

# Mechanisms and prevention by proline-specific proteases

**CHILL-HAZE FORMATION** | An understanding of the chemistry involved in the formation of chill-haze has led to the development of a unique enzymatic route to increase the colloidal stability of beer. In this approach, proline-rich, haze-active proteins are selectively degraded by a proline-specific protease.

## COMPUTER MODELLING STUDIES

suggested that the resulting low molecular weight peptides formed during this hydrolysis are unable to form precipitating networks with the polyphenols present. Using a food grade version of the proline-specific enzyme, the reliability of the new method was confirmed in a number of 20 hl experiments.

As predicted, the beers developed using these enzymes (now commercialised as Brewers Claretex) showed an excellent colloidal stability. In addition, adverse effects on beer foam stability are minimised due to the high selectivity of the enzyme. Industrial application of the new enzyme approach will greatly simplify the brewing process and offer significant cost advantages – by eliminating bulk powders such as polyvinylpyrrolidone (PVPP) and silica hydrogel, expensive dosing and powder regenerative equipment are made redundant and handling, storage and disposal problems are avoided.

## Proteins in chill-haze formation

During beer fermentation and maturation, colloidal precipitates are formed that may lead to chill haze in bottled beer. These colloidal precipitates represent aggregates between proteins and polyphenols that are extracted from malt and hops. In the beer

making process the larger part of these precipitates is formed during the cold maturation phase. The remaining polyphenols or proteins that could lead to chill haze formation in packaged product are typically removed by PVPP or silica hydrogel treatments prior- or post-filtration.

It has been established for many years that only so-called 'haze active proteins' can aggregate with polyphenols. The literature available implicates the high proline contents of these haze activity proteins in the precipitate forming process (1). In beer, the haze active proteins predominantly originate from the proline-rich alpha-gliadin (hordein) fraction of malted barley.

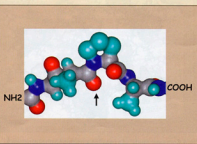
Proline is a distinctive amino acid in many aspects (2). One of its unique features is its side chain that is bound to the amide nitrogen hereby introducing a five membered pyrrolidine ring structure into the peptide chain (Fig 1). Where other amino acids each contribute two rotatable bonds to a peptide

chain, proline contributes only one due to this ring structure. Additionally the presence of the bulky pyrrolidine ring restricts the rotational freedom of the amino acid preceding the proline. As a result proline residues impart a rigid and extended conformation upon a peptide chain in such a way that the pyrrolidine ring is maximally exposed to the solvent. Multiple prolines in a sequence will give rise to even higher rigidity and polyprolines are believed to adopt only a single preferred conformation in solution known as the 'polypyrrolidine II helix'.

Because of the pyrrolidine ring structure, the peptide bond preceding proline lacks the hydrogen on the amide nitrogen. As a result the carbonyl oxygen preceding the ring structure (indicated by an arrow in Figure 1) has become a stronger hydrogen bond acceptor. The latter feature explains that despite their hydrophobic nature, polyprolines are often located in solvent exposed regions of proteins. It also explains why this carbonyl oxygen easily engages into hydrogen bonding with the hydroxyl groups of polyphenols.

It is generally assumed that the formation of large protein-polyphenol aggregates is mainly driven by hydrophobic interactions (3, 4). Other interactions, including the above mentioned hydrogen bonding interactions, may further stabilise the aggregates. The hydrophobic interactions are

**Fig. 1**  
Computer model representing a proline residue in a peptide chain. The hydrophobic five membered pyrrolidine ring structure is clearly visible. The hydrogen bonding carbonyl oxygen (red) immediately preceding the pyrrolidine ring structure is indicated by an arrow. Amide nitrogens are shown in blue



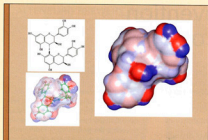


Fig. 2 Computer models of the catechin dimer procyanidin B3. Hydrophobic patches on the polyphenol molecule are shown in white. The phenolic hydroxyl groups which might be involved in hydrogen bonding with proline-rich proteins are shown in red (oxygen) and blue (hydrogen)

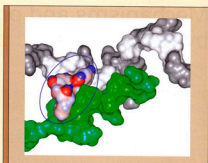


Fig. 3 Computer model of a polyphenol bridge (encircled) cross-linking two peptide chains via proline residues present in each of these chains. The proline residues are shown in green and white

caused by the exposure of the hydrophobic proline pyrrolidine ring that tends to stack face-to-face with the aromatic rings present in polyphenols. Although side chains of other amino acids such, as for example, phenylalanine exhibit a hydrophobic ring too, the restricted mobility, the extended nature and the solvent exposure of proline rich regions are believed to be decisive for their unique polyphenol binding ability.

### Polyphenols in chill-haze formation

Polyphenolic substances such as the flavonoids are present in most plants being concentrated in their seeds, fruit skin or peel.

bark and flowers. Although subject to some debate, it is recognized that such polyphenols add to the oxidative and flavour stability of beers (5, 6, 7).

In polyphenol-based flavonoids two benzene rings on either side of a 3-carbon ring form the common theme. Additional hydroxyl groups, sugars, oxygen and methyl groups create various classes of flavonoids such as flavanols, flavanones, flavones, flavan-3-ols, anthocyanins and isoflavones. Haze-forming polyphenols have at least two binding groups, each of which has at least two hydroxyl groups on an aromatic ring (8). Catechin represents the monomeric building block of the more complex polyphenols found in beer. Both procyanidin B3 and prodelphinidin B3 represent catechin dimers. Procyanidin C2 incorporates three catechin moieties. Together the latter three polyphenols represent some 80% of the phenolic content in barley (9). Three-dimensional computer models for the major polyphenols found in beer were built. The model of the catechin dimer procyanidin B3 (Fig 2) clearly shows the presence of large hydrophobic patches interrupted by polar hydroxyl groups.

Taking all available data into account, the possible ways how proline-rich peptides could interact with procyanidin B3 were explored using computer modelling techniques. The molecular details of proline-polyphenol interactions have been studied

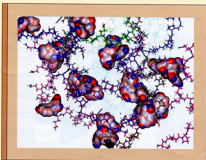


Fig. 4 Computer model of a possible protein-polyphenol network

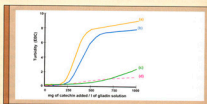


Fig. 5 Model experiment showing haze formation obtained by combining different gliadin preparations with various levels of catechin. Wheat gliadin was used as such (a) or after a pre-incubation with either the neutral protease from *B. amylioliquefaciens* (b) or the proline-specific oligopeptidase from *F. meningo-septicum* (c). The haze formed by adding just the buffer used for dissolving catechin to non-hydrolysed gliadin is shown by (d)

led by titrating a proline rich peptide with different polyphenols and identifying the products formed by NMR (10, 11). Based on their results, hydrophobic interactions were maximized through a face-to-face stacking of the proline pyrrolidine and the procyanidin B3 aromatic rings. The degrees of freedom were significantly reduced when we tried at the same time to form a hydrogen bond between the carbonyl oxygen preceding the proline and one of the hydroxyl groups of the polyphenol. The data obtained showed that the binding of procyanidin B3 covers about three amino acids and indicated that more procyanidins B3 could be bound to the peptide by ensuring that the polyphenol binding sites were at least 3 amino acids apart. Our modelling experiments also revealed that a single procyanidin B3 molecule bound to a proline containing peptide can bind a second proline containing peptide chain. The implication of this observation is that via this second binding site, procyanidin B3 can cross-link peptide chains into complex networks (Fig 3).

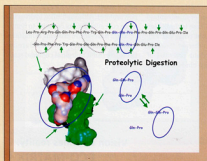
Diffusion experiments have indicated that yet another mechanism drives network formation. It was observed that prior to dimerization and precipitation proline-rich peptides become already increasingly coated with polyphenols (11). Above a certain polyphenol loading, polyphenol-polyphenol self-associations can be expected to add to the stability of protein-polyphenol complexes. So cross-linking of peptide chains occurs concurrently via direct polyphenol bridging and via polyphenol-polyphenol self-association. A computer model of a possible peptide-polyphenol network resulting from such interactions is shown in Figure 4. Such peptide-polyphenol networks can be expected to precipitate as chill haze under low temperature conditions.

### Chill-haze prevention in beer making

Among the various options available, the use of adjuncts is the most likely to make a significant contribution towards lowering the chill haze potential of beer. The protein fractions present in maize and rice have relatively low proline contents, diluting the level of haze-active proteins that are extracted from the malt. However, chilling the beer to approx 0°C in a maturation vessel, adding PVPP and/or silica hydrogel and filtering the precipitate from the beer continues to be

the industry standard for chill haze prevention. There are a number of disadvantages of the use of PVPP. These include high in-use costs, high capital costs if PVPP is regenerated and an inherent lowering of the natural antioxidant potential of beer as PVPP selectively binds with any

polyphenols present (PVPP resembles polyproline). Although silica hydrogel is more cost effective, its capacity to remove residual haze-active proteins is limited so that beers clarified by this method are only likely to exhibit marginal colloidal stability (13).



**Fig. 6** Schematic representation of 2 hordein derived peptide sequences and possible polyphenol bridging sites (encircled). Arrows indicate the potential cleavage sites of a proline-specific protease. The double arrow illustrates the weak binding of polyphenols to oligopeptides containing a single proline residue only

The use of an acidic proteolytic enzyme such as papain presents an alternative beer stabilising option. In this approach the residual haze-active proteins are degraded so that formation of the protein-polyphenol network occurs less effectively. However, none of the enzymatic activities present in papain can efficiently hydrolyse proteins with high proline contents. As a result papain is sometimes overused, diminishing beer foam quality.

### The hypothesis

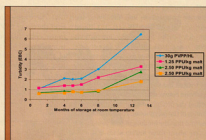
As outlined above, beer haze formation strongly depends on a hydrophobic interaction between polyphenols and proline-rich proteins. By reducing the length of these proline-rich regions via hydrolysis with a

suitable proteolytic enzyme, the formation of precipitating networks should be effectively prevented. Although there was a possibility that the very short oligopeptides generated would still bind polyphenol, it was thought that the resulting small complexes would be so soluble that no precipitation would occur upon cooling. Challenging this hypothesis would require the availability of an enzyme that can cleave proline-rich proteins into very small fragments. To avoid hydrolysis of beer foam proteins, the protease of choice should be highly specific.

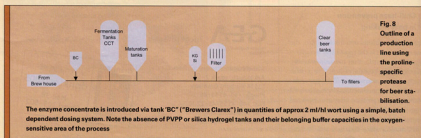
### Challenging the hypothesis

To test our hypothesis we started out with a model experiment in which chill haze formation was limited by slowly adding

catechin to a stirred solution of gliadin, a proline-rich wheat protein (14). The haze formed was followed using a calibrated tannometer. Upon the addition of increasing amounts of catechin to a solution of intact gliadin, the haze of the solution increased before levelling off (Fig. 5). The experiment was repeated but this time we used a gliadin solution that had been pre-hydrolysed with a neutral protease from a *Bacillus* species ("PNR" from DSM Food Specialties). The latter enzyme is unable to cleave peptide bonds involving proline but focuses its attacks on peptide bonds involving leucine and phenylalanine residues, neither of which have been implicated in polyphenol-protein interactions. Despite long-term incubations with sufficiently high enzyme



**Fig. 7** Colloidal stability of bottled beers stored at room temperature for 1, 4, 5, 8, 13 and 14 months. The beers were produced at 20hl scale and stabilized with either PVPP (30 g/hl) or the proline-specific endoprotease in concentrations of 1.25 and 2.5 enzyme units (PPU)/kg malt. Duplo's of the 2.5 PPU/kg malt dosage illustrate the reproducibility of the enzyme method. Turbidities were measured using a calibrated tannometer



**Fig. 8** Outline of a production line using the proline-specific protease for beer stabilisation.

The enzyme concentrate is introduced via tank "BC" ("Brewers Claes") in quantities of approx 2 ml/hl wort using a simple, batch dependent dosing system. Note the absence of PVPP or silica hydrogel tanks and their belonging buffer capacities in the oxygen-sensitive area of the process

levels, the pre-digestion of gliadin with the neutral protease hardly affected the level of gliadin-catechin haze formed. However upon pre-hydrolysing the gliadin with a proline-specific enzyme (prolyl oligopeptidase from *E. meningosepticum*, (15), haze formation was suppressed to a level as recorded for the non-hydrolysed gliadin solution i.e. without catechin addition. These results clearly support the hypothesis that only a proline-specific protease can effectively prevent precipitating networks of proline-rich proteins and catechin (16).

Figure 6 shows the amino acid sequences of two different proline-rich hordein-derived peptides and illustrates the results obtained in this model experiment. For clarity, arrows have been added to indicate potential cleavage sites for a proline-specific protease, while sites where the two peptide chains can be cross-linked by polyphenols have been circled. Evidently incubation of these hordein-derived peptides with a proline-specific protease will yield a mixture of many, very small peptide fragments.

The results of the experiment show that these very small peptide fragments cannot precipitate with catechin. We assumed that with small peptide fragments it is difficult to get a large protein-polyphenol network and the small peptide fragments are much more water soluble than the intact haze-active proteins.

In contrast with the many potential cleavage sites for a proline-specific protease, the neutral protease cannot be expected to introduce even a single cut in the two hordein sequences shown. Likewise papain is unable to cleave these hordein sequences. Although papain is known to prefer cleavage next to a.o. arginine residues, hydrolysis of the arginine containing peptide bonds is prevented by the many proline residues present in the hordein sequences shown.

### Large scale brewing experiments

Having demonstrated the profound impact of a proline-specific protease on haze formation, we continued our studies with an enzyme more akin to practical beer brewing

considerations. In earlier studies we identified a proline-specific endo-protease secreted by the microorganism *Aspergillus niger* (17). In contrast with known proline-specific proteases, the *A. niger* derived enzyme is food-grade, has an acid pH optimum, is a pure endoprotease and is available on an industrial scale. Various laboratory and microbrewing tests were performed in order to challenge this enzyme in the complex environments of cereal mashing, hop additions and boiling, fermentation, maturation, filtering and bottling.

Convinced of the quality of the new enzyme, various 20 hl trials were set up at the Institut Français des Boissons de la Brasserie Malterie (IFBM) in Nancy, France. Four pure malt beers were brewed under exactly the same conditions. Each brew was produced from 300 kg of barley malt and hop pellets.

The fermentation process with a bottom yeast strain was at 12°C until 5 Plato and at 14°C until the end of fermentation. Following cold maturation for 5 days at 1°C

+/- 1°C, the beers were carbonated to 5.2 g/l and pasteurized at 60°C for 20 min.

In three of the four trials, two different concentrations of the *A. niger* derived proline-specific protease were used. The enzyme was added at the beginning of fermentation and no PVPP or silica hydrogel was used.

A 'control' brew (without enzyme) used 30g/l of PVPP (Polyclear AT) added with the filter aid. After bottling and pasteurisation samples were stored for up to 13 months at ambient temperature with haze readings being taken at various intervals.

After 13 months storage at room temperature the haze of the enzyme-treated beers proved to be significantly lower than that of the control in which PVPP was used (Fig. 7). The results of these semi-industrial scale beer brewing experiments validated the results of the model experiments, and confirmed the hypothesis that chill haze can be effectively prevented by using a proline-specific protease.

Further semi-industrial scale trials have also demonstrated that the use of the proline-specific protease results in negligible effects on taste and beer foam stability (16). The very low proline content of foam active beer proteins (18) in combination with the very high specificity of the enzyme used, provide an acceptable explanation for this observation. Since polyphenols are not removed in this enzymatic approach, it is not surprising that we have also been able to confirm enhanced polyphenol levels in the bottled beer.

### Considerable simplification of beer production

The use of a proline-specific protease during the beer fermentation phase offers a number of significant advantages—in particular, the benefits to be gained on the beer processing side are very interesting. For example, upon the addition of a small volume of concentrated enzyme solution to the fermentation tanks (approx 2 ml/hl wort 12° Plato), only minimal time is required in the cold maturation vessel. Additionally the beer filtration capacity is increased because less precipitate is formed.

Most importantly, however, bulk powders like PVPP or silica hydrogel could be

completely eliminated. Apart from the obvious handling, storage and disposing considerations, the use of PVPP or silica hydrogel powders demands great care. For example, during PVPP pre-hydration, there is a significant risk of oxygen ingress during powder additions and in the cleaning and sterilisation of the equipment used. The use of the enzymatic route would abolish the requirement for such stringent oxygen control.

DSM's protein-specific protease enzyme has now been commercialised by DSM Beverage Enzymes under the name "Brewers Clarex" and an outline of a production line using the enzymatic route for chill haze prevention is shown in Fig. 8. Because of its simplicity, the enzyme route could provide an attractive option in case production volumes need to be raised temporarily.

Finally the newly devised enzyme option allows manufacturers to initiate planning for the elimination of powders from the beer brewing process, in line with growing acceptance that Kieselguhr-free filtration will gain in popularity in the coming years.

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