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125th Anniversary Review: The role of proteins in beer redox stability

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Flavour stability is usually approached through inhibition of reactive oxygen species (ROS). It may be possible to suppress ROS, but never to entirely eliminate them in packaged beer. The role of proteins in ROS suppression seems to have been lost in the compliant acquiescence to supply haze-free bright beer. Proteomics allows beer polypeptides to be finely resolved, identified and correlated with beer quality and stability. This has already produced a broader view of what stabilizes beer foam. No doubt it could do the same for beer stability and the broader roles that proteins, such as LTP1, can have in redox reactions and free radical suppression. Cysteine oxidation and reversibility is central to cellular signalling in biological systems. Thiol chemistry is also integral to beer redox stability. We can, and should, extrapolate the recent biological findings to the simple pleasure of creating a high-quality beer.

Keywords: beer proteome; LTP1; oxidation; redox; thiol proteins; thioredoxin; flavour stability

Could this mean the world is changing?

It is always a pleasure to read of researchers who do not waver from the economic imperative, making money. It goes a long way towards correcting public misconceptions. In late 2010, Pier Righetti and his colleagues *(1)* spoke about their ground-breaking proteomic work in beer to *Science Daily (2)*. They identified 20 barley proteins, 43 proteins from yeast and two related to corn, representing the largest-ever picture of the beer proteome. The journalists were impressed that the inspiration for the researchers came from a Belgian series, *Les Maîtres de l'Orge (3)* that chronicles the fortunes and saga of a family of brewers. They mention that beer ranks only behind water and tea in the list of the world's most popular drinks. That sounds a tad fanciful, but even so, the juxtapositioning of family fortune and commercial significance does heavily underscore '*economic opportunity*'. It is tempting to think (like a journalist) that this 'may give brewers a powerful new ability to engineer the flavour and aroma of beer'. Underscore '*economic advantage*', an opinion that many researchers will be happy to share.

In this review we infer that making money and high-end research using the relatively new field of proteomics are not exclusive. Proteomics is the study of proteins and function. Proteins contribute sensory expression and stability to beer and probably flavour stability. In the past it has been the problematic side of proteins that has received the publicity, mindful of a similar view, in a different context, about evil persisting and good being forgotten in Act 3 of William Shakespeare's *Julius Caesar*. This review is not about these problems *per se* and we will steer well away from physical stability. It is more a story of goodwill towards proteins and reinterpreting old stories, like Righetti using the metaphor of Jean van Hamme's, *Les Maîtres de l'Orge*.

The following piece appeared as the *ASBC Journal*'s editors' pick in the ASBC newsletter 2011 *(4)*:

> A method for quantification of thiol groups directly in beer was established based on fluorescent detection using the maleimide-derived probe ThioGlo 1. Marianne Lund and Mogens Andersen *(5)* show in their paper entitled 'Detection of Thiol Groups in Beer and Their Correlation with Oxidative Stability' that measurement of thiol compounds (both proteins and smaller molecules) using an agent called ThioGlo offers a good prediction of the flavour stability of beer.

Lund and Anderson *(5)* quantified beer thiols and compared these data with beer stability measurements obtained with electron spin resonance (ESR). Thiol concentrations between 13.6 and 46 μM glutathione equivalents in several different beers tallied with beer stability estimations, but not directly with protein thiols, even though they were part of the net thiol estimations.

Typically fresh beer contains many reduced protein thiols (PrSH), meaning that these proteins, in our vernacular, are in a reduced (redox) state *(6,7)*. However they are lost as beer ages. Peroxide then appears on cue once these thiols are oxidized and no longer visible using specific staining techniques. The reduced redox state can be restored by sulfite, which is a reducing agent. Whatever is happening, it is reversible. The conundrum is 'Can we use this information to construct more flavour stable beers?' And how can we fit the data of Lund and Anderson into

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some kind of explanation. It is with these data in mind that we have approached this review.

Conundrums and thiol chemistry

A conundrum is a riddle, the answer to which involves a pun or play on words. Or it can mean anything that puzzles. Two major clinical trials in the past three years are at odds. One says that lowering serum low-density lipoprotein levels does not always decrease heart attacks and the second says the high levels of high-density lipoproteins do not guarantee fewer heart attacks or strokes. Diet control is therefore sound, or, depending on your lipoprotein levels, a waste of time!

Beer proteins stabilize beer flavour and improve quality; is this also a conundrum? After all, reactive proteins are commonly considered a problem. Yet six-row malt dominates in North America, whereas in Australia it is almost exclusively two-row varieties, despite the significant protein differences. Proteins can be part of the root cause of physical instability *(8–11)*, and this is a view that has persisted. Or could it be that these stabilization steps are tossing out the 'problems' and the 'solutions'? Michael Lewis wrote some time ago that the protein character of beer is defined in the brewhouse *(12)*. He no doubt was not thinking of flavour stability at the time. More likely he was referring to physical stability and sensory character. (Is this a creeping conundrum as we see the craft brewers redefine old and lost styles and forge ahead with robust sometimes hazy beers?) However, returning to Michael Lewis, defining beer protein from the extremes in the brewhouse, and considering the time at which this was written, his was the *Zeitgeist* of the times. Protein and beer quality did seem to be at odds, although the positive role proteins play in foam stability was recognized early on *(13,14)*. Beer proteins have also been implicated in hydrogen peroxide formation *(15,16)*; from the 1970s, the largely indiscriminate removal of protein was in full flight *(17)*.

Approaches to beer proteomics

Proteomics opens up another view and defines protein from a broader and more collective functionality. It is about the entire protein complement of a cell or tissue *(18)*, which in our case is a complete and unbiased understanding of beer proteins. Proteomics is about streamlining established approaches – the separation and the identification methodologies – and making the most of the new biology, which allows sequencing with very small amounts of protein, and further understanding protein functions holistically. The search for the initiating steps in particle formation or antioxidant proteins in packaged beer was never easy and 'fraught' one way or another with qualifications: malt cultivars, malting style, brewhouse operation and so on. However, an understanding of the lineage of proteins, based on sequence data, provides a broader database on which to search for underlying threads. This can be easier and faster in the long run than searching for a magic bullet.

Proteomics approaches vary, but all need to end up using mass spectrometry (MS) and micro-sequencing techniques (Table 1). Righetti and his colleagues *(1)* used a combinatorial peptide ligand library to scan each beer for a large number of proteins. The method uses activated beads that can recognize and bind specific peptides. The captured polypeptides are amplified and sequenced. Others have kept to two-dimensional electrophoresis (2-DE) separation combined with mass spectrometry and sequence analysis (Table 1).

Table 1. Approaches to beer proteomics

Samples	Proteomics methodology	Protein species resolved and identified	References
Beers (including gluten-free beers)	Beers were degassed and protease-digested. The resultant peptides were analysed by HPLC, MS and database matching. Undigested beers were also analysed with LC, MS and database matching.	Seventy-nine proteins were identified, with lipid transfer family (LTP) and α-amylase/trypsin inhibitor the most abundant. Variants of hordein were delineated in detail.	*(19)*
Beers	Proteins were extracted by solid-phase extraction using C_{18} chromatography, and analysed by SDS–PAGE, 2-DE, immunoblotting, MS and database matching.	Dominant proteins (serpins, LTP); minor species (hordeins, α-amylase/trypsin inhibitor).	*(20)*
Beers	Proteins were extracted by ProteoMiner (combinatorial hexapeptide ligand library bead), then analysed by 2-DE, MS and database matching.	Twenty protein species originated from barley, 40 from yeast and two related to corn were identified.	*(1)*
Beers	Beers were firstly desalted and lyophilized. Proteins were then solubilized and analysed by 2-DE coupled with MS and database matching.	A total of 199 protein spots were resolved; 85 of them were identified, belonging to 12 protein species.	*(21)*
Barley, malt and beer	Beers were degassed and lyophilized. Proteins were solubilized and analysed by 2-DE coupled with MS and database matching.	Forty heat-stable and water-soluble proteins from barley, 41 from malt and 30 from beer were identified. Seven beer protein species were from barley, two of yeast origin.	*(14)*

The beer proteome

Proteomics, if you want to be dismissive, is something of a fishing expedition. However, as in the past, it starts with the posing of formal questions *(22)*. Iimura *et al. (23)* correlated proteins identified by 2-DE with beer foam stability across different cultivars and different modification conditions. They identified three protein 'spots' that correlate with foam stability. They extended these findings and their general applicability by showing that one was protein Z from barley and another was yeast thioredoxin. The third was barley dimeric α-amylase inhibitor-1 (BDAI-1). Both BDAI-1 and protein Z were foam-positive factors. However, yeast thioredoxin was reported as a novel foam-negative factor, so there emerges a more comprehensive and collective view of foam stability.

Identification of the complete protein composition in beer was reported in 2005 using 2-DE coupled with mass spectrometry and database matching *(1,14,21)*. It is not yet possible to identify every protein in beer, but this data collection will proceed apace if the application in other areas of science is anything to go by (Table 2). Like all methodologies, the devil is in the detail, and all protein analyses depend on the initial extraction. The protein extraction step is the key. It is easy enough to imagine that changes in protocol may lead to differences between datasets. This can only be resolved as more work is published.

An obvious feature of the beer proteome is the dominant presence of two families of proteins, the serine protease inhibitor (serpin) family, which includes protein Z, and the lipid transfer family (LTP), both of which are derived from barley. Their abundance is more evident in one-dimensional polyacrylamide gel electrophoresis *(7)*. In addition there are up to 20 barley proteins and 40 yeast proteins *(1)*.

The cysteine contents can be calculated from the primary sequences of the individual proteins from open databases such as Swiss-Pro/TrEMBL and Uniprot (http://www.uniprot.org) via the bioinformatics Resource Portal (http://au.expasy.org). The 9 kDa LTP1 contains eight cysteines, representing 9% of the total polypeptide sequence. The 7 kDa LTP2 also has eight cysteines (12%). As shown by the sequence alignment in Fig. 1, cysteine residues have been conserved amongst cereal LTPs. The cysteines in the native forms of these proteins form inter- and intra-molecule disulfide bonds *(24–27)*. These proteins are denatured and linearized during the brewing process. The 2-DE gel data from beer samples *(14)* showed more multiple spots for LTP than these two identified isoforms. This may be explained by the secondary structure and polymorphism of the proteins and glycation variants resulted from brewing conditions *(7,28)*.

The multiple forms of beer LTP, owing to the glycation process, have been well revealed by proteomics *(14)*. Glycation with sugars such as glucose and xylose often occurs with glycine and lysine residues via the Maillard reaction *(14,29)*. D-Glucose reacts with a free amino group in amino acids (e.g. lysine), giving a Schiff base that rapidly rearranges to form a more stable (1-deoxy-D-fructose-1-yl)-amino acid derivative called an Amadori compound *(30,31)*. Barley LTP1 contains nine glycines and four lysines, while LTP2 contains 11 glycines and three lysines, which is about 13% of the whole protein. To a lesser extent, approximately 8 and 6% of barley serpin is glycine and lysine. It is likely that such a biased presence of these two residues plays a significant role in the multiple-spot pattern for the beer proteins. Interestingly, glycation itself could hinder proteomic characterization, since the typical enzyme for proteomic study is trypsin, and protein glycations occur mostly at the epsilon amino group of lysine. The glycation of

Table 2. Proteomic identification of beer proteins

Beer proteins	Functionality	Abundance	Glycation	References
Beer proteins originating from barley malt with defined functionality (11)				
LTP1	Foam, flavour stability, antioxidant	Abundant	Yes	*(1,21,35)*
LTP2	Foam, flavour stability, antioxidant	Abundant	Yes	*(1,21,35)*
Protein Z4 (Serpin)	Foam	Abundant	Yes	*(1,21,35)*
Protein Z7 (Serpin)	Foam	Abundant	Yes	*(1,21,36)*
Protein ZX (Serpin)	Foam	Abundant	Yes	*(1)*
α-Amylase inhibitor BDAI-1	Haze, foam	No	Unknown	*(1,14,23)*
α-Amylase/trypsin inhibitors (CMa, CMb, CMd, CMe)	Haze	No	Unknown	*(1,14)*
Hordein γ	Haze	Abundant	Unknown	*(1,14)*
Beer proteins originating from barley malt with unknown functionality (9)				*(1)*
Thionin, thionin DB4, RNA-binding protein blt801, hordoindoline (A, B1, B2), calmodulin, 40 S ribosomal protein S7, barwin				
Beer proteins originating from brewing yeast with defined functionality (1)				
Thioredoxin-2	Flavour stability, antioxidant	No	Unknown	*(1,21)*
Beer proteins originating from brewing yeast with unknown functionality (42)				*(1)*
Profilin, glycine-rich RNA-binding protein blt801, protein VEL1, cell wall mannoprotein CIS3, cell wall protein ECM33, pre-mRNA leakage protein 1, phosphoglycerate mutase 1, glucan 1,3-β-glucosidase, mitochondrial protein NCA3, glyceraldehyde-3-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase 2, alcohol dehydrogenase 1, alcohol dehydrogenase 4, protein UTH1, coproporphyrinogen-III oxidase, probable family 17 glucosidase SCW4, probable family 17 glucosidase SCW10, box C/D snoRNA protein 1, saccharopepsin, phosphoglycerate kinase, enolase 1, enolase 2, protein TOS1, protein SIM1, glucan 1,3-β-glucosidase 1/II, probable glycosidase CRH1, pyruvate kinase 1, hydroxymethylglutaryl-CoA synthase, 1,3-β-glucosyltransferase GAS3, invertase 1, invertase 2, glucose-6-phosphate isomerize, triosephosphate isomerase, protein TBF1, probable transporter SEO1, long-chain fatty acid CoA ligase 4, tRNA wybutosine synthesizing protein 1, protein EGT2, glucan 1,3-β-glucosidase, α-galactosidase, uncharacterized protein YOR020W-A, uncharacterized protein YGR237C				

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H. vulgare LTP2    --C--EPAQLAVCASAILGGTKPSGECCGNLR---------AQQGCLCQYVKDPNYG-HY 46
T. aestivum LTP2   -AC--QASQLAVCASAILSGAKPSGECCGNLR---------AQQGCFCQYAKDPTYG-QY 47
H. vulgare LTP1    LNCGQVDSKMKPCLTYVQGGPGPSGECCNGVRDLHNQAQSSGDRQTVCNCLKGIARGIHN 60
T. aestivum LTP1   -DCGHVDSLVRPCLSYVQGGPGPSGQCCDGVKNLHNQARSQSDRQSACNCLKGIARGIHN 59
                      *    : :  * : : .*. ***:**..::         .::   *:  *.   * :
H. vulgare LTP2    VSSPHARDTLNLCGIPVPH-------C---- 66
T. aestivum LTP2    IRSPHARDTLTSCGLAVPH-------C---- 67
H. vulgare LTP1     LNLNNAASIPSKCNVNVPYTISPDIDCSRIY 91
T. aestivum LTP1    LNEDNARSIPPKCGVNLPYTISLNIDCSRV- 89
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Figure 1. Multiple sequence alignment of thiol LTP showing conservation of cysteine residues. The amino sequence alignment was performed with ClustalW2 (www.ebi.ac.uk/Tools). The conserved cysteine residue is in bold. The barley (*Hordeum. vulgare*) LTP1 (P07597) and LTP2 (P20145), wheat (*Triticum. aestivum*) LTP1 (P24296) and LTP2 (P82900) were retraced from the protein database, UniprotKB (www.uniprot.org). Asterisks denote identical amino acid residues among LTPs. Dashes indicate a gap. Semicolons and dots denote conserved and semi-conserved amino acids, respectively. Cysteine residues are highlighted.

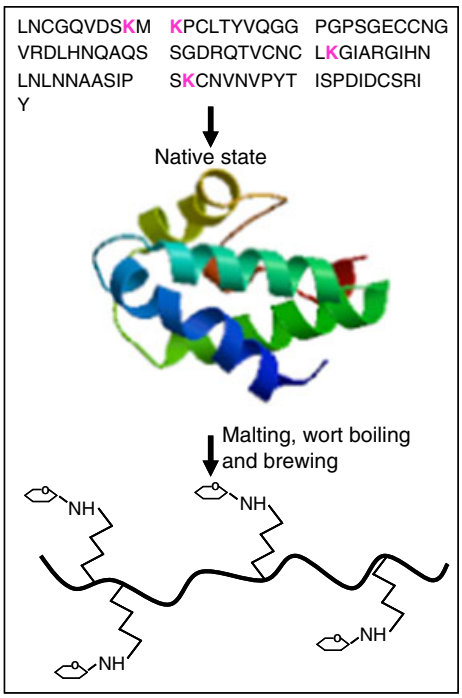

Figure 2. Denaturation and glycation of barley LTP1.

lysine would render proteins resistant to trypsin cleavage, leading to less peptide coverage and even failure to identify target proteins. Using a protein structure modelling tool, SWISS-Model *(32–34)*, the native structure of barley LTP1 and its glycation after brewing is illustrated in Fig. 2.

The 42 kDa barley serpins BSZ4, BSZ7 and BSZx *(28)* contain two cysteines, or one or none at all. These data pose the question: what do serpins do in beer? Research findings have provided a wealth of evidence that beer serpins have lost their capacity as protease inhibitors, but are involved in foam stability *(14)*. The α-amylase inhibitor BDAI-1 (16 kDa), α-amylase/trypsin inhibitors (16 kDa) and hordein γ (33 kDa) are all notably cysteine-rich *(21)* and also very high in proline. Each of the amylase or amylase/trypsin inhibitors has 10 cysteines. Hordein contains eight cysteines and hordeins are linked with an unfavourable trait, beer haze.

The cysteine content will more or less reflect the protein band pattern as shown in the case of LTP in the schematic in Fig. 3. Most proteins are almost certainly denatured or linearized after wort boiling and fermentation. In Fig. 3 *(7)*, the 40 kDa molecular weight serpin from beer under nonreducing conditions shows two bands side by side, which when the conditions were made reducing merged into one band. On the other hand the notional 10 kDa molecular weight LTP1 presents as one band under reducing and nonreducing conditions. This indicates that the smaller protein is linearized and will not compress into a more compact form, whereas the large protein does still have this ability.

Yeast thioredoxin (TRX, 14 kDa), has four cysteines and is a proven antioxidant in cellular systems, where it operates in cascades, dependent on reduced cellular coenzymes *(37)*. As shown in Table 2, many beer polypeptides of yeast origin are principal enzymes in the glycolytic pathway, namely glyceraldehyde-3-phosphate dehydrogenase and enolases. These proteins are soluble enzymes in the yeast cytoplasm, and presumably they end up in finished beer because of yeast proteolysis.

It is unlikely these glycolytic enzyme fragments could contribute to a redox buffering system in the beer, whereas all of the cysteine-rich proteins are much more likely candidates, especially in the light of Lund and Andersen's findings *(5)* and our own.

Oxidative modification of protein thiols

The beer proteomic data prompted our focus on thiol proteins. Together with many small thiol compounds, such as cysteine in fresh beer, as Lund and Anderson have reported *(5)*, we hypothesized that upon exposure to oxidants such as hydrogen peroxide, the redox active molecules, small and large, might work together in a cycle. Cysteine thiols can be modified, by reactive oxygen species and reactive nitrogen species, to produce a series of oxidized derivatives (Fig. 4). Small thiol-containing molecules (R′SH) such as glutathione in turn can change the activity and structural conformation of protein thiols (PrSH) by redox reactions (Fig. 5). They can be progressively modified and many are reversible. Thiols will react with almost all of the reactive oxygen species, which makes them especially important as far as beer ageing is concerned. They most commonly react with H_2O_2 in biological systems. This is exclusively through the protein thiolate (PrS^-), and hence the reaction is pK_a-dependent *(38,39)*. Only protein cysteines with a low pK_a should be reactive and it is true that cysteines such as this have been identified in specific catalytic proteins. The lowering of the pK_a is due to the specific microenvironment of the cysteine residue in the protein. However it seems that a low pK_a in itself may not explain peroxide reactivity entirely. It is possible to quote examples that are really beyond the scope of this review, but two will be mentioned in passing. The low-pK_a active site cysteine, of the protein code named PTP1B, by hydrogen peroxide, occurs at a rate no faster than for low

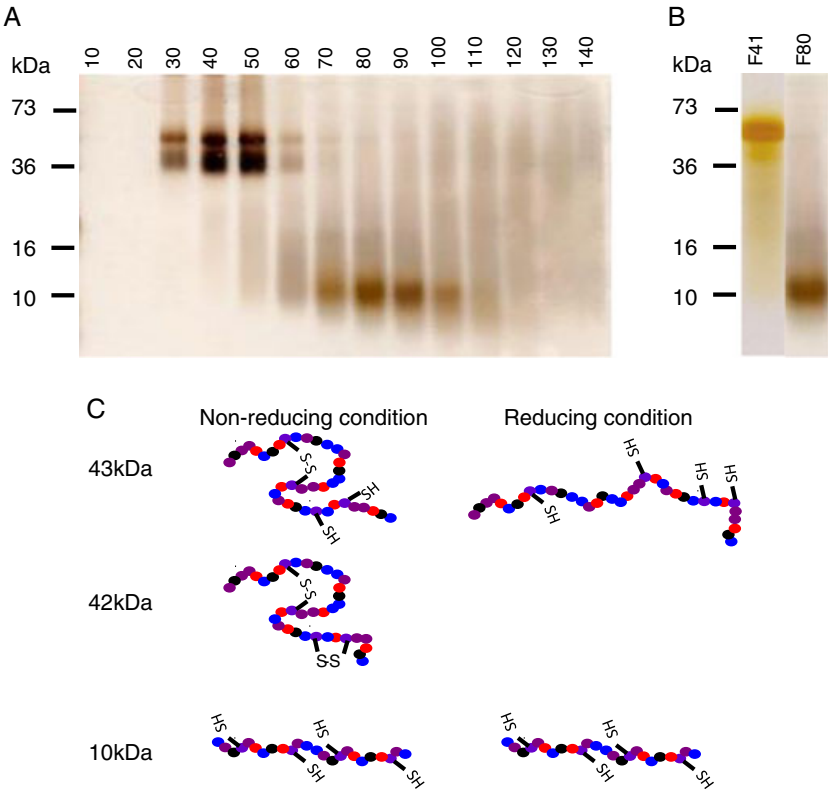

Figure 3. Beer protein analysis was performed under reducing and nonreducing conditions. The electrophoresis profiles in (a) were obtained under nonreducing conditions on SDS–PAGE gels. Each lane represents protein fractions from size exclusion chromatography of total beer proteins. The protein profiles in every tenth fraction of 300 fractions were obtained using 15% nonreduced SDS–PAGE and visualized by silver staining. (b) The protein profile in reduced SDS–PAGE. Fractions 41 (F41) and 80 (F80) were fractionated in reduced SDS–PAGE and stained with silver nitrate in the same way as in (a). (c) A schematic illustration of intrapeptide disulfide bond formation. Proposed disulfide bonds or free thiols in the 43 kDa serpin (fraction F40) and the 10 kDa LTP1 (fraction F80) under nonreducing and reducing conditions

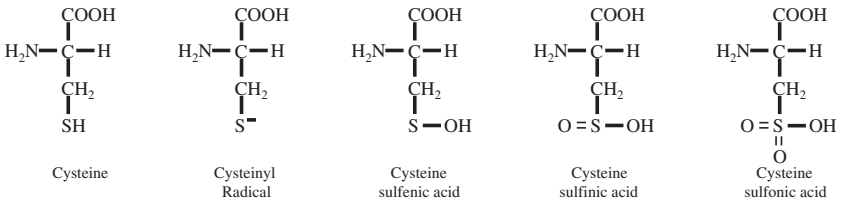

Figure 4. Cysteine and oxidized derivatives.

molecular weight thiols (rate constant approx 20 $M^{-1} s^{-1}$). Other proteins with redox-sensitive thiols, such as Cdc2B and GAPDH, have faster rate constants *(40)* for oxidation by hydrogen peroxide of 160 and 500 $M^{-1} s^{-1}$. These, however, are still many orders of magnitude less than those displayed by peroxidases *(40)*, which are around 10^6–10^7 $M^{-1} s^{-1}$. These enzymes have remarkable catalytic mechanisms involving sophisticated triads or tetrads that communicate between peroxidatic cysteines and H_2O_2 in their active site and achieve these very high rates *(41–43)*. There is obviously, albeit based on a very selective snapshot of just two proteins, a broad range of turnover rates for nominal peroxidases. We are ever mindful that extremely slow turnover rates (peroxide destruction rates) may be all that is required to keep reactive oxygen species (ROS) in check in packaged beer.

The reaction of the thiolate ion (PrS^-) with H_2O_2 involves a two-electron oxidation of the thiolate to the oxyacid, sulfenic acid (PrSOH). Despite the instability and the high reactivity of sulfenic acids, they occur in a remarkably large number of proteins *(44–46)*. They are extremely important in cell signalling and they seem to be transiently stable and operate in reversible cycles of oxidation and reduction *(47)*. PsSOH can further react with another protein thiol (RSH) to form a PrSSR. This disulfide bond of the intermediate, can be reduced to the original PrSH by RSH or sulfite or further oxidized into irreversible thiosulfinate and thiolsulfonate. With this thiol-cycling hypothesis in mind, we set out to find evidence using a series of biochemical assays.

Beer thiols and beer aging

To identify beer proteins that are redox active – that can rapidly be oxidized or reduced – beer samples were reacted with MPB (*N*′-3-maleimido-propionyl biocytin), a maleimide derivative linked to biotin. This reagent reacts with free thiols, which can subsequently be visualized with an avidin-linked horseradish peroxidase. In fresh beers, the prescient LTP1 stained strongly

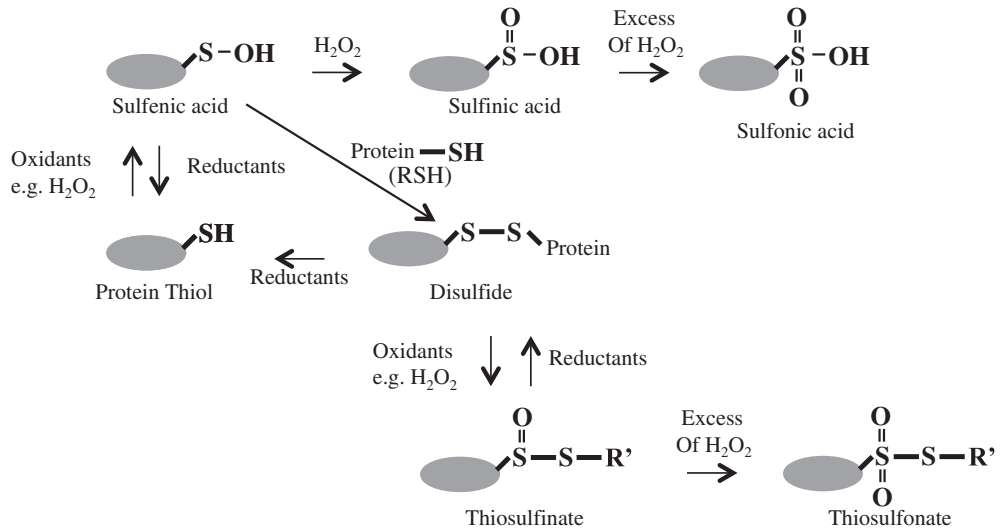

Figure 5. Oxidative modification of protein thiols.

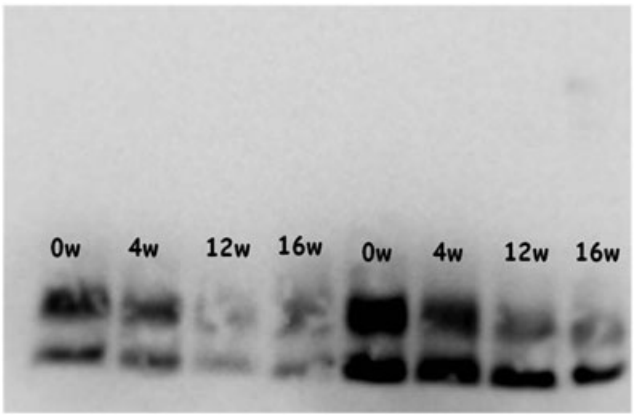

Figure 6. Identification of beer proteins with free thiols. Proteins from two separate lots of fresh lager beer were labelled with MPB (*N*'-3-maleimido-propionyl biocytin), which specifically stains protein thiols, before fractionation by SDS–PAGE. Only those proteins with thiols stained. Samples were removed at zero (0w) up to 16 weeks (16w) of storage of bottled beer at 60°C.

for free thiols (Fig. 6), presumably because the compact disulfide-bonded structure of LTP1 was unfolded and the free thiols of LTP1 were reduced in the fresh beer and accessible to the thiol-specific stain, MPB. However, the staining markedly decreased during ageing up to 16 weeks.

Thiol and hydrogen peroxide measurements during the oxidative forcing test (OFT) *(48)* showed that peroxide accumulation was only detected when the thiols were practically exhausted (Fig. 7A and B). The timing of this changeover depended on the original thiol level and in this regard reflects the work of Lund and Andersen *(5)*. Less thiol means a shorter lag-time before changeover (see lag-times Fig. 7A and B). Peroxide accumulation, in each case, started when the thiol loss reached a minimum value. The protein thiol levels stayed much the same up until the changeovers were reached and then they began to decline.

Following the line of reasoning in Fig. 5, we further tested for the presence of cysteine sulfenic acid using the sulfenic acid-modifying chemical dimedone (Fig. 8). Dimedone reacts specifically with sulfenic acid residues and does not react with thiols. If an essential reactive cysteine sulfenic acid is inactivated by dimedone, peroxide accumulation ought to, it could be argued, accelerate, hence reducing the antioxidative capacity of beer.

With relatively fresh beer, dimedone significantly reduced the waiting time observed using OFT; it not only reduced the lag time before peroxide appearance, but also accelerated peroxide accumulation and almost tripled the final concentration. Treatment with *N*-ethyl maleimide (NEM) also reduced the waiting time, but the peroxide level that was reached at the end of the experiment was much the same as for the control, pointing to a difference between small molecules and large molecules, that is proteins. Aged beers showed progressively shorter lag-times in OFT tests than fresh beer, as aged beers have a brief or even no lag time when hydroxyl radicals are measured in force tests using electron paramagnetic resonance techniques.

Inferring that sulfenic acid is part of a protein-mediated response from inhibitor (dimedone) studies is a first step, but a more substantive proof of modified protein(s) is highly desirable. PrSOH can be visualized with a 'sulfenic blot' technique. NEM blocks free thiols. After removal of excess unreacted NEM, Pr-SOH is reacted with arsenate to convert PrSOH back to PrSH. The putative PrSOH originating thiols are finally tagged with MPB. The putative protein sulfenic acids are then identified on a Western blot with avidin and peroxidase development.

The data in Fig. 9 were obtained with a green hopped beer that showed excellent flavour stability, even after prolonged storage, yet there was no residual sulfite remaining. Such long-term stability, in the absence of a tried and true standby, seemed like a good place to detect a last line of ROS defence. The dimedone-treatment of this beer prior to OFT resulted in persistent higher level of peroxide compared with the untreated control (Fig. 9A). However there was no lag period before peroxide detection and the kinetics of appearance were quite different from any observed previously. The staining for the presence of protein sulfenic acids in these samples is shown in the accompanying blot (Fig. 9B). The control samples incubated without dimedone showed strong and consistent staining in the 10 kDa LTP1 region for up to 6–8 h indicative of the presence of protein sulfenic acid residues, while the dimedone-treated sample exhibited far weaker staining intensity indicative of far fewer protein sulfenic acid residues, consistent with dimedone blocking, which began to decline markedly

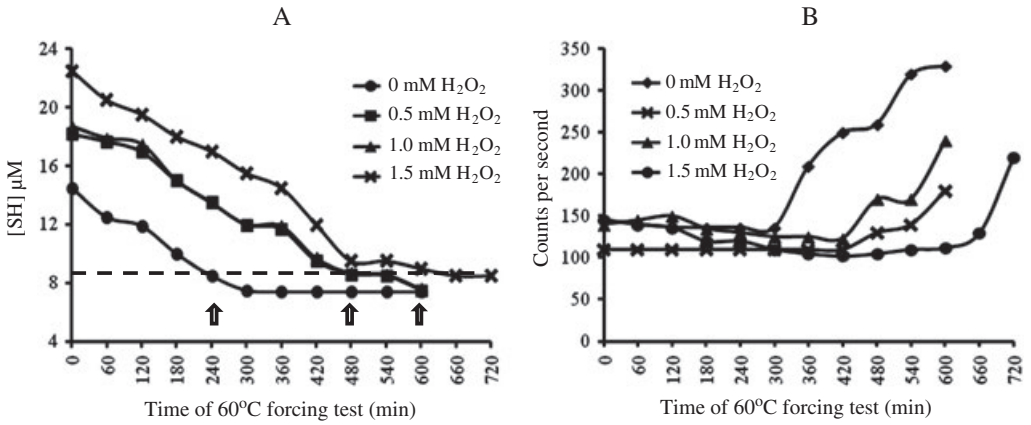

Figure 7. Measurements of total thiol and hydrogen peroxide in beers during an oxidative forcing test (OFT). (A) Total thiol measurement. Beer samples were incubated at 60°C and regularly sampled to test for the DTNB[5,5-dithiobis(2-nitrobenzoic acid)]-reactive complement. The readings were converted to thiol equivalents. The four sets of data describe beers that had a different total thiol reactivity to start, as hydrogen peroxide had been dosed into the wort at the outset of the fermentation, as described in the legends. The arrows indicate when the base level DTNB reactivity was reached. (B) Measurement of H_2O_2. Hydrogen peroxide was assayed during the oxidative force test of the beers, as described in the legend, with the sensitive luminol chemiluminescence method. Luminescence counts were plotted for samples withdrawn over a 12 h incubation.

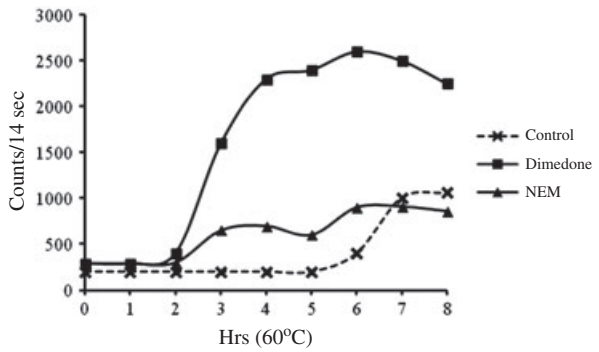

Figure 8. The formation of peroxide in fresh lager beer incubated at 60°C OFT. Putative hydrogen peroxide levels are expressed in counts per 14 s. Hydrogen peroxide was measured using the luminol chemiluminescence assay. One sample was treated with dimedone and one was treated with *N*-ethyl maleimide (NEM). The control received no additions. Data from Rogers and Clarke *(6)*.

after 2 h. The persistent presence of the prescient protein sulfenic acids in the control up to 6–8 h may signal that a functional protein thiol dependent peroxidase system is operating, while the subsequent loss of blot intensity can be explained by exhaustion of reductants resulting in a consequential inability of the system to regenerate PrSH from PrSOH and the rapid oxidation of the reactive sulfenics to sulfonic acid moieties and beyond. The accumulation of the prescient sulfenic acid stain in the control may signal the exhaustion of reductants and a consequential inability of the system to regenerate Pr-SH from Pr-SOH. The subsequent loss of blot intensity can be explained by the rapid oxidation of the reactive sulfenics to sulfonic acid moieties and beyond. Recall that sulfenic acid is very reactive. We suspect from other data that the accompanying protein denaturation at this time accelerates irreversible oxidation. The limited staining of proteins from dimedone-treated beer can be readily explained provided dimedone reacts rapidly with Pr-SOH once it is formed. This would prevent re-formation of Pr-SH and would progressively shut down flux through the cycle.

The nature and the identity of the proteins that could participate in antioxidant reactions is now, to us, no longer a *cause célèbre*. LTP, being the most dominant candidate because of its abundance, and thioredoxin derived from brewing yeast can play a pivotal role in the process.

Beer antioxidant thiol-proteins – LTP1 and yeast thioredoxin

As shown previously, LTP survives the brewhouse, retaining redox thiols that appear to be redox active in the packaged beer. These are also reactive and protective in tests designed to measure their effectiveness against inhibition of yeast growth in the presence of reactive oxygen species. Wu *et al. (7)* sought to see if this protein could protect sensitive entities against reactive oxygen species. This might go some way to ensuring that LTP1 was a redox protein that could function in a reduction cascade. A micro-titre culture of yeast is convenient, simple to set up, allows multiple tests to be carried at the same time and has been used in the evaluation of LTP's antioxidant capability. The assay is a biological screening system to measure the antioxidant capacity of compounds using the oxidant-induced cell cycle arrest response of *Saccharomyces cerevisiae*. Alternative methods using the nonphysiological free radical compounds such as diphenylpicrylhydrazyl (DPPH) and azinobis ethylbenzothiaziline-6-sulfonate (ABTS) only give an indication of the ability of a compound to scavenge oxidants *(49)*, whereas this yeast based method can also measure the ability of a compound to elicit cellular resistance to the damaging effects of oxidants.

The screening assay was established against a panel of six physiologically relevant oxidants, ranging from reactive oxygen species (hydrogen peroxide, cumene peroxide, linoleic acid hydroperoxide), to a superoxide-generating agent (menadione), reactive nitrogen species (peroxynitrite) and the thiol-oxidizing agent (diamide). The antioxidants ascorbate and gallic acid displayed scavenging activity and induced the resistance of cells against a broad range of oxidants using this assay. Lipoic acid, which showed no scavenging activity and thus would not be detected as an antioxidant using a nonphysiological screen, was however identified in this assay as providing resistance to cells against a range of oxidants. The 96-well format is very high throughput. The results of the challenge tests, with and without LTP present, are shown in Fig. 10. All of the antioxidants that were screened affected yeast growth significantly, but in all cases, LTP rescued the cells, particularly against hydrogen peroxide and menadione (superoxide).

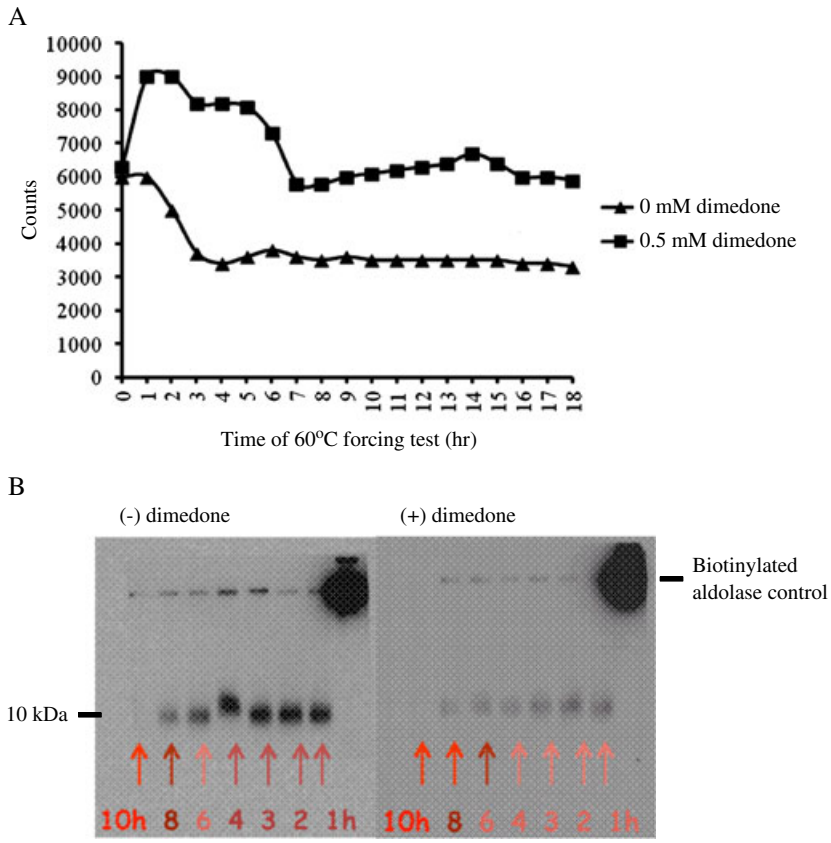

Figure 9. The effects of dimedone on the beer peroxide level and protein sulfenic acid residues. (A) The effects of dimedone on beer peroxide and protein sulfenic acid residues. The lager beers treated with and without dimedone were incubated at 60°C and measured for their peroxide content by a lumonol assay. The *y*-axis (hydrogen peroxide formation) is expressed in counts per 14 s. (B) The effects of dimedone on beer thiol proteins. The proteins from the treated beer samples were fractionated in SDS–PAGE and stained using a method that specifically visualizes sulfenic acid as described by Saurin *et al. (45)*.

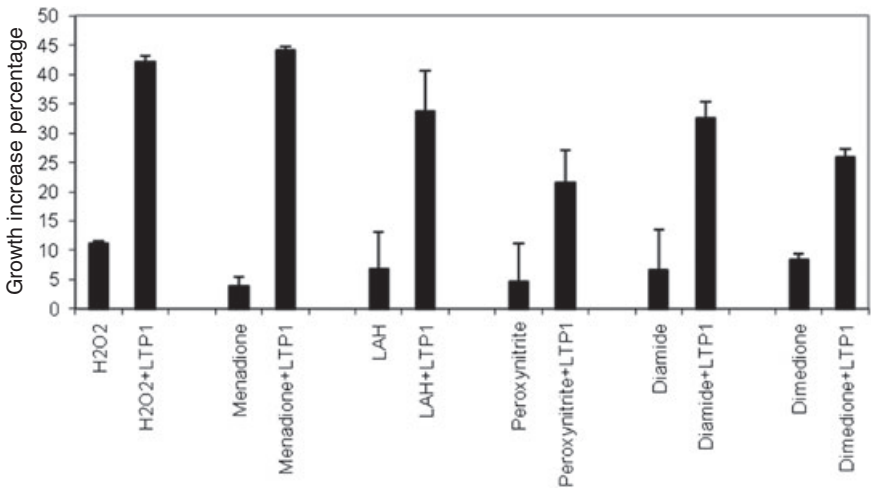

Figure 10. Antioxidant activities of the beer thiol-protein LTP1. Antioxidant activity of LTP1. Antioxidant activities of the 10 kDa LTP1 against six oxidants were measured using a *Saccharomyces cerevisiae* based assay. Addition of LTP1 at 2 mg/mL increased yeast growth by 31% against the buffer control at the end of a 4 h treatment under 4 mM H_2O_2.

Yeast thioredoxin (TRX) is another thiol-rich candidate ripe for consideration as a functional element of anti-ROS cascades. At one time we anticipated that TRX, which is secreted by yeast during fermentation, could be linked to beer stability. That was in fact the start of our move into beer proteomics. Yeast cells protect themselves against oxidative stress with interlinking processes that can transfer electrons within cells to where they are needed to quench reactive oxygen species. More contentiously, they can transfer reducing equivalents to outside the cell, where presumably they may carry out different or perhaps the same defence strategies. The trouble with this view, at least on face value, is that, outside the cell, the rest of the machinery for recycling oxidized TRX is just not present. Yet it might be possible - if yeast happens to secrete small thiols like cysteine as some mammalian cells do - this might be how LTP is reduced during fermentation after probably being oxidised in the wort and in the kettle. However, it is

always possible that other proteins can, albeit ineffectively, perform this task.

At the outset, we admit we have not discovered the link between TRX, beer protein and staling. However, our study of TRX expression and secretion has made us aware of the possibilities for protein participation in anti-staling mechanisms, and has drawn us into learning more about beer proteins and the role of SH groups in staling and, in a leap of faith, to use some of the protein characteristics as a key guiding light to improve beer stability. We also held to the belief that TRX could aid beer stability when we found that exogenous peroxide stimulates TRX formation, and accelerates the accumulation of TRX in the extracellular pool. Peroxide ramps up the intracellular TRX levels. When the western blots were quantitated by densitometry, the data showed a gradual decrease over time in TRX inside the cell and an obvious increase of TRX outside the cell (Fig. 11). This was true for each of the peroxide challenge tests.

Taking together the data of beer proteomics and thiol proteins, we propose that there could be a thiol-based cycle operating in beer that involves oxidized thiols and reversible reduction after peroxide destruction using sulfite or some reductant molecules (Fig. 12). In model 1, protein thiol (Pr′SH) is oxidized by oxygen, leading to protein disulfides (Pr′SSR) with the help of a small molecular reductant (RSH). Localized sulfite antagonizes the reaction, reversing the intermediate to the original thiols (Pr-SH) for re-entry into the cycle. The pathway is sensitive to inhibition by *N*-ethylmaleimide. In model 2, PrSH reacts directly with hydrogen peroxide and the thiol is oxidized to the sulfenic derivative, i.e. PrSOH. This route is sensitive to inhibition by dimedone, and *N*-ethylmaleimide. The sulfenic acid

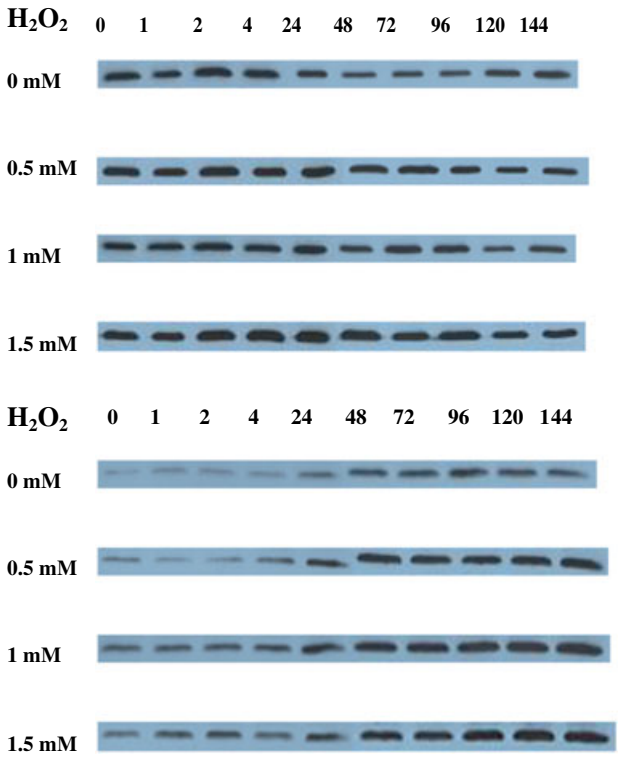

Figure 11. TRX production and secretion under peroxide challenge conditions. The levels of TRX, intracellular and extracellular, were estimated for 144 h by western blot analysis of cell extracts and cell-free medium, in a lager yeast strain growing in wort supplemented with exogenous H_2O_2 (0–1.5 mM) . Top panel – the intracellular TRX levels. Lower panel – the secreted thioredoxin. Data from Rogers and Clarke *(6)*.

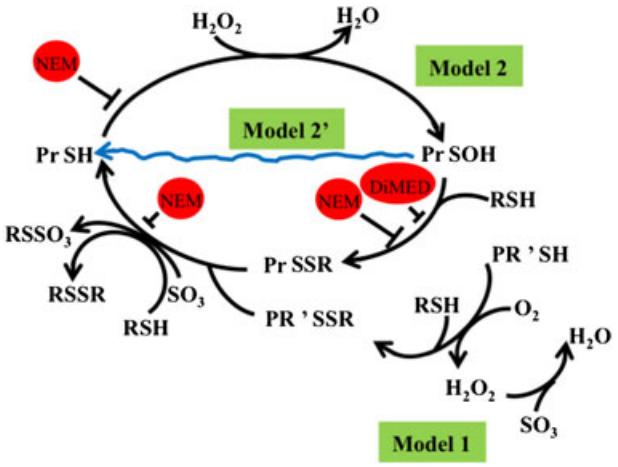

Figure 12. Three protein-dependent routes for peroxide quenching.

is reactive and will react with a small RSH compound, forming a disulfide. As in model 1, the original thiol can be reformed by sulfitolysis using sulfite as the electron donor, or by the participation of another reactive thiol compound, which might include a number of cascading reactions. PrSOH can also be returned to Pr-SH by a strong reductant (model 2′, shortcut). Thioredoxin, for instance, will effect this reduction, as will other possible reductants. This thiol-cycling process would clearly provide a sustainable source of reducing power for beers and mitigate in a direct fashion against staling by destroying peroxides.

Summary

Our studies mentioned in this review started as a contemporary, quotidian, plot-less investigation to solve a nagging problem caused by thread formation in commercial lagers. The solution was simple enough: reduce sulfite levels below 10 ppm and the problem disappears. Sulfite and catalytic copper levels promote sulfitolysis of disulfide bridges, leading to mixed products, including at least one thiol variant. At the time it was believed that these reactive thiols could re-oxidize and generate extended thread-like structures. However, what was surprising was that attempts to solubilize the particles were best performed with reduced yeast thioredoxin. Thiothreitol and mercaptoethanol, which are normally enough, were nowhere near as effective. This was something of a moment-of-truth revelation for us. It showed that beer protein thiols were redox active and that large protein molecules, such as thioredoxin, can take part in redox reactions in beer. It belied the perception that beer proteins operated as independent entities in solution, and it underscored the opposite behaviour, i.e. that they are capable of peroxide generation when nearby sulfhydryl groups are oxidized, and of peroxide destruction in the peroxidase mode.

This begs the question of which proteins are in what category. Generally we would expect cysteine-rich proteins with high levels of modification, such as glycation modification, to be in the latter group and as we have reported these proteins post brewhouse show little tendency to form intermolecular disulfide bridges as expected for pro-oxidative behaviour. However the serpins do appear to be able to do this and may therefore be pro-oxidants. The bifurcation of proteins, even based on this simple discriminatory test, provides a rationale for the findings of Lund and Andersen *(5)*, that protein thiols

are not linked *per se* to beer stability, while small molecule thiol compounds are linked. If some proteins are reductively functional, while many others are not, it might explain the seeming impasse.

Proteomics seems to be an excellent means of accounting for thiol-rich participants in antioxidative pathways. At any rate it gives a broader picture of the beer complement, which has only really just started. With this information it ought to be possible to begin to correlate beer stability against a greater database. Our choices so far, of LTP and thioredoxin are certain to be extended. The model we have proposed for an antioxidative thiol cascade is based on biological models, and if beer proteins did not form complexes and decorate we would probably dismiss this possibility out of hand. However, since they do and even based on the simple observations above about the effectiveness of thioredoxin in solubilizing beer particles, it seems that we simply cannot underestimate the versatility of protein chemistry. The results of Iimure *et al. (21)* point the way to a more comprehensive view, as they have opened up the stability of beer foam with their pioneering proteomics study.

Lastly, what of the relevance to beer makers? Well, barley breeders might have something to say about this and even now it seems that there are plans for cultivars that have higher levels of LTPs as well as thioredoxin (Goldsmith, M., personal communication). One way or another, by plant breeding and/or by the use of yeast genetically modified organisms, it may be possible to identify and amplify the levels of desirable proteins in beer. However, if we count in lifetimes, we doubt it. There may be other options, however.

Hioe *et al. (50)* reported that hop cones, with an inordinate extra load of leaf material, in some cases showed improvements in beer stability and in the destruction of peroxide that forms in the brewhouse operations. Green hopped beer also showed remarkable stability, judged by the sustained appearance of protein thiols, as well as from sensory evaluation over years.

Auditing of beers, the use of tests such as the challenge test we have described in this review, could be used very effectively to deconstruct beers, especially the very stable ones, and hopefully one could create a synthetic cascade in a test tube. What a rapid means of testing for stability. Surely, oh surely, as we thought after our thioredoxin epiphany, there must be more to redox and flavour stability than this.

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