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Aldehyde-Induced Xanthine Oxidase Activity in Raw Milk

CHARLOTTE L. STEFFENSEN, HENRIK J. ANDERSEN, AND JACOB H. NIELSEN*

Department of Animal Product Quality, Danish Institute of Agricultural Sciences, Research Centre Foulum, P.O. Box 50, DK-8830 Tjele, Denmark

In the present study, the aldehyde-induced pro-oxidative activity of xanthine oxidase was followed in an accelerated raw milk system using spin-trap electron spin resonance (ESR) spectroscopy. The aldehydes acetaldehyde, propanal, hexanal, *trans*-2-hexenal, *trans*-2-heptenal, *trans*-2-nonenal, and 3-methyl-2-butenal were all found to initiate radical reactions when added to milk. Formation of superoxide through aldehyde-induced xanthine oxidase activity is suggested as the initial reaction, as all tested aldehydes were shown to trigger superoxide formation in an ultrahigh temperature (UHT) milk model system with added xanthine oxidase. It was found that addition of aldehydes to milk initially increased the ascorbyl radical concentration with a subsequent decay due to ascorbate depletion, which renders the formation of superoxide in milk with added aldehyde. The present study shows for the first time potential acceleration of oxidative events in milk through aldehyde-induced xanthine oxidase activity.

KEYWORDS: Aldehydes; milk; oxidation; xanthine oxidase; ascorbate; ascorbyl radical; ESR

INTRODUCTION

Spontaneous oxidation in milk, giving rise to off-flavor formation, is an increasing problem in some countries. The mechanisms behind spontaneous oxidation are far from completely understood. Factors normally believed to be responsible for the oxidative stability of raw milk, e.g., concentrations of polyunsaturated fatty acids, transition metals, vitamin E, and carotenoids, cannot alone explain the accelerated oxidation in milk (*1*). During oxidation of milk, a number of aldehydes are expected to accumulate, leading to aldehyde-derived off-flavors (*2*). However, spontaneously oxidized milk does not contain the amount of aldehydes normally registered in other foods with similar lipid composition. Recently, Nielsen et al. (*3*) showed that the inherent pro-oxidative enzyme activity in milk might be critical in initiation of oxidative reactions. One of the dominating pro-oxidative enzymes in milk is xanthine oxidase, which is activated during cooling of raw milk.

In the presence of O_2, xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine, which is further oxidized to uric acid (*4*). During this reaction, oxygen is reduced into the reactive oxygen species superoxide and hydrogen peroxide (*4*), which are known to be able to initiate further oxidative reactions (*5*). The potential antioxidant uric acid is also formed during the catalyzed oxidation of the purines, hypoxanthines and xanthine, and uric acid has in fact recently been shown to display antioxidative activity in milk (*6*). Consequently, xanthine oxidase-catalyzed oxidation of these purines results in the formation of both pro-oxidative and antioxidative species. However, certain aldehydes, e.g., acetaldehyde and propanal, have also been shown to be potential substrates for xanthine oxidase (*7*). In contrast to the above-mentioned purines, these aldehydes do not oxidize upon formation of known antioxidative compounds. Thus, the characteristic milk enzyme, xanthine oxidase, might be an obvious candidate in the initiation of oxidative processes in both raw and pasteurized fresh milk, as it is not deactivated completely during the pasteurization of milk (*8*). Consequently, a further understanding of the role of xanthine oxidase as a potential contributor to oxidative processes in milk is highly relevant.

In the present study, spin-trap electron spin resonance (ESR) spectroscopy was used to detect and identify radical species formed during the reactions of different aldehydes with xanthine oxidase in an accelerated milk system. Among the aldehydes studied, acetaldehyde, propanal, hexanal, *trans*-2-hexenal, *trans*-2-heptenal, and *trans*-2-nonenal are all potential oxidation products in milk (*2*, *9*), and they have been shown to act as substrates for xanthine oxidase in pure systems (*7*, *10*), while the branched aldehyde 3-methyl-2-butenal is known to be formed by *Streptococcus lactis* var. *Maltigenes* via leucine (*11*). These experiments were carried out to obtain a further understanding of the potential role of xanthine oxidase in oxidative reactions in milk. Moreover, the influence of aldehyde-induced oxidase activity on oxidation of inherent ascorbate in milk was monitored by measuring ascorbate degradation and by monitoring the formation of ascorbyl radicals by ESR spectroscopy.

To the authors' knowledge, aldehyde-induced xanthine oxidase activity in milk and the potential role of aldehydes as critical compounds in the propagation of oxidative processes in milk have never been considered previously.

MATERIALS AND METHODS

Milk and Chemicals. Fresh raw bovine milk obtained from the production herd at Research Centre Foulum was stored for less than 12 h at 5 °C before use. UHT milk (141 °C for 4 s) was bought in

* To whom correspondence should be addressed. Phone: (45) 89 99 11 63. Fax: (45) 89 99 15 64. E-mail: JacobH.Nielsen@agrsci.dk.

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retail trade. Xanthine, xanthine oxidase, 5,5-dimethyl-1-pyroline-*N*-oxide (DMPO), and activated charcoal were obtained from Sigma Chemical Co (St. Louis, MO). The spin trap DMPO, used to detect highly reactive radicals, e.g., superoxide, was diluted to 0.8 M and filtered through activated charcoal before use. Acetaldehyde, propanal, hexanal, *trans*-2-heptenal, *trans*-2-nonenal, 3-methyl-2-butenal were purchased from Aldrich (Sigma-Aldrich, Steinheim, Germany), and *trans*-2-hexenal was purchased from Fluka Chemie AG (Buchs, Switzerland). All other chemicals were of analytical grade. All solutions were prepared within the same day and made up in 50 mM phosphate buffer, pH 7.4, and with an ion strength of 0.16 M.

ESR Experiments. A Bruker EMX X-band ESR spectrometer equipped with an ER 4119HS cavity (Bruker Analytische Messtechnik, Rheinstetten, Germany) was used to record ESR spectra at 25 °C. The magnetic field was modulated with a frequency of 100 kHz using 2 G field modulation amplitude, 20 mW microwave power, a receiver gain of 2×10^6, a sweep time of 84 s, and a time constant of 20.48 ms.

Hexanal-Induced Oxidation in Raw Milk. Upon addition of hexanal to a total concentration of 40 mM in a solution of 80% raw milk, containing 80 mM DMPO, hexanal-induced formation of reactive organic radicals in milk was followed using ESR spectroscopy, as mentioned above. The reaction was followed for 30 min, and as control an identical reaction mixture where hexanal was replaced with buffer was used.

Aldehyde-Induced Xanthine Oxidase Activity in UHT Milk. Xanthine oxidase and different aldehydes, expected to act as substrates for xanthine oxidase, were added to UHT milk known to display zero enzyme activity, to display the effect of xanthine oxidase without interference from other enzyme systems. The reaction mixtures were made by addition of acetaldehyde, propanal, hexanal, *trans*-2-hexenal, *trans*-2-heptenal, *trans*-2-nonenal, or 3-methyl-2-butenal to a final concentration of 20 mM in a solution of 75% UHT milk containing 80 mM DMPO and 0.5 μM xanthine oxidase. Complementary mixtures without added xanthine oxidase were used as controls. ESR spectra were recorded 10 min after addition of the enzyme.

The relative formation of aldehyde-induced ascorbyl radicals in raw milk was followed upon addition of different aldehydes using ESR spectroscopy. Solutions of 20 mM acetaldehyde, propanal, hexanal, *trans*-2-hexenal, *trans*-2-heptenal, *trans*-2-nonenal, or 3-methyl-2-butenal and 2 mM acetaldehyde in buffer were mixed with raw milk in the ratio 1:9. ESR spectra were repeatedly recorded during a 50 min incubation time at 25 °C after addition of the aldehyde. As control the aldehyde was replaced with buffer. The relative concentration of ascorbyl radicals in the raw milk was determined by the height of the ESR signal of the ascorbyl radical.

Analysis of Ascorbate and Dehydroascorbate. Quantification of ascorbate was carried out after derivatization of dehydroascorbate using HPLC separation and fluorescence detection (HP1100, Agilent Technologies, Palo Alto, CA) based on a modification of the method of Tessier et al. (*12*).

Ascorbate and dehydroascorbate were isolated from the raw milk by precipitation of the protein with metaphosphoric acid (0.56% w/v). After centrifugation (15000*g* for 10 min), 150 μL of the supernatant containing dehydroascorbate and ascorbate was oxidized for 10 min by adding 30 μL of iodine solution (4.92 mM I_2 in 0.16 M KI) in order to measure the total concentration of ascorbate and dehydroascorbate. The concentration of dehydroascorbate was measured by substituting water for the iodine solution. Subsequently, the solutions were derivatized in the presence of 150 μL of phosphate buffer, pH 5.4 (1.63 M), and 60 μL of dimethyl-*p*-phenyldiamine (DMPD) (1 g/L DMPD was solubilized in 1 mL of HCl prior to addition of water). HPLC analysis of the dehydroascorbic derivatives was performed on a Hypersil ODS column (250 × 4 mm) with MeOH/phosphate buffer, pH 7.4, 80 mM (1:1 v/v) as mobile phase (flow 0.6 mL/min) and fluorescence detection (λ_{ex} 360 nm, λ_{em} 440 nm).

The amounts of ascorbate in raw milk and raw milk with added 20 mM hexanal were monitored during incubation at 25 °C for 120 min.

RESULTS AND DISCUSSION

Purine-induced xanthine oxidase activity accelerates the oxidation of ascorbate in raw milk (*3*). However, the levels of hypoxanthine and xanthine in raw milk are very low, and the end product, urate, is found to be an antioxidant in milk (*5*). Consequently, the potential pro-oxidative role of purine-induced xanthine oxidase activity in milk could be questioned.

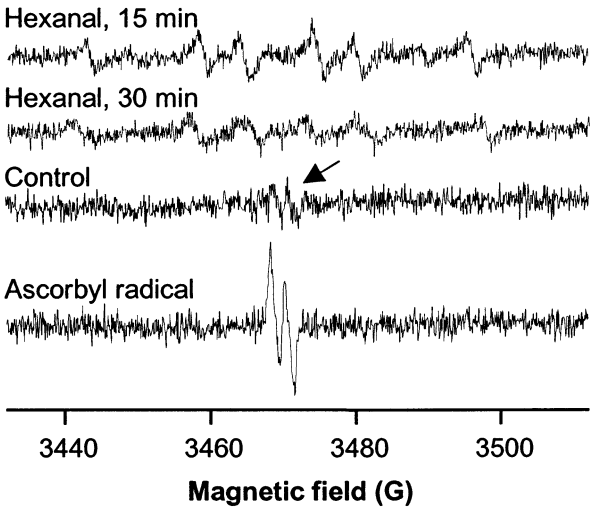

Figure 1. Typical ESR spectra measured at ambient temperature in an 80% raw milk solution measured 15 and 30 min after addition of 40 mM hexanal using 80 mM DMPO as spin trap. Control shows the small ascorbyl radical ESR signal observed throughout the incubation time, shown by the arrow. The two-line ESR signal of ascorbyl radical observed in a 2 mM ascorbate solution is shown for comparison.

Figure 1 shows typical ESR spectra of accumulated organic radicals in a milk system upon addition of hexanal, using DMPO as spin trap. The observed ESR signal changed over time; i.e., it became slightly broader. Without addition of hexanal, a small two-line ESR signal ($a^H = 1.8$ G) characteristic of the ascorbyl radical was found (*13*), and the signal remained unchanged during the entire incubation time. To verify the identity of the ascorbyl radical in milk, an ESR signal of the ascorbyl radical seen in a solution of ascorbate in buffer is also shown.

The obtained ESR spectra did not support a direct formation of superoxide radicals in raw milk upon addition of hexanal, to indicate the involvement of xanthine oxidase. However, the observed formation of organic radicals upon addition of an aldehyde to raw milk shows that the aldehyde stimulates propagation of oxidative processes in the system. It is not surprising that no superoxide is trapped by DMPO in the raw milk, as the formed superoxide readily may react with other milk constituents found in high concentrations (e.g., ascorbate, as discussed later, urate, cysteine residues, etc.). Superoxide can also dismutate to hydrogen peroxide either spontaneously or through enzymatic reactions, e.g., by superoxide dismutase, which is known to be present in milk (*14*). Recently, hydrogen peroxide was shown to enhance oxidative processes in milk through activation of inherent peroxidases, e.g., lactoperoxidase, resulting in protein oxidation and enhanced oxidation of ascorbate and urate (*3*, *5*). Consequently, even though no superoxide was detected in the present upset, xanthine oxidase is an obvious mediator of aldehyde-induced oxidation, which give rise to the DMPO-trapped radicals.

Figure 2 shows the spin trap ESR spectra obtained upon addition of aldehydes to an otherwise enzyme-silent UHT milk system supplemented with xanthine oxidase in an amount normally present in raw milk (*15*). The spectra all include the typical DMPO−OOH ESR signal, with characteristic hyperfine splittings, $a^N = 14.3$ G, $a^H = 11.7$ G, $a^H = 1.25$ G (*16*), arising from DMPO-trapped superoxide. The signal was weak when

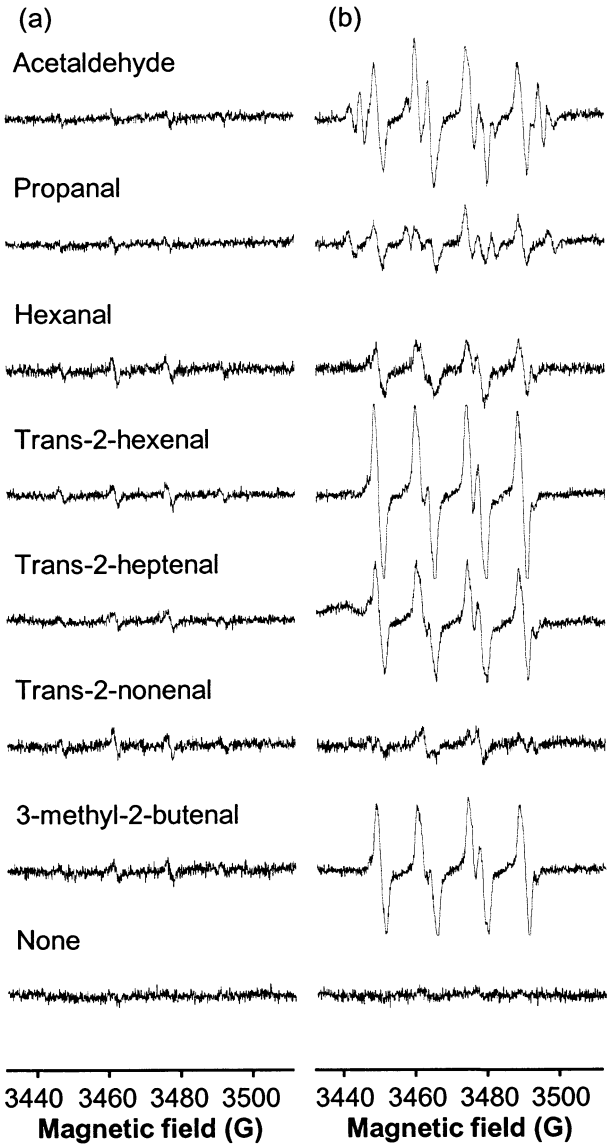

Figure 2. Typical ESR spectra measured in UHT milk solutions containing (a) 75% UHT milk, 80 mM DMPO, and a 20 mM concentration of the respective aldehydes; (b) 75% UHT milk, 80 mM DMPO, 0.5 μM xanthine oxidase, and a 20 mM concentration of the respective aldehydes.

trans-2-nonenal was used as substrate. When acetaldehyde or propanal was added, other DMPO-trapped organic radicals were detected along with superoxide. A weak DMPO−OH signal with hyperfine splittings, $a^N = 14.95$ G, $a^H = 14.95$ G, caused by DMPO-trapped hydroxyl radicals (*17*) could be seen when 80 mM DMPO and a 20 mM concentration of an aldehyde were added to UHT milk without xanthine oxidase, which indicates that the added aldehydes contained radicals or hydroperoxides that can be cleaved homolytically, with subsequent formation of hydroxyl radicals in the UHT milk.

The DMPO-trapped superoxide, detected in the present UHT milk system upon addition of both xanthine oxidase and any of the examined aldehydes, supports that the reaction responsible for generation of organic radicals in milk in the presence of aldehydes involves enzymatic oxidation of the aldehyde:

$$\text{aldehyde} + 2O_2 \xrightarrow{\text{xanthine oxidase}} \text{corresponding carboxylic acid} + 2O_2^{\bullet -} \quad (1)$$

The radicals detected along with superoxide when acetaldehyde or propanal was used as substrate for xanthine oxidase in UHT milk have earlier been detected in pure aldehyde-induced xanthine oxidase systems, as reported by Bosser and Berlin (*18*), and are thus not a consequence of the milk matrix but are more likely radical products from the reaction between aldehyde and xanthine oxidase.

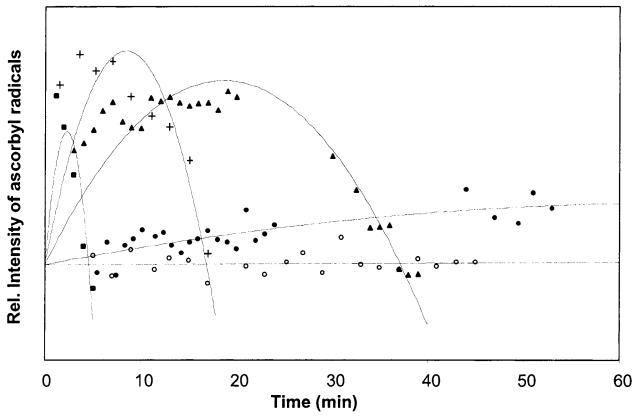

Figure 3. Kinetic traces for ascorbyl radical formation in 90% milk with different aldehydes added [hexanal (20 mM, +), *trans*-2-heptenal (20 mM, ▲), *trans*-2-nonenal (20 mM, ●), acetaldehyde (2 mM, ■), and buffer (control, ○)] as measured by ESR spectroscopy at ambient temperature.

The above results support that aldehydes detectable in oxidized milk [e.g., hexanal (*9*)] and aldehydes already known to be substrates for xanthine oxidase in pure systems (*4*, *7*, *10*), as well as the branched aldehyde 3-methyl-2-butenal, can act as substrate for xanthine oxidase in a milk model system resulting in superoxide, hereby indicating a pro-oxidative effect of these aldehydes in raw milk.

The superoxide formed by, e.g., xanthine oxidase activity is known to oxidize ascorbate to the ascorbyl radical (*15*). Ascorbyl radicals are easily detectable by ESR spectroscopy, which is why it is possible to follow the oxidation of ascorbate as an indirect evidence of superoxide formation in raw milk.

Figure 3 clearly shows a steady-state concentration of ascorbyl radicals in raw milk during an incubation time of 45 min at 25 °C. However, upon addition of aldehydes, e.g., hexanal and *trans*-2-heptenal, to milk, the initial ascorbyl radical concentration increases rapidly 3-fold and decays subsequently below the limit of detection, as also seen in **Figure 3**. The developments in ascorbyl radical concentration found upon addition of propanal, *trans*-2-hexenal, and 3-methyl-2-butenal closely resemble the development after addition of hexanal and are thus not shown. These findings resemble those of Nakamura (*15*), showing that addition of xanthine to milk results in a 5-fold increase of the ascorbyl radical concentration level. It is proposed that the increase in the concentration of ascorbyl radicals upon addition of aldehydes is a result of oxidation of ascorbate, either directly by superoxide or indirectly by lactoperoxidase (*3*). The subsequent decrease in the ascorbyl radical concentration is due either to interaction of ascorbyl with the lactoperoxidase system, as reported by Nakamura (*15*), or more obviously to the well-known disproportionation of the ascorbyl radicals (*19*):

$$2\ \text{ascorbate}^{\bullet} \rightarrow \text{ascorbate} + \text{dehydroascorbate} \quad (2)$$

That the subsequent decrease in the concentration of ascorbyl radicals is likely to be due to disproportionation of the ascorbyl radicals is supported by data which show that the inherent

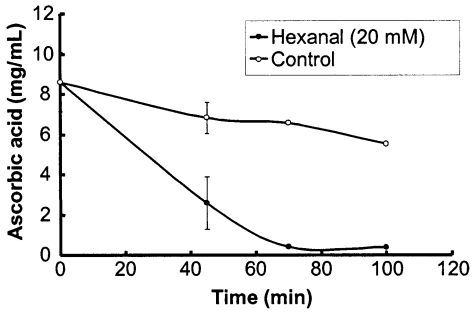

Figure 4. Oxidation of ascorbate in raw milk, with and without addition of 20 mM hexanal at 25 °C. Data are mean ± SD from three independent experiments.

ascorbic acid in milk with added hexanal was almost completely oxidized after approximately 80 min of incubation with hexanal (**Figure 4**), while the steady-state concentration of the ascorbyl radical was reached after less than 20 min of incubation in an identical system (**Figure 3**).

In the absence of hexanal, the decrease in the amount of ascorbate was less than 25%, corresponding to the known slow ongoing oxidation of ascorbate in the milk (*13*, *20*).

Addition of 20 mM acetaldehyde to milk eliminated the ascorbyl radical, while addition of 2 mM acetaldehyde showed a very fast depletion of ascorbyl radical (**Figure 3**). This is in agreement with the previous experiments where acetaldehyde was found to be the most optimal substrate for xanthine oxidase among the aliphatic aldehydes (*4*). When *trans*-2-nonenal was added to milk, only a slight increase in the concentration of ascorbyl radicals was observed (**Figure 3**). This is consistent with the very slow superoxide formation found in xanthine oxidase-enriched UHT milk with added *trans*-2-nonenal (**Figure 2**). These results indicate that hydrophobic aldehydes react with xanthine oxidase at a slower rate than the more hydrophilic aldehydes.

In conclusion, it is found that several aldehydes detectable in milk can propagate oxidation in accelerated milk model systems. Aldehydes are found to be oxidized by xanthine oxidase upon reduction of oxygen to superoxide. Superoxide is able to oxidize ascorbate directly or to dismutate to hydrogen peroxide, known as a substrate for peroxidases in milk. The delicate antioxidative balance found in all biological systems might thus be even more sensitive in raw milk and in pasteurized dairy products, as aldehydes, being secondary oxidation products, can propagate and thereby accelerate oxidation in milk through the xanthine oxidase enzyme system, potentially with off-flavors or functional changes as a result.

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