior to measurement, the centrifuge tube containing the sample was inverted three times to achieve homogeneous distribution of particles. The sample was transferred into a precooled turbidimeter cuvette, and the haze measurement was carried out within 5 s using signal averaging. An air purge system provided in the turbidimeter was used to flush the optical compartment with dry nitrogen gas (99.9997%) to prevent condensation on the outside of the sample cell while measuring cold samples. The turbidimeter was calibrated according to the supplier's instructions with Formazin Primary Standards (20, 200, 1000, 4000, and 7500 NTU). Instrument calibration was verified by using precalibrated Gelex Secondary Standards. If the reading was not within 5% of the assigned value for the standard, the turbidimeter was recalibrated.

RESULTS AND DISCUSSION

A series of trials was performed to assess the impact of protein concentration, polyphenol concentration, time, and temperature on the extent to which haze was developed in model systems (**Figure 1**). The protein content of beers can be as high as 7 g/L and that of polyphenols as high as 300 mg/L, so the concentrations employed in this study are of the relevant order of magnitude (*16*).

An initial problem concerned the inherent insolubility of the gliadin protein since molecules of gliadin in solution spontaneously combine with one another through hydrogen bonding. This results in the exposure of hydrophobic groups making the protein insoluble in water, absolute alcohol, and other neutral solvents (*17*). We were unable to achieve the degree of dissolution of gliadin that was reported by others (e.g., *7*). As a consequence, the approach taken was to achieve the highest solubility possible at ambient temperature (ca. 20 °C), then to filter and assess protein by u.v. spectrophotometry, correcting for discrepancies arising from the relatively low u.v. absorbance displayed by gliadin (see Materials and Methods). All concentrations quoted in this work are based on this approach. This may impact the differences observed between the present work and that reported elsewhere.

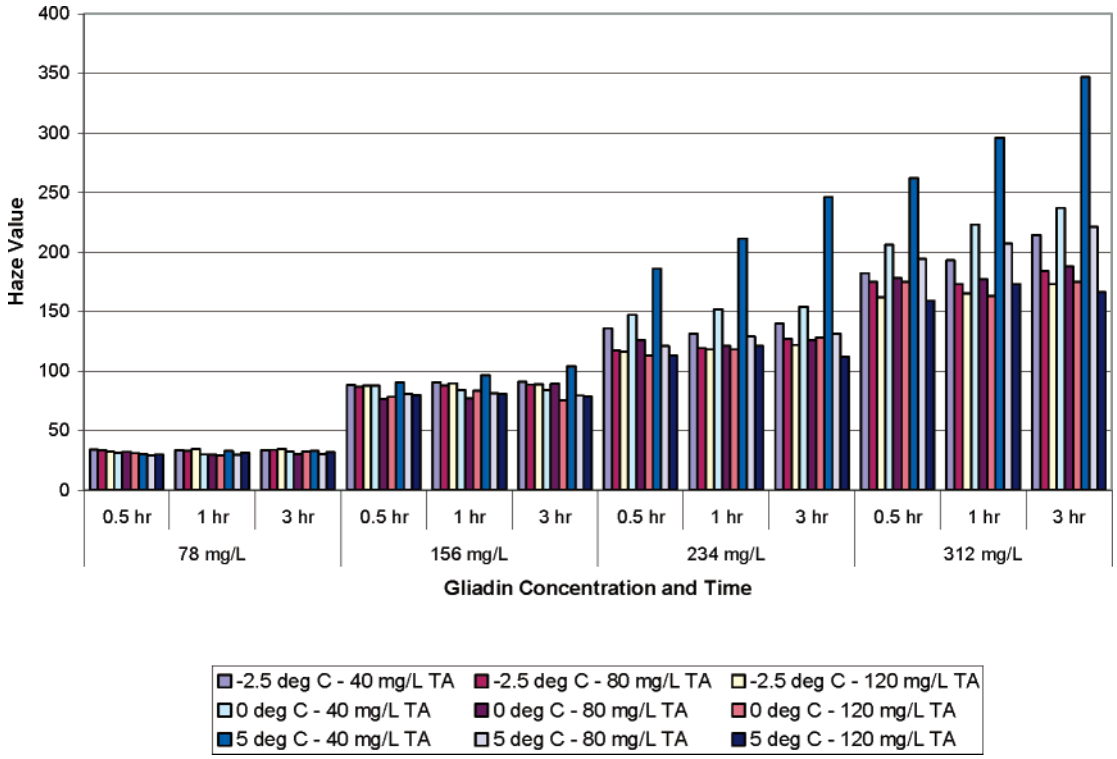

Figure 1. Haze values developed in a model system. Variables were gliadin concentration, tannic acid (TA) concentration, time, and temperature, as explained in the Materials and Methods. All values shown are the means of duplicates with a spread of less than ±5% of the indicated number.

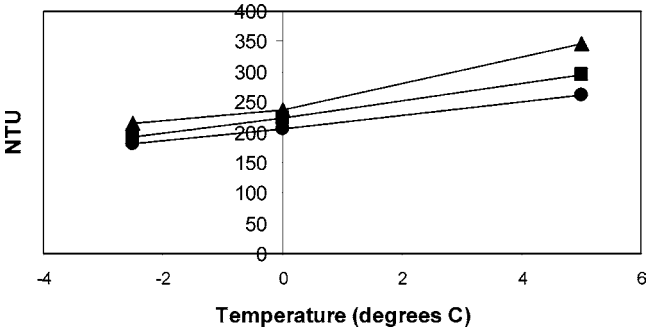

Figure 2. Relationship between turbidity development and temperature at varying time intervals: ▲, 3 h; ■, 1 h; and ●, 0.5 h. Tannic acid was present at 40 mg/L, and gliadin was present at 312 mg/L.

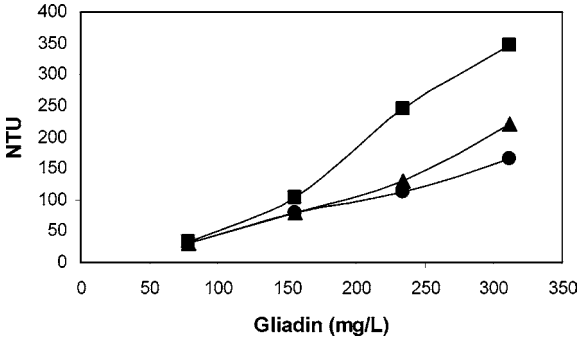

Figure 3. Relationship between turbidity and gliadin concentration at fixed levels of tannic acid: ●, 120 mg/L; ▲, 80 mg/L; and ■, 40 mg/L. Incubation was at 5 °C for 3 h.

In all instances, the amount of haze produced in model systems was greater as the protein concentration was increased (**Figure 1**). On this basis, it should be inferred that the risk of colloidal instability would be manifestly reduced by all incremental reductions in the level of haze-sensitive protein.

In contrast, it can be seen that levels of tannic acid in excess of 40 mg/L do not elevate the haze level. Indeed, the greatest turbidity was obtained with the lowest addition rate of tannin, and this was most notable at the highest temperature employed and least apparent at the lowest temperature. In almost all instances, the bulk of the haze formation was complete within 0.5 h, the more so as the temperature is increased. The exception was at the highest protein concentrations coupled with the lowest tannic acid concentration.

When the findings of the present study are compared with those described in our prior paper (*4*), in which we studied haze development in a commercial beer stored at very low temperatures, we find significant differences. In studies with beer, there is a simple inverse relationship between extent of haze development and temperature over the range −2.5 to +2.5 °C, with most of the haze development occurring rapidly (within 1 h). By contrast, we now find that, at any given combination of gliadin and tannic acid, there is no greater haze at the lowest temperature. Indeed, and unmistakably so at the lowest tannic acid concentration, the extent of haze formation increases both with time and (especially) with temperature over the range −2.5 to +5 °C (**Figure 2**).

The differences observed between haze development in beer and that in the model described in this paper suggest that a simple system with gliadin and tannic acid does not embrace all of the complex interactions that can occur between the various constituents of beer. The model will need to be built upon in order to fully understand the underlying mechanisms of haze formation in beer, but as suggested by others (*12*), this type of system does allow inroads to be made in addressing a highly complex phenomenon.

According to classic crystallization theory (*18*), nucleation of particles is promoted at lower temperatures, whereas particle growth is encouraged at higher temperatures. It seems therefore that in the prior studies on beer (*4*) the event principally governing the development of turbidity was the initial formation (nucleation) of particles, which presumably comprised colloidal materials present in the beer, including tannoids and polypeptide degradation products formed via proteolysis of hordein. By contrast, in the present work, haze development seems to primarily reflect particle growth. We infer that the prime components of the model system (gliadin and tannic acid) are so weakly soluble and/or the sample solution is sufficiently concentrated such that they readily nucleate under all temperature conditions tested but are especially prone to interact to form larger light-scattering entities as the temperature is raised above 0 °C. Particle growth is a thermally activated process in which rates increase with temperature according to an Arrhenius relationship (*19*). At higher temperatures, the probability that two molecules will collide is higher. In addition, the higher solubility of particles at higher temperatures may reinforce this observation. This may at first appear to be paradoxical; however, the solubility of crystalline particles depends on their size, with small particles being more soluble than large ones. In a system of crystalline particles of diverse sizes, the small ones can dissolve and have their mass subsequently transferred to the larger particles, which grow (Ostwald ripening). In this way, the mean particle size in the sample increases and larger light-scattering entities are formed.

Whereas there is an invariable increase in turbidity development as the gliadin concentration is increased (**Figure 1**), insolubilization bears an inverse relationship to tannic acid concentration over the range tested, especially when the gliadin concentration exceeds 156 mg/L. The relationship exists for all conditions of time and temperature explored but is particularly evident at the highest temperature and time frame tested (**Figure 3**).

The behavior observed in this model system would be consistent with tannic acid molecules being present in excess even at the lowest concentration examined in the first set of experiments (40 mg/L), leaving a proportion of the tannic acid molecules unable to find a gliadin molecule to which to bind. Reference to **Table 1** shows that at this concentration of tannic acid there is still an approximate 3-fold molar excess to tannic acid over the highest gliadin concentration employed. Accordingly, a second set of experiments was performed in which the tannic acid concentration was varied over the range 0−40 mg/L. In this trial, only one temperature was used (5 °C). As for the first set of trials, haze formation was essentially complete within 0.5 h, but data are presented for a set incubation time of 3 h (**Figure 4**). As found in all of our experiments, no haze was developed in the absence of tannic acid. Haze development was highest at 20 mg/L tannic acid for the highest gliadin concentration and 5−10 mg/L for the lowest gliadin concentration. The decrease in haze as tannic acid concentrations increase beyond the maximum haze-generating concentrations is likely to be due to an increased likelihood that the polyphenol molecules will react with fewer protein molecules, with a lesser degree of cross-linking and particle formation. Alternatively, there may be a shift in the size of the particles formed: Instead of many small particles, fewer larger ones occur, with less net light scatter. It is not possible on the basis of the present study to differentiate between these or other explanations.

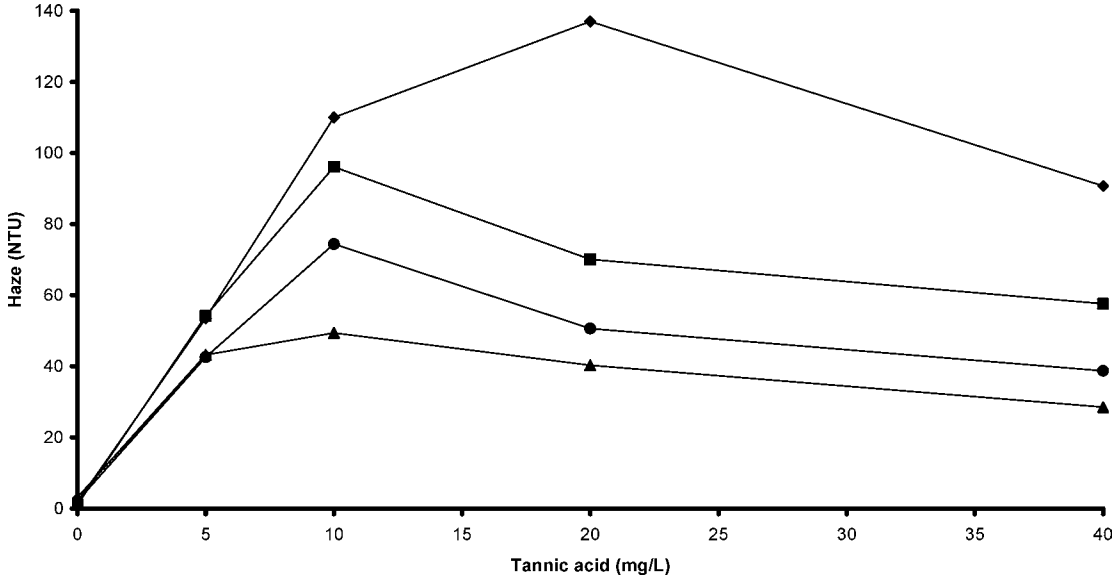

Figure 4. Haze values developed at lower tannic acid concentrations. The symbols ♦, ■, ●, and ▲ indicate 312, 234, 156, and 78 mg/L gliadin, respectively.

Under conditions where turbidity formation is at its highest, the molar ratio of tannic acid to gliadin is approximately 1:1. The number of tannic acid molecules able to bind to a gliadin molecule could be very limited because the large molecules of tannic acid may hinder each other sterically in binding to gliadin. In this case, an addition of more tannic acid will not result in significantly more particle formation since the molecules will be unable to find a gliadin molecule to bind to, whereas the addition of gliadin would lead to additional particle formation. Haze formation would continue to increase with increasing concentration of gliadin until tannic acid becomes limiting. These inferences concur with those of Siebert (*9*).

Let us assume that at around 10−20 mg/L (6−12 μM) tannic acid the potential protein binding sites in a tannic acid molecule (typically six to nine, http://www.ansci.cornell.edu/plants/toxicagents/tannin/chemical.html, accessed 08.26.2005, page published 09.17.2005) are fully occupied by gliadin (within prevailing steric constraints), leading to large tannic acid−gliadin complexes. If more tannic acid molecules are added, more binding sites of tannic acid stay empty because there are not enough gliadin molecules available. This presumption is supported by the observed increase in turbidity with increased gliadin concentrations. On the basis of this theory, haze values will keep increasing with increasing gliadin concentrations until all of the gliadin binding sites on all of the tannic acid molecules are occupied. From then on, an addition of both tannic acid and gliadin would lead to increased haze formation.

The findings in the present study are somewhat at variance with those reported by Siebert and co-workers (*7*−*12*). However, there is one substantial difference in the experimental approach: Siebert (e.g., *8*) made incubations at higher temperatures (routinely 25 °C but sometimes as high as 80 °C or even 100 °C). As temperatures greater than 5 °C did not enter into our study, it is difficult to draw any conclusions that can satisfactorily explain the differences observed. Ambient temperature is inherently relevant from the perspective of haze development in prolonged storage of beer under wholesale or retail conditions, although ambient temperature will vary enormously depending on local seasonal climate. However, the use of very high temperatures is of dubious consequence, especially when considering events occurring at the low temperatures employed in downstream stabilization processes in commercial breweries.

Siebert and co-workers found, then, that increasing the concentration of tannic acid over the range 40−80 mg/L had a profound impact on haze at all concentrations of gliadin (*8*). By contrast, we generally found that precipitation was maximal at 40 mg/L tannic acid, and furthermore, we found a much greater impact of changes in protein concentration on haze formation than was reported by Siebert. For example, Siebert et al. (*10*) varied gliadin over the range 0−500 mg/L and found that, at a tannic acid concentration of 40 mg/L, the highest turbidity attained was less than 50 NTU. We have been unable to achieve sufficient dissolution of gliadin to render protein concentrations in solution of greater than 312 mg/L, but reference to our data reveals that even at 156 mg/L protein, the haze values developed were much higher than reported by Siebert. This is most likely due to enhanced nucleation processes at the lower temperatures.

One of the consequences of employing higher temperatures may be an increase in the availability of polyphenol-binding sites. Because 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 could be exposed by heating. Heating has been shown to increase the hydrophobicity of proteins (*20*). Siebert found that synthetic peptides (polyproline, a mixed copolymer of proline and glycine in a 2:1 molar ratio) produced only slightly higher haze values at a temperature of 80 °C than was found at 25 °C. These synthetic compounds have no particular secondary structure, and they would be expected to show similar properties irrespective of which way the molecule is folded or unfolded.

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