The Protein Character of Beer is Defined in the Brewhouse

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ABSTRACT

The behavior of proteins during mashing was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate protein subunits. Proteins dissolved at somewhat different rates during the "protein rest" stage of mashing to yield a typical and reproducible SDS-PAGE pattern by the end of this period. Sephadex gel filtration (followed by SDS-PAGE) of selected fractions suggested that some of the subunits were substantially aggregated in the wort. Protein subunits precipitated at different rates and to different extents during the remainder of the mash temperature program, and wort at the end of mashing was generally dominated by only two subunits in the molecular weight range of 30,000 to 40,000 (plus some presently uncharacterized material at or below 16,000). Subunits lost during mashing could be recovered in the redissolved precipitated protein, although the precipitate also contained irreversibly aggregated high-molecular-weight species. Such species also appeared after mashing in polyvinylpyrrolidone-treated wort; this treatment also prevented the precipitation of any subunits originally dissolved during the protein rest. Gallic acid (but not tannic acid, which had general protein-precipitating action) added to polyvinylpyrrolidone-treated wort caused selective protein precipitation similar to that caused by the mash temperature program. After boiling there were only two major protein subunits remaining in the wort; these remained unchanged through fermentation and finishing and were present in all the commercial beers we examined.

Key words: Beer, Haze, Mashing, Precipitation, Protein, Wort

We have shown previously that in a temperature-programmed mash protein first dissolves during the protein rest and is then lost by precipitation as the mash temperature approaches 70° C (5). The precipitate is over 60% protein, with some polyphenol and carbohydrate, and accounts quantitatively for the protein lost during mashing (9). Protein is additionally lost during kettle boiling and wort cooling in the form of hot and cold breaks, but the amount precipitated is much less than in mashing (5). The protein complexes that are precipitated in mashing may be the origin of the "top dough" which affects lautering efficiency and extract recovery (7). Calcium affected the amount of protein that dissolved during mashing but was not involved in protein precipitation; precipitation was more efficient in thicker mashes (9). Precipitation of protein occurs in the presence of polyphenols at conversion temperatures; protein aggregation may precede interaction with polyphenols, which are necessary for a rapid precipitation in mashing (8).

Although our previous work demonstrated that from one-third to one-half of the dissolved protein precipitates in mashing, we could not demonstrate that all proteins precipitate equally or whether protein precipitation is a selective process. If this were true, then variation in precipitation (which we have previously shown to occur) could account for variations, e.g., in foam and haze stability of beer. It seems, then, that the soluble protein complexes of wort warrant more specific examination. This paper utilizes sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis (PAGE) to trace specific protein subunits through the brewhouse processes from the initiation of the protein rest to the end of the kettle boil. During these processes, the protein composition of beer is defined. However, other results support previous speculation that aggregations of protein-containing species occur and may play a significant role in the colloidal character of beer.

EXPERIMENTAL

Haze Measurement

Haze is reported in ASBC formazin turbidity units (FTU); haze was measured in a standardized radiometer haze meter, type UKMID, using a 200-ml sample read at 20° C in 55-mm cuvettes. Samples were diluted 1:1 (extract:deionized water) to keep haze readings within the scale of the instrument. The method, Beer 26, "Formazin Turbidity Standards" (*ASBC Methods of Analysis*), was initially used to standardize the haze meter (1). AMCO AEPA-1 turbidity standards, from Advanced Polymer Systems, Inc. of Redwood City, CA, were also used for haze standardization and, in addition to providing excellent correlation to formazin standards, had a much longer shelf life.

Brewhouse Trials

Mash trials were conducted according to ASBC method Malt 4, "Extract Determination, Fine Grind" (1), except that the mash temperature program was modified to rise at a rate of 1.5°C/min between the protein rest and the conversion phases of mashing. Two-rowed malt (Klages) was used; the water to malt ratio was 8:1 (400 ml water plus 50.0 g malt). A six-cup, stainless steel, ASBC standard mashing bath provided continuous agitation and temperature control. Before boiling, wort volume was measured to allow dilution with deionized water to the original volume after boiling.

In certain trials the cold water extract (CWE) was mashed. CWE was produced by extracting finely ground malt with water (1:8 ratio) for 30 min at 22°C. This extract was filtered, and the filtrate was subjected to a normal mash temperature program.

To simulate kettle boiling, filtered wort was brought to a full boil in a 750-ml Erlenmeyer flask for 15 min. Whole Cascade hops, which were calculated to yield 18 ASBC bitterness units, were added at the beginning of heating. Following the boil, the wort was cooled to 20°C in a constant temperature bath and filtered.

Post-Brewhouse Trials

For fermentation, an actively growing culture of *Saccharomyces carlsbergensis* (UCD strain #C-421) was pitched at a rate of 50 mg dry basis per 100.0 ml of wort. Fermentation flasks were fitted with air locks and held at 15°C for seven days. Conditioning consisted of moving the flask to a 0°C room, storing it for seven days, and filtering the beer at 0°C.

All samples were taken at the end of the processing stage identified in the text.

Protein and Polyphenol Analysis

Protein was determined using the Bradford method (2) standardized with known concentrations of bovine serum albumin (Sigma Chemical Co., St. Louis, MO). The dye reagent (Coomassie Brilliant Blue G-250) was from Bio-Rad Laboratories, Richmond, CA. Coomassie Brilliant Blue R-250 was used to stain protein bands on SDS polyacrylamide gels after electrophoresis.

ASBC methods Beer 35, "Total Polyphenols" (International Method) (1) was used to determine the polyphenol content of the wort and beer samples.

Gel Filtration

Wort and beer samples were fractionated on the basis of molecular weight by using a Sephadex G-100 gel packed into a K16/70 column (Pharmacia Fine Chemicals, Piscataway, NJ). Specifications for the column used were: column diameter, 1.67 cm; cross-sectional area, 3.80 cm^2; bed height, 45 cm; bed volume, 118 ml; void volume, 55.3 ml; and flow rate, 0.2 ml/min. A Sigmamoter peristaltic pump (model AL4F, serial no. 12324) set at an input rate equal to the column flow rate was used to introduce the eluant. The eluant was a 0.1 *M* phosphate buffer, pH 5.5, which contained 0.02% sodium azide as a bacteriostat. Fractions were collected in an LKB Redirac collector, which was calibrated in a time mode to separate 1.0-ml samples. The void volume was determined using a solution of blue dextran 2000 (2 mg/ml). A graph of fraction number versus log molecular weight of known protein standards was used to estimate the molecular weight of proteins.

Electrophoresis

The discontinuous SDS-PAGE system of Laemli (4) was used to characterize protein subunits of macromolecules in selected samples. A 0.75-mm, 10% polyacrylamide slab gel was found to give optimum results. Sample size was 0.10 ml, and separation was done at a constant 250 V. Separation was ended when the tracking dye reached the bottom of the slab. As in any exclusionary method, separation of protein on the gels occurred only in the area between the gel top and the tracking dye in the front, referred to as 0 and 1.0 relative distance traveled, respectively. The exclusionary limits for our 10% gel were approximately 70,000 to 16,000. Protein was always noted in the tracking front throughout processing (and is shown in some figures) and comprised protein species at or below 16,000. Molecular weights were determined using proteins of known molecular weight to construct a standard separation; there was a linear relationship between relative distance traveled and the log of molecular weight.

RESULTS AND DISCUSSION

Processing Samples

SDS is an anionic detergent that denatures proteins by disrupting hydrogen bonds and hydrophobic and hydrophillic interactions. 2-Mercaptoethanol reduces bonds such as disulfide linkages and, upon boiling with SDS, the mixed reagents assure disaggregation of proteins into stable subunits. Association with SDS gives each protein subunit a uniform negative charge density; the total charge is proportional to the length of the denatured protein chain. The charged proteins move in response to an electric field through a polyacrylamide gel slab of known porosity; this separates the proteins on the basis of their molecular weight. This technique is useful for examining the subunits of proteinaceous macromolecules. SDS electrophoresis has been previously shown to be selective enough in its analysis of polypeptides to allow varietal identification of barley (10).

Lewis and Wahnon reported that proteins dissolve and then precipitate during mashing (9). To examine in more detail the behavior of proteins in mashing, samples taken at frequent time intervals were separated by SDS-PAGE (Fig. 1). At least 11 distinguishable bands of protein subunits dissolved at different rates during the first 30 min of mashing at 40°C. Six of these bands disappeared by the end of mashing; the remaining protein was primarily contained in two bands of 30,000–40,000 mol wt, although four rather faint bands were also present. Lewis, Krumland, and Muhleman observed that more protein was lost in mashing than in boiling (6). SDS-PAGE revealed that during boiling, only the four faint protein bands disappeared (Fig. 2). From the end of boiling to the end of conditioning, the protein fraction of wort and beer was characterized by the presence of those two protein subunit bands that dominated in wort after mashing. These bands have a molecular weight of approximately 30,000 to 40,000 and were identical in hopped and nonhopped worts and beers. These major bands not only comprised the majority of protein in our worts and beers, but were present identically in all the domestic and imported beers we examined. The similarity of protein subunits in a wide variety of beers suggests that variations

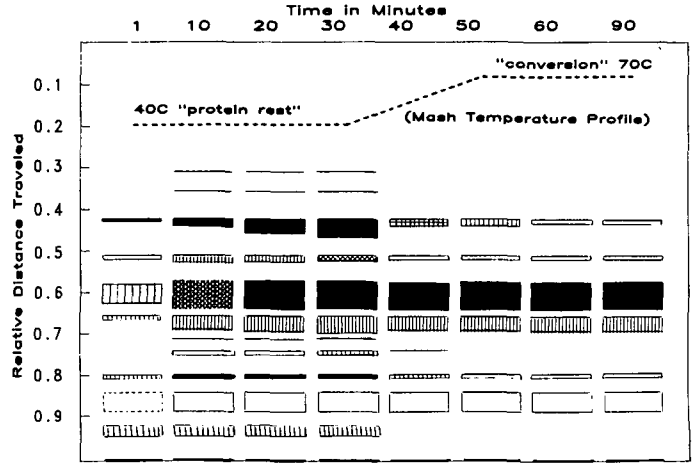

Fig. 1. Separation on a sodium dodecyl sulfate polyacrylamide gel of protein subunits present in mash samples taken at the times shown. The mash temperature profile used is shown.

in those beer qualities derived from the colloidal behavior of protein is caused by the variable nature of complexes and aggregates formed from these subunits.

Hejgaard (3) used immunoelectrophoretic techniques to analyze a barley protein referred to as protein Z; its molecular weight was estimated to be approximately 40,000. This protein was thought to be much less affected by brewing processes than any other soluble barley protein and was found in partly modified forms in beer. Because of their dominance in beer and similar molecular weights, it is likely that the two broad bands we identified in Figure 2 include protein Z. We previously speculated on the existence of a compound that enhances the colloidal stability of beer (6). Because protein Z is thought responsible for solubilization of other proteins (incuding β-amylase), it could also be involved in the inhibition of interactions resulting in protein precipitation, e.g., chill haze in beer.

The SDS-PAGE method disrupts aggregates of proteinaceous subunits that may occur in beer and reveals only the subunits themselves. In contrast, Sephadex gel filtration allows the separation of the proteins in their normal (aggregate) state in beer. Sephadex gel filtration (Fig. 3), before and after mashing, revealed a similar molecular weight distribution to that reported previously (8). The proteins separated ranged in molecular weight from approximately 4,000 to more than 150,000. Protein precipitation in mashing preferentially removes larger molecular weight aggregates from the wort.

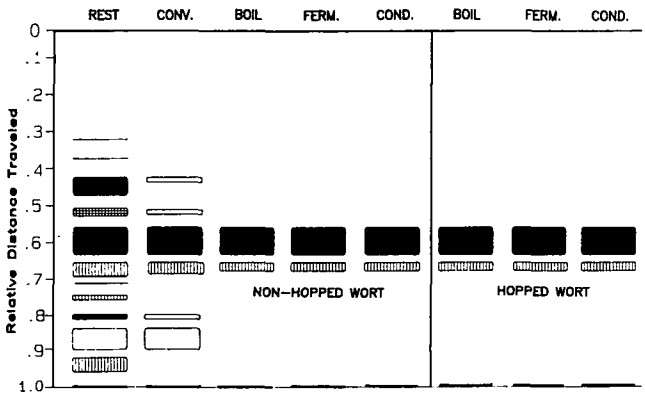

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoretic separation of protein subunits in samples of hopped and unhopped wort taken from the indicated beer processing stages.

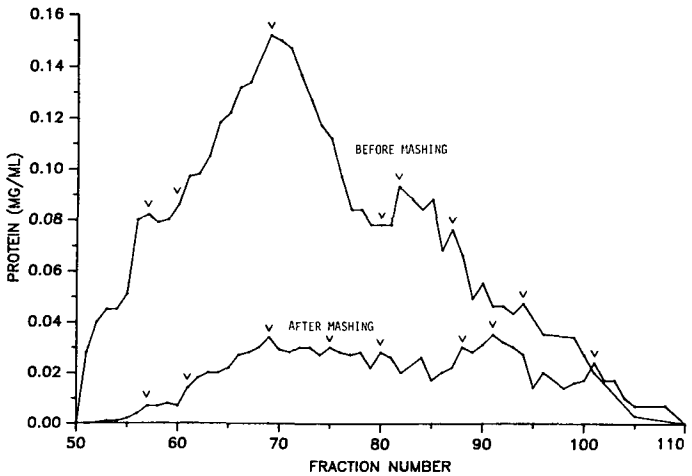

Fig. 3. Separation on Sephadex G-100 of proteins in wort samples taken before and after mashing. Fractions selected for sodium dodecyl sulfate *polyacrylamide gel electrophoretic analysis are indicated with a* **V**.

The proteins in the Sephadex fractions could comprise either different autonomous protein species or different aggregates of lower molecular weight subunits. When the proteins of selected Sephadex fractions were separated on SDS-PAGE, the bands separated ranged from approximately 70,000 (at the top of the gel) to 16,000 (at the bottom of the gel) (Figs. 4 and 5). That there was some separation of autonomous species is shown by the appearance of lower molecular weight subunits (0.7 and higher) in the later fractions (#80 onward) and higher molecular weight subunits (lower than 0.4) in the early fractions (#63 and earlier) (Figs. 4 and 5). However, some of the subunits, especially those near 0.4, appeared in every fraction; this suggested that substantial aggregation occurred and that the potential for the subunits to aggregate survived the mashing process.

We have demonstrated earlier that protein disappearing from wort during mashing is lost by precipitation rather than by enzymatic action. Therefore, those proteins lost from solution should appear in the precipitate. This proved to be the case (Fig. 6); the protein lost during conversion and the protein lost during boiling (hot and cold breaks) appeared specifically on SDS gels of the redissolved protein precipitates. Because there appeared to be no formation of new, low-molecular-weight protein subunits, these results further supported the previous argument (9) that protein is

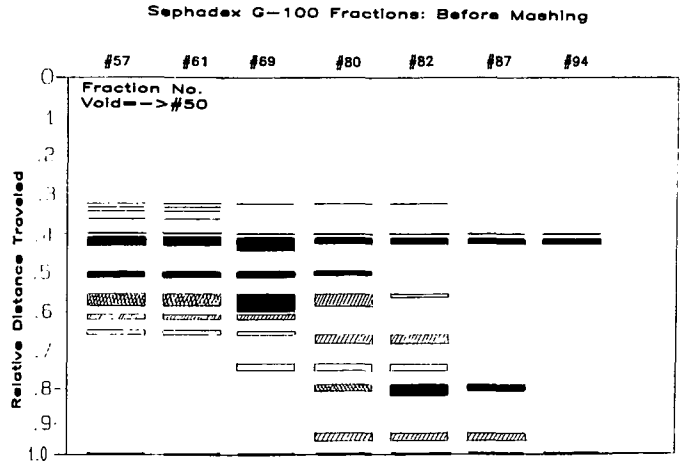

Fig. 4. *Sodium dodecyl sulfate polyacrylamide gel electrophoretic separation* of protein subunits present in selected fractions from the "Before Mashing" Sephadex G-100 gel filtration shown in Fig. 3.

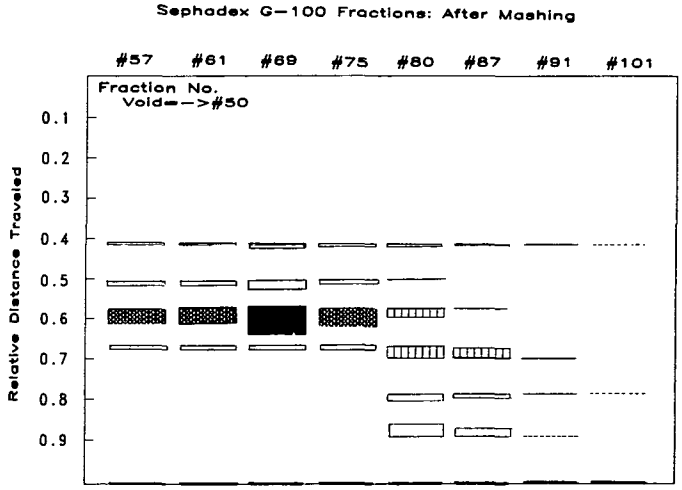

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoretic separation of protein subunits present in selected fractions from the "After Mashing" *Sephadex G-100 gel filtration shown in Fig. 3.*

lost by precipitation not by action of proteolytic enzymes. However, the precipitates formed during mashing and boiling were not completely dissociated by normal treatment of the samples (i.e., boiling in a solution of 2-mercaptoethanol and SDS); significant amounts of high-molecular-weight material appeared at the top of the SDS gels. The precipitate samples were treated in varying ways with SDS and 2-mercaptoethanol to be sure that weak bonds and disulfide bonds would have the greatest chance of dissociation. That we were unable to resolubilize all of the precipitated protein indicates that the precipitates contain newly formed aggregated species that either are bound together by bonds stronger than hydrogen, hydrophobic/hydrophillic, and disulfide bonds, or that they consist of new complexes which resist the actions of SDS and 2-mercaptoethanol. How these interactions during conversion and boiling occur, their nature, and possible significance remains a topic for further investigation. This study also indicated that the dominant protein subunits at 0.6 and 0.7 mobility (30,000–40,000 mol wt) were precipitated to some extent in mashing and boiling.

Mash Affectors

Mash proteins react differently to temperature and, depending on mash temperature, more or less protein is lost from wort by precipitation (9). The protein band patterns were characterized for three isothermal mashes conducted for 90 min at 50, 60, and 70° C, respectively (Fig. 7). The band patterns were relatively constant throughout the mashes. Whereas all three mashes yielded wort of identical protein composition after boiling, there were considerable differences in the proteins present in wort during mashing, depending on mash temperature. Generally, fewer protein bands were present in wort made at mash temperatures above 50° C, and wort from the 70° C mash contained very little of those protein bands normally precipitated during boiling (Fig. 7). Although the proteins remaining in beer are merely those not lost by boiling (because boiling precipitates atypical wort proteins), the mash temperature profile markedly affects the number and kind of proteins that are (at least temporarily) in solution during mashing. If we assume that those proteins in solution are those that can react during mashing (e.g., with polyphenols or enzymes or carbohydrates), then the mash profile may have a significant effect on wort nitrogenous components.

The effect of calcium ions on protein dissolution in mashing, described in previous work (9), was examined by SDS-PAGE; the same bands were present in wort whether or not calcium was added.

When Lewis and Serbia (8) treated cold water extract (CWE) with a large amount of polyvinylpolypyrrolidone (PVPP) to remove polyphenols, they observed increased wort haze, much less protein precipitation, and the formation of larger protein aggregates when the treated CWE was mashed. We repeated this study using SDS-PAGE to reveal details (Fig. 8). Untreated and PVPP-treated CWE samples contained the same protein subunits at the end of the protein rest; during the mash temperature incline to conversion temperature (70° C), some proteins wholly or partly precipitated in the untreated CWE in a way identical to that shown for the whole mash in Figure 2. In contrast, no subunits completely precipitated in the PVPP-treated CWE, and wort after conversion had the same basic protein composition as wort at the end of the protein rest. In addition, higher molecular weight proteins appeared at the top of the gels of PVPP-treated CWE (Fig. 8) that were highly reminiscent of the proteins recovered from the mash precipitates in Figure 6. These observations agree with and expand those of Lewis and Serbia (8).

These data from PVPP-treated mashes suggest that potentially precipitable wort proteins are less susceptible to precipitation in the absence of polyphenols, and under these conditions form high-molecular-weight aggregates. These aggregates may involve only proteins but could include other species such as carbohydrates. Whether lack of precipitation in PVPP-treated wort results from the absence of polyphenols or from the formation of the large aggregates, which could act as a protective colloid, is not clear, but obviously those materials removed by the PVPP treatment significantly affect protein behavior. Furthermore, protein precipitation in mashing emerges as a significant mechanism for preventing polyphenols advancing through the brewhouse into beer. Curiously, PVPP-treated CWE after conversion (Fig. 8) is very similar, in terms of its protein subunits, to the composition of the mash precipitate shown in Figure 6.

Gallic acid and tannic acid, when added to PVPP-treated CWE, caused precipitation of wort proteins (8). Using the SDS-PAGE method, we found tannic acid lacked specificity and precipitated

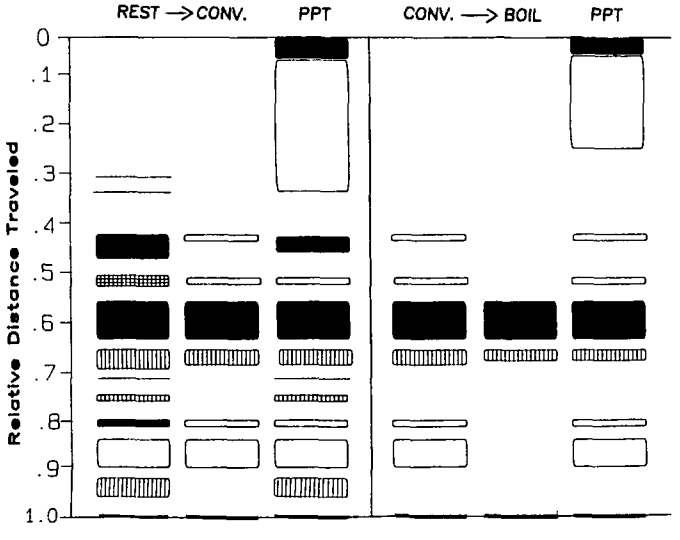

Fig. 6. Separation on a sodium dodecyl sulfate polyacrylamide gel of proteins present in the proteinaceous precipitate (PPT) formed during mashing (conversion) and boiling.

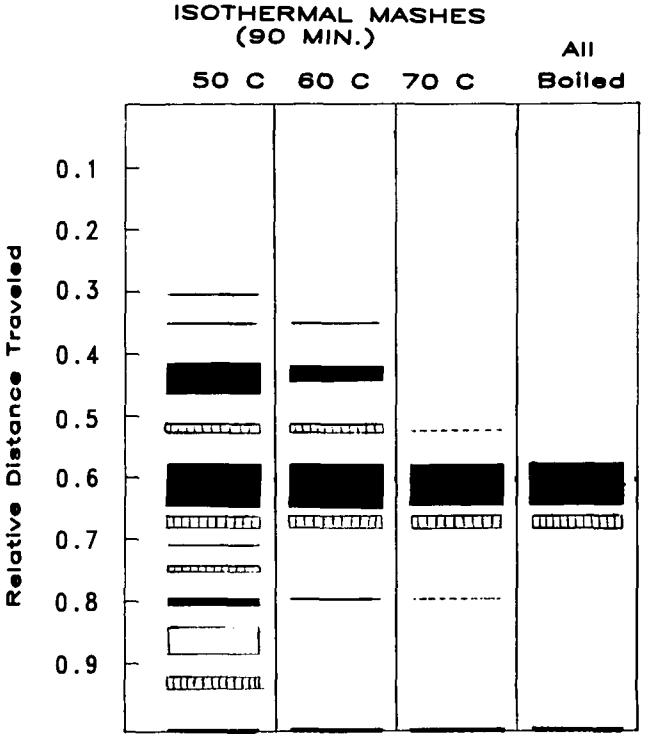

Fig. 7. Separation on a sodium dodecyl sulfate polyacrylamide gel of protein subunits present in wort from three isothermal mashes (50, 60, and 70° C). All worts were identical after boiling.

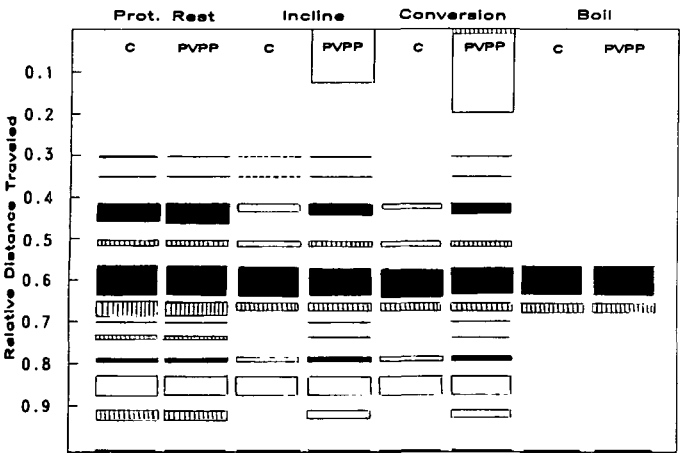

Fig. 8. Effect of polyvinylpyrrolidone (PVPP)-treatment of cold water extract on the protein subunits remaining in the wort during a temperature-programmed mash as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. At each stage, the treated sample (**PVPP**) is compared to its control (**C**).

large amounts of all the proteins present (Fig. 9). Gallic acid, on the other hand, precipitated specific proteins, primarily those that normally precipitate during the mash temperature program. Gallic acid treatment also caused formation of soluble species of high molecular weight which remained at the top of the SDS gel. With the exception of this high-molecular-weight material, gallic acid-treated CWE contained the same protein subunits as normal wort at the end of mashing. Although gallic acid has not been detected in malt (it is present in hops), and such simple molecules are not generally thought to react with proteins in brewing, Lewis and Serbia (8) suggested that gallic acid is a good model for the phenolic materials that precipitate wort proteins. The SDS-PAGE method confirms and specifies that work.

Summary

The SDS-PAGE method confirmed the general pattern of protein behavior we previously identified in mashing and provided additional support to our suggestion that proteins can aggregate before reaction with polyphenols under the influence of the mash conversion temperature or boiling. The method, however, permits us a more specific view of the nature of the proteinaceous make-up of wort and beer, showing that the brewing process, especially mashing (and boiling), controls both the amount and kinds of proteins that survive into beer. There are at least two subunits in the 30,000–40,000 mol wt range (plus uncharacterized material at or below 16,000); aggregates of these protein subunits account for those beer qualities we attribute to the presence of protein (e.g., foam and chill haze).

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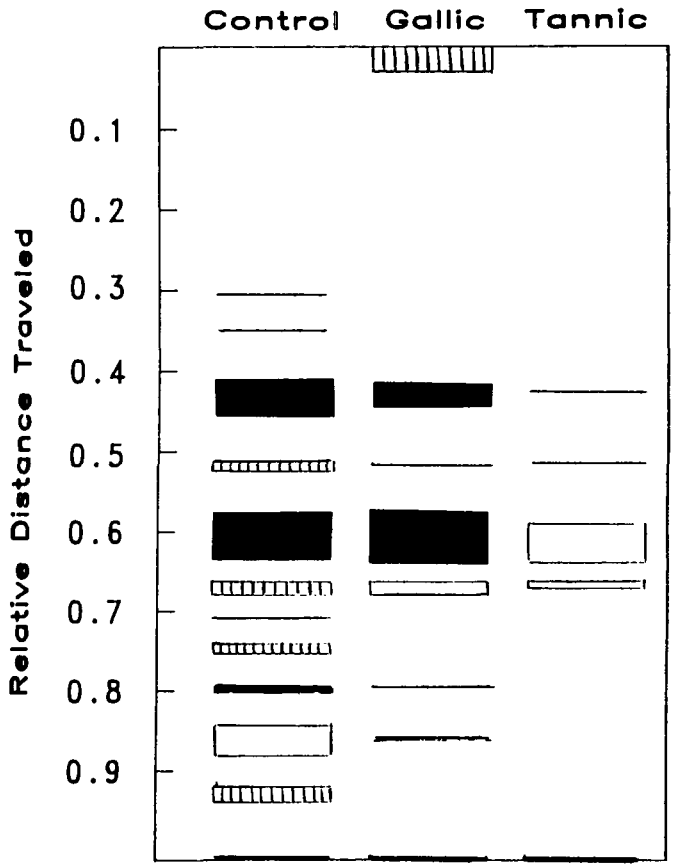

Fig. 9. Effects of precipitation by gallic acid and tannic acid on the protein subunits present in cold water extract, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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