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Use of Nitroxide Spin Probes and Electron Paramagnetic Resonance for Assessing Reducing Power of Beer. Role of SH Groups

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Intensity of EPR spectra of stable organic free radicals, nitroxides, is decreasing with time if the radicals are dissolved in beer. The process is determined by a chemical reaction of nitroxide reduction by components naturally present in beer. Kinetics can be described as a simple irreversible first order with respect to both nitroxide and one reducing agent. Effective concentration of the reducing agent and the corresponding reaction rate constant has been determined. It is demonstrated that the nitroxide reduction is sensitive to the presence of solvent-accessible SH groups of proteins present in beer. It is proposed that quantitative analysis of reduction kinetics of small water-soluble nitroxide radicals such as TEMPO and TEMPOL can be used to assess the reducing power of beer. The effect of accelerated aging of beer achieved at elevated temperatures on nitroxide reduction kinetics is demonstrated.

KEYWORDS: Beer; EPR; nitroxides; reducing power; spin traps; antioxidant pool

INTRODUCTION

One of the long-standing problems of the brewing industry is deterioration of beer with time. Many researchers in the field agree that a chain of oxidation processes that involves oxygen and oxygen-depended radicals is the likely mechanism of beer spoilage. Detailed chemical nature of these processes appears to be complex and so far only a few simplified models have appeared in the literature (*1*). For a long time, it has been thought that beer spoilage is related to its redox stability and that beer shelf life is determined by the amount and properties of reducing species present. To characterize the effect of these species, several methods were suggested, including those based on dicholorophenol-indophenol (DCPI), silver nitrate with ammonium hydroxide, dipiridyl, iodine, and so forth. Review of these methods and examples of applications can be found in ref 2.

Although evaluation of beverages with redox indicators can be widely utilized in research or industry, these methods may yield contradictory results if different indicators are employed. For example, Kaneda et al. demonstrated that while the reducing activity of beer stored at 37 °C and measured by a redox indicator 1,1-diphenyl-2-picrylhydrazyl (DPPH) decreased by 15% after 5 days, the use of another well-known indicator DCPI pointed to an increase of the beer "reducing power" (*3*). This contradiction could be explained by different properties of the indicators. It was suggested that DPPH reacts mainly with polyphenols, while DCPI is more selective for the products of polyphenol oxidation and also reductones and melanoidines (*3*).

Recently, some new criteria for evaluation of beer properties have been suggested. For example, Bright and co-workers introduced so-called antioxidant potential for malts (*4*). These authors defined the antioxidant potential as an inverse of the amount of the malt required for 50% inhibition of a Fenton-type reaction of linoleic acid hydroperoxides with a leuco form of a dye resulting in formation of methylene blue. The reaction was carried out in the presence of hemoglobin. Araki and co-workers introduced another parameter, the total reactive antioxidant potential, which is sensitive to polyphenols naturally occurring in beer (*5*). This potential was defined through optical measurements of peroxy radicals. Formation of these radicals can be partially inhibited by antioxidants already present in beer or added with hop (*6*, *7*).

Prediction of beer shelf life can be based on the use of spin traps and EPR (*8*). Initially, a spin trap molecule is not paramagnetic, but upon reacting with a short-lived free radical it forms a relatively stable spin-adduct giving rise to a characteristic EPR spectrum. The rate of radical-mediated processes of beer deterioration is relatively low below room temperature. For example, for beer stored at 0 °C only small, micromolar level concentrations of spin-adducts were accumulated after 5 days of the experiment (*9*). At elevated temperatures and in the presence of air, kinetics of adduct

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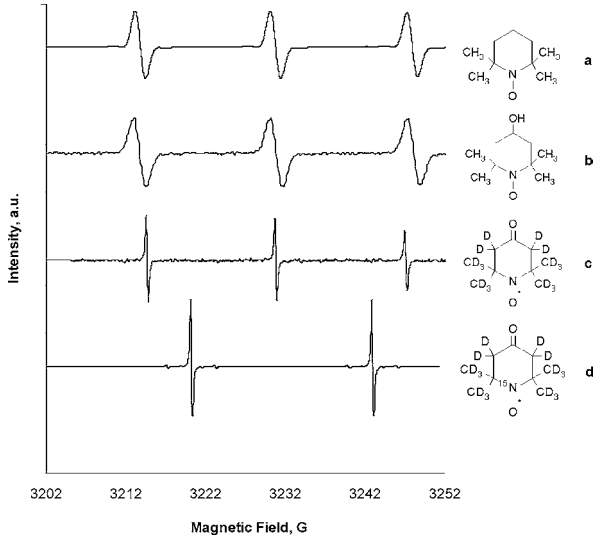

Figure 1. Structure and solution EPR spectra of nitroxides utilized in beer experiments described here: (a) TEMPO (2,2,6,6-tetramethylpiperidine 1-oxyl), (b) TEMPOL (4-hydroxy-TEMPO), (c) perdeuterated-TEMPONE (4-oxo-TEMPO-d_{16}), (d) ^{15}N-substituted perdeuterated-TEMPONE (4-oxo-TEMPO-d_{16}-^{15}N).

formation has a lag period, which is determined by the content of antioxidants in beer. The appearance of the spin-adduct EPR signal coincides with the moment of time when main antioxidants are almost completely depleted. This lag period can be used as an indicator of beer flavor stability and correlated with the beer age (*10*−*12*). Later, the same authors have suggested another index, which is related to the quantity of OH• radicals generated after 120 min of the temperature-forcing test (*13*).

In this paper, we describe the use of stable organic free radicals called nitroxides (**Figure 1a**−**d**) for assessing redox properties of beer. Nitroxides, which are available commercially, have already found many applications in chemistry, biology, and experimental medicine. These molecules are known as spin probes because their EPR spectra are very sensitive to physical properties of microenvironment such as viscosity, polarity, presence of other paramagnetic species, and so forth. (*14*). At low concentrations, nitroxides are generally nontoxic and have an antioxidant activity. These properties even motivated several groups to initiate the use of nitroxides as experimental anticancer drugs and antidotes for gamma radiation (*15*). Although even the smallest nitroxides are much larger than the diatomic oxygen, some of the properties of these molecules are very similar. For example, for nitroxides the density of unpaired electron on the O-atom is similar to that of the molecular oxygen. Such properties of O−N bonds in nitroxides as length, fundamental vibrational frequency, and dissociation energy are similar to those of O_2. As the result, nitroxides compete with oxygen in some redox and free valence reactions (*16*).

While nitroxide radicals are relatively stable at ambient conditions in aqueous solutions, they can be used as mild reagents, producing a nonparamagnetic hydroxylamine in the reaction with ascorbic acid and other reducing agents. The process can be easily monitored as a disappearance of the nitroxide EPR signal with time and can be used in nontransparent media. Nitroxide radical TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl) also reacts with peroxy radicals, but only slowly with a typical rate constant of 10^4−10^5 M^{-1} min^{-1}. This rate constant is about 5 orders of magnitude lower than that with alkyl radicals (*16*). While standard redox potentials of DPPH and DCPI are 1.2 and 0.67 V, the redox potential of TEMPO is near 0.4 V (*16*).

We have found that nitroxides EPR spectra are decreasing with time when the radicals are dissolved in beer. Here, we describe EPR experiments with different nitroxide radicals, TEMPO, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl), perdeuterated TEMPONE (perdeuterated 4-oxo-2,2,6,6-tetramethylpiperidin-1-oxyl, or PDT), and also N^{15}-labeled PDT, to assess reducing power of beer on the basis of quantitative analysis of kinetics of the EPR signal reduction. This allowed us to measure important redox parameters of beer such as effective concentration of reducing agent and the corresponding reaction constant. Our data indicate that the mechanism of nitroxide reduction in beer is likely to involve solvent-exposed cysteines in protein molecules. An additional advantage of our method is that the nitroxides we are using are water-soluble while previously utilized DPPH is not (only in water−ethanol mixtures). Thus, one could use our nitroxide method for evaluating redox stability of nonalcoholic beverages.

METHODS

Miller Genuine Draft (MGD) beer was provided by Miller Brewing Co. (Milwaukee, WI) and Tiger Lager Beer was provided by Asia Pacific Breweries (Singapore). To force anaerobic oxidation, some beer samples were stored at 32 °C in their original packaging (bottles or cans) over different time periods before the measurements.

Nitroxides TEMPO, TEMPOL (Aldrich, Milwaukee, WI), perdeuterated TEMPONE (PDT), and N^{15}-perdeuterated TEMPONE (Cambridge Isotope Laboratories, Andover, MA) were added to beer from aqueous stock solutions. Other chemicals including Ellman's Reagent (DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)), EDTA, NaOH, Na_2HPO_4, pepsin from porcine gastric mucosa (pepsin A), cysteine, and miscellaneous solvents were purchased from Sigma-Aldrich and used as received.

In some experiments, 8 mg of solid Ellman's reagent was dissolved in 0.2 mL of ethanol and added to 10 mL of a fresh, <1-month old, beer stored in the refrigerator. Then, the pH of solution was adjusted up to 7.5. Final concentration of Ellman's reagent was 10^{-3} M. Solution was incubated for 1.5 h to allow Ellman's reagent to react with SH groups.

When required, beer samples were treated with pepsin (20 mg/mL of dry pepsin powder in fresh beer). Solutions were purged with nitrogen for 2 min and incubated for 30 min in a closed flask at $T = 37$ °C.

Following initial treatments of beer samples, the nitroxide reduction experiments were conducted. Typically, a beer solution was drawn with a syringe into a gas-permeable Teflon (poly(tetrafluoroethylene)) capillary (PTFE, 0.81-mm i.d., 0.86-mm o.d.; Zeus Industrial Products, NJ) and the ends of the capillary (length ca. 3 cm) were closed by crimping. The capillary was inserted into a standard EPR quartz tube. Oxygen content in beer was regulated by flux of nitrogen, air, or oxygen around the gas-permeable capillary in this tube. At room temperature, gas re-equilibration of a solution inside such a capillary occurred with a time constant of ca. 3−4 min.

EPR spectra were taken with a Varian (Palo Alto, CA) Century Series E-112 X-band (8.8−9.5 GHz) spectrometer installed at the University of Illinois EPR Research Center (IERC) or a Bruker (Bruker BioSpin Gmbh, Rheinstetten/Karlsruhe, Germany) Elexsys Series E500 CW-EPR X-band (9−10 GHz) spectrometer installed at the Biophysics Laboratory, Division of Bioengineering, National University of Singapore. The spectra were recorded with microwave power 0.5−5 mW, modulation amplitude 0.04−1.0 G, and modulation frequency 100 kHz. For TEMPOL spectra, the amount of nitroxide was calculated from double-integrated EPR intensity. To simplify the analysis of TEMPO spectra, however, we have also used peak-to-peak amplitude of the EPR signal as the line width was constant except for the initial 2−3 min. Kinetics of the nitroxide reaction in beer with excess of nitroxide (100 μM) was analyzed as irreversible process of first order in respect to both nitroxide and reagent in beer according to the equation

$$\frac{1}{a-b}\ln\frac{b(a-x)}{a(b-x)} = kt \quad (1)$$

where a and b are the initial concentrations of both reagents ($a \neq b$), k is the second-order rate constant, and x is the decrease of nitroxide concentration. Parameters a, b, and k were found using software SigmaPlot (SPSS Science, Chicago, IL) by fitting the entire kinetics curve. At lower concentrations of nitroxide (10 μM), kinetics was pseudo-first order with respect to the nitroxide.

In our initial experiments, it was found that foaming of beer upon opening of cans/bottles complicated mixing of exact volumes of beer with nitroxides. This uncertainty was later avoided by weighting the beer samples and calculating the nitroxide concentration per unit of weight of the solution or by degassing the beer under vacuum just before the experiments.

RESULTS

EPR spectra of nitroxides in beer consisted of three spectral components due to hyperfine splitting on the ^{14}N spin $I = 1$ (**Figure 1a−c**). The peak-to-peak amplitude of these components was almost equal indicating that the nitroxide molecules tumble freely in solution.

When other paramagnetic molecules are present in solution, spin−spin interactions with nitroxides could broaden the nitroxide EPR spectra. For molecular oxygen, which is paramagnetic, the spin−spin interactions are dominated by Heisenberg spin exchange, yielding a concentration-dependent broadening effect that is useful for oxygen measurements in solutions and at specific sites of biomolecules (*17*).

For aqueous solutions, the oxygen broadening is ca. 170 mG/mM. Deoxygenation of beer inside a gas-permeable capillary resulted in line-narrowing effects. Upon removal of oxygen, the shape of the individual spectral components becomes primarily determined by partially resolved hyperfine coupling from the protons of the radical molecule (**Figure 2**). The spectra in this case were recorded with a better digital resolution, smaller time constant, modulation frequency of 25 kHz to avoid undesirable broadening effects, and a small modulation amplitude (0.04 G). EPR spectra and oxygen broadening effect could be simulated using the methods described elsewhere (*18*).

To improve signal-to-noise ratio of detected EPR signals in experiments with beer, we have used perdeuterated TEMPONE (PDT) because of a substantial line narrowing arising from an isotopic substitution. The hyperfine interactions with deuterium spins are not resolved for this nitroxide. During the deoxygenation of the beer solution, the peak-to-peak width of the Lorentzian component of the PDT spectrum decreased from ca. 230 mG to ca. 100 mG and remained the same over 10 h of the experiment. The latter simplifies the line shape and facilitates the analysis of spectra. After oxygen removal, we have observed a progressive decrease in the nitroxide spectra in time. The effect was reversible after addition of oxygen and it means it was determined by nitroxide reduction to hydroxylamine (*16*).

To verify the reaction order, we have monitored kinetics for almost 10 h at a relatively low initial concentration of the radical (10 μM). All this time, the beer sample was under nitrogen atmosphere and at room temperature. The resulting experimental kinetics demonstrates that the process is pseudo-first order with respect to the nitroxide.

At higher (100 μM) initial concentrations of the nitroxide, the reduction of the corresponding EPR signal has eventually reached a plateau as there was no reducing agent left at the end (**Figure 3**). The observed kinetics could be described by the simplest possible mechanism as the first-order process with respect to both nitroxide and just one reagent in beer. Residual,

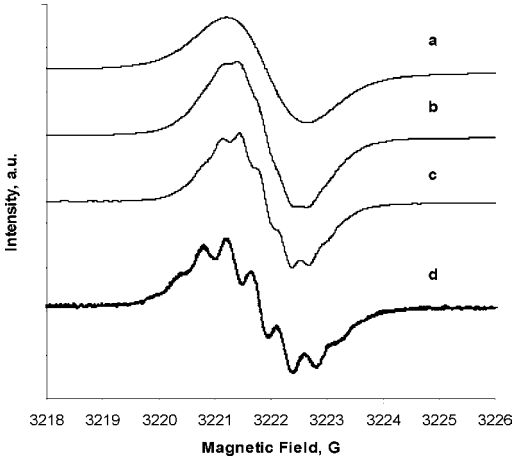

Figure 2. Effect of deoxygenation on the central ($m_I = 0$) nitrogen hyperfine component of TEMPOL EPR spectrum in MGD beer at room temperature: (a) initial, (b) after 3 min nitrogen flow, and (c) after 10 min nitrogen flow; (d) least-squares simulation of the spectrum 1c.

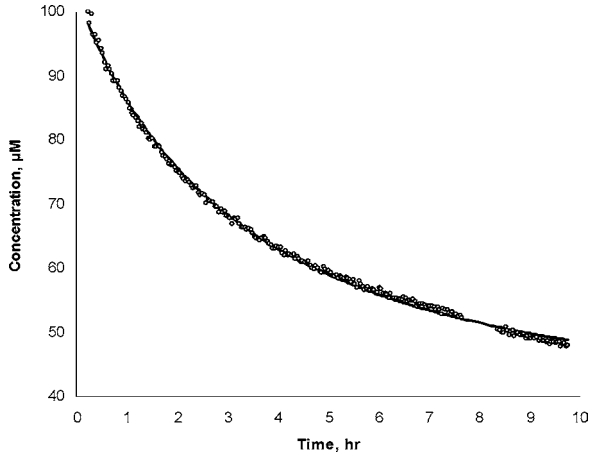

Figure 3. Kinetics of reduction of PDT in MGD beer at room temperature–experimental data and exponential fit.

a difference between the experimental data points and exponential fit, shows that this kinetic model describes the data exceptionally well. In fresh MGD, the rate constant of the reaction with PDT was 54.8 ± 0.5 M^{-1} min^{-1} and the concentration of the reducing agent was 61.7 ± 0.2 μM.

Similar experiments were also conducted with another nitroxide TEMPOL and a different sample of MGD beer. Again, observed kinetics of TEMPOL reduction could be explained by a mechanism according to which there is effectively just one agent irreversibly reducing the nitroxide. The rate constant was ca. 2.25 times higher than with PDT (123 M^{-1} min^{-1}). The concentration of the reducing agent in this experiment (60 ± 1 μM) was essentially the same as the one found previously in the experiment with PDT. Similar results were observed for TEMPO in MGD beer. This indicates an excellent reproducibility of the nitroxide reduction method carried out with different radicals one week apart with different samples of the same brand of beer. For TEMPO in Tiger beer, the concentration of the reducing agent was less than half of what was measured for MGD.

One of the important questions is the nature of compounds determining the redox power of beer. Intermediate organic free radicals (possibly of a semiquinone type) formed in the beer oxidation process are not stable in solutions, and we were able to detect those by EPR only from freeze-dried samples of MGD

at liquid nitrogen temperature (77 K). The spectra (not shown) disappeared after addition of water to the sample.

Another option for a reducing agent was ascorbic acid. Addition of ascorbic acid to beer at 60 °C resulted in a very rapid reduction of nitroxides (*19*). The rate constant of TEMPONE reduction by ascorbic acid in aqueous solutions at room temperature is about 450 M^{-1} min^{-1} (*20*). On the basis of Figure 5 given in ref **19**, we calculate the second-order rate constant to be equal to 2×10^3 M^{-1} min^{-1}. The increase of the rate constant can be easily explained by a temperature dependence of this reaction (*20*). The ascorbyl radicals formed in the reaction with nitroxide give rise to a characteristic two-line EPR spectrum overlapping with the central spectral component of the ^{14}N-containing nitroxide. To avoid this overlap and to increase the sensitivity, we have utilized ^{15}N-substituted perdeuterated TEMPONE and a 10-fold concentrated MGD solution prepared by initial liophylization. EPR spectrum of a ^{15}N-substituted nitroxide has only two major components (**Figure 1d**) because the nuclear spin of ^{15}N is $I = 1/2$, and there should be no overlap of spectral lines at the region characteristic of the ascorbic acid radical. Nevertheless, no EPR signal of ascorbyl radical was observed in this experiment. Furthermore, independent chemical analysis conducted by the Miller Brewing Co. (Milwaukee, WI) demonstrated that there was no detectable ascorbic acid in MGD.

Recently, several authors argued that SO_2 and sulfites are the main antioxidants in beer (*10*, *27*). Concentration of sulfite can reach 100 μM, and this compound is capable to delay the formation of free radicals in beer (*21*). Development of stale cardboard flavor connected with unsaturated aldehyde *trans*-2-nonenal is also prevented by sulfites (*22*). Thus, one could hypothesize that the sulfite present in beer may react with nitroxides. However, our direct experiments with sodium sulfite and nitroxides did not support this hypothesis. Moreover, comparison of the standard redox potentials indicated that this reaction is possible only at a relatively high pH.

Another plausible explanation was that SH-containing substances might be the cause for the observed nitroxide reduction. Cysteine only slowly reacts with TEMPO in water with the second-order rate constant by at least a factor of 500 less than that for the ascorbic acid as determined in separate experiments with aqueous solutions at pH $\sim$ 7. This reaction is affected by the presence of Fe ions and oxygen (*16*). Nevertheless, when cysteine was added with 10^{-3} M EDTA to beer at anaerobic conditions, an increase in the rate of nitroxide reduction was observed. After addition of cysteine, the reaction still could be described by reduction with one reducing agent. In the presence of 5×10^{-2} M cysteine, the effective first-order rate constant was twice as large as the control. Initially, the effective rate constant increased with the concentration of cysteine, but then it reached a constant value (**Figure 4**). The insert in this figure shows this dependence in the inverse coordinates. The effect of saturation demonstrates that the reaction has a catalytic mechanism.

The product of the second-order rate constant and the concentration of reducing agent yields the pseudo-first-order rate constant, which can be useful for characterizing beer and for assessing the effect of different additives/storage conditions. For TEMPOL and MGD, this rate constant was 7.4×10^{-3} min^{-1}.

In the experiments with TEMPO and Tiger beer, the rate constant was 3.2×10^{-3} min^{-1}. The rate constant in the Tiger beer which was filtrated through a 0.22-μ Millex-GP Syringe Driven Filter (Millipore Corp., Bedford, MA) was essentially the same as for nonfiltrated beer and was not affected by

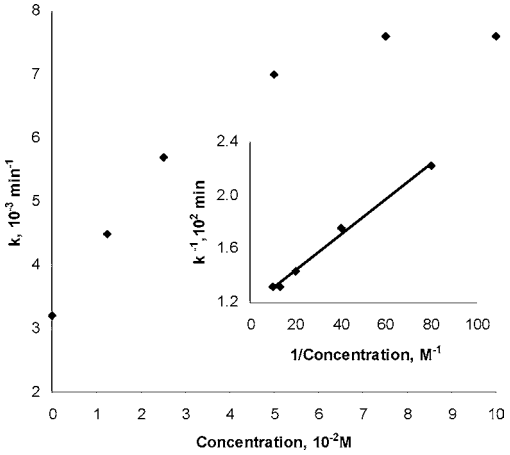

Figure 4. Effective pseudo-first-order rate constant of TEMPO reduction as a function of L-cysteine concentration in Tiger beer. The insert shows these data in inversed coordinates.

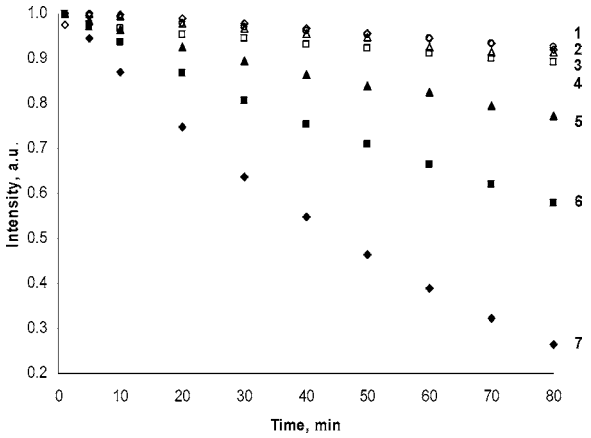

Figure 5. Kinetics of TEMPO reduction in Tiger beer: (1) filtrated, molecular weight cutoff of the filter (MWCO) 30 K, (2) beer + Ellman's reagent, (3) beer + pepsin + Ellman's reagent, (4) boiled beer, (5) control beer, (6) beer + L-cysteine (5×10^{-2} M), (7) beer + pepsin.

centrifugation (12 min at 10 000 rpm) to any significant degree. This means that the reduction is not determined by yeast impurities. Still, when beer was filtrated with a Centrifugal Filter Unit (MWCO 30K, Whatman plc, Maidstone Kent, U.K.) under the same conditions, the effective first-order rate constant was only 9×10^{-4} min^{-1}. These and other related kinetics are shown in **Figure 5**. When beer was preliminary purged with nitrogen for 2 min and then kept at a boiling temperature for 10 min in a closed flask, it became cloudy and the rate constant decreased by more than 2-fold versus the control. Addition of pepsin gave a strong opposite effect. The rate constant after this treatment increased to 1.7×10^{-2} min^{-1}, that is, more than a factor of 5 versus what was observed for TEMPO in a fresh beer. Thus, the decrease in radical concentration in the latter experiments was much more significant than in the untreated beer. In control experiments, it was demonstrated that pepsin alone practically does not react with nitroxide.

We have also studied effects of SH-specific Ellman's reagent on the rate of nitroxide reduction in beer. At pH 4−5, Ellman's reagent is not soluble and when added up to 10^{-3} M concentration it did not affect the rate of nitroxide reduction in beer. When pH was raised to 7.5 by addition of NaOH, this reagent became soluble, turning the solution color to a bright yellow. After treating beer sample with the Ellman's reagent this way for 1.5 h at room temperature, nitroxide TEMPO was added

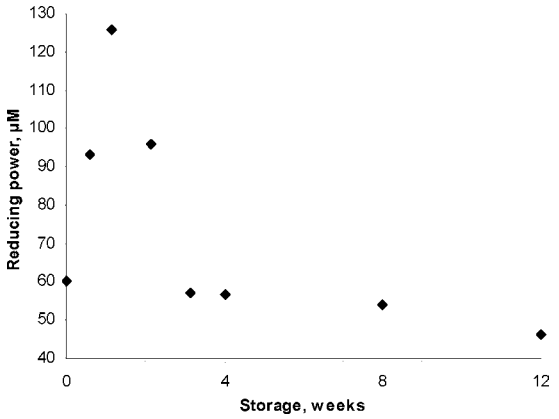

Figure 6. Beer reducing power as determined from TEMPOL experiment vs duration of MGD beer storage at elevated temperature of 32 °C.

and the nitroxide reduction experiment was conducted as before. The rate constant in this experiment was 1×10^{-3} min^{-1} which is less than a third of what was observed for TEMPO in a fresh beer.

When the beer solution was treated with Ellman's reagent following the treatment with pepsin, the rate constant was 1.4×10^{-3} min^{-1}, that is, only slightly higher than that after the Ellman's treatment without pepsin. All these data demonstrate that TEMPO can be reduced by SH groups of unpaired cysteines of proteins present in beer and that these cysteines should be accessible to the nitroxides. The facts that the second-order rate constant in this reaction is much higher than the one obtained for aqueous cysteine solutions and that the rate reaches a plateau at a high concentration of the exogenious cysteine indicate that beer might contain a catalyst which is relatively stable to temperature and pH treatments.

In the next series of experiments, we have investigated the effect of accelerated aging of bottled beer at 32 °C on the effective concentration of the reducing agent. The effective concentration of the reducing agent first increased and then decreased by 10−15% upon storing the beer at the elevated temperature (**Figure 6**). The same decrease was reported earlier by Chapon and co-workers, who used Fe/dipyridyl complex to measure reducing power of beer (*23*). They determined that the concentration of reducing agents was 200 μM, which is higher than our estimates based on the nitroxide kinetics. It is quite likely that the same cysteine groups react with both nitroxides and the Fe/dipyridyl complex, although this complex is a much stronger oxidant and reacts less selectively with some additional components.

DISCUSSION

Oxidative stability of beer and its shelf life are certainly affected by oxygen content and the EPR spectra of nitroxides could be used to evaluate the efficiency of oxygen removal. Simultaneously to the process of oxygen removal, the EPR experiments allowed us to observe the reduction of nitroxides in beer by its endogeneous components that occurred with the effective first order with respect to both a nitroxide and a reducing agent. The effective value of the reducing agent concentration and the rate constant of the reaction with the nitroxide can be determined with high accuracy thus providing us a quantitative method to characterize redox power of beer. This chemical reaction of nitroxides in beer can be explained by a catalytic reduction with participation of SH groups of cysteines. The facts that kinetics of nitroxide reduction was affected by the state of proteins in beer (aggregation and denaturing upon boiling and fragmentation upon pepsin treatment) and accessibility of cysteines to nitroxides provide strong arguments for this hypothesis.

We would like to speculate that during beer storage proteins are partially hydrolyzed and cysteines become more accessible to low molecular weight species in solution, thus increasing the reducing power of beer. Thiols are efficient antioxidants because of their ability to react with free radicals producing thiyl radicals (*24*). In this case, thiols may participate in one-electron oxidation. The process can be both nonenzymatic and enzymatic (*25*). Thiyl radicals accumulated in high concentrations can be prooxidants and it is important to reduce them back to thiol. The latter can be done by ascorbic acid (*26*). Also, ascorbate and cysteine can be prooxidants in beer (*27*). Thiyl radicals are able to react with unsaturated fatty acids (*28*). Sulfur-containing amino acids can participate in photosensitive reactions with isohumulones in beer and can be responsible for lightstruck flavor due to formation of 3-methyl-but-2-ene-1 thiol (*29*). Reactions of thyil radicals result in formation of sulfenic acids and disulfide-S-oxides, thus influencing oxidative stress not only in beer but also in biological systems (*30*). Though the content of proteins, amino acids, and even sulfur compounds in beer has been studied (*31*−*33*), we were not able to find literature data on the cysteine content. Nevertheless, it is known that free thiol groups, measured with Ellman's reagent, are lost during mashing (*34*). This process is catalyzed by mash constituents and results in a formation of hydrogen peroxide (*35*). Heating to 45 °C increases hydrogen peroxide formation by malt proteins that are rich in thiols (*36*). Data on the main oxidoreductases involved in the malting and brewing can be found in ref *37*. All this explains the relationship of redox properties (reducing power) and radical oxidation processes in beer.

Mild and selective reaction of nitroxides with protein SH groups can be used to characterize the initial state of the SH groups in the system, which is extremely important for all subsequent steps of redox and radical reactions in oxidative stress. Combination of kinetics of nitroxide reactions in beer and measurements of lag time (*38*) and new kinetic parameters with spin traps would provide more comprehensive description of factors and components influencing oxidation and storage processes of beer (*39*).

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