Oxidation of Ascorbate in Raw Milk Induced by Enzymes and Transition Metals

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The effect of xanthine oxidase, lactoperoxidase, and transition metals [Fe(III), Cu(II)] on the oxidation of ascorbate in raw milk was investigated. Data clearly showed that iron(III) (200 μM) does not accelerate ascorbate oxidation in raw milk in concentrations relevant for raw milk. In contrast, addition of copper(II) (10 μM) to the raw milk accelerated oxidation of ascorbate. Furthermore, both xanthine oxidase and peroxidase activity were found to accelerate ascorbate oxidation dramatically in raw milk, indicating that xanthine oxidase and lactoperoxidase might be some of the most obvious candidates for mediation of ascorbate oxidation in raw milk. The present data are discussed in relation to using the fate of ascorbate in raw milk as an indicator of the oxidative stability of the milk.

Keywords: *Ascorbate; oxidation; milk; lactoperoxidase; xanthine oxidase*

INTRODUCTION

Spontaneous oxidation of raw milk has been reported to be a major problem in raw milk produced in certain countries, for example, Canada and Sweden (*1*, *2*). Despite intensive studies of potential causes for the initiation and propagation of spontaneous oxidation, the underlying mechanisms are still a matter of dispute (*2*). Recent investigations of factors traditionally known to affect oxidation in raw milk, for example, content of polyunsaturated fatty acids, transition metals, activity of pro-oxidative enzymes (xanthine oxidase and lactoperoxidase), antioxidants (tocopherols, ascorbate, carotenoids, etc.), have not been able to explain the susceptibility of milk from specific herds to oxidation (*2*). However, milk from cows with high milk yield compared with milk from cows with low milk yield is more susceptible to oxidation due to lower concentrations of antioxidants (e.g., tocopherols and carotenoids) in milk from high-yielding cows (*3*). Most studies on spontaneous oxidation have identified this quality defect using sensory analysis and/or detection of primary and secondary oxidation products, as lacking sensitivity of previously available/used analytical methods has not made it possible to predict spontaneous oxidation before the deterioration has taken place. Nowadays, however, more sensitive analytical methods should make it possible to measure the fate of specific biomarkers, for example, antioxidants known to influence the formation of spontaneous oxidation, which can subsequently be used to predict the quality of milk before the deterioration has taken place.

Ascorbic acid, ascorbate in milk, is known for its antioxidative activity through direct scavenging of singlet oxygen and other reactive oxygen species (superoxide, peroxyl, and hydroxyl radicals) as well as potential regenerator of α-tocopherol and urate from α-tocopheryl radicals and urate radicals, respectively (reviewed in ref *4*). However, ascorbate can also exert in vitro pro-oxidant properties through reduction of Fe(III) to the more reactive species Fe(II).

Both the multiple antioxidative and pro-oxidative properties of ascorbate result in oxidation of this species. Consequently, degradation of ascorbate might be a parameter that is suitable as an early marker of oxidative stress of the milk. Oxidation of ascorbate leads to generation of the ascorbyl radical, which can be regenerated to ascorbate either through reaction with an identical species or disproportionation to dehydroascorbate (*5*). Dehydroascorbate is unstable and undergoes further degradation by poorly understood reactions, most probably to oxalic acid and L-threonic acid.

Ascorbate is known to oxidize during the storage of raw milk; however, knowledge regarding the parameters mediating this reaction is rather limited. High levels of transition metals (iron or copper ions) in the milk obtained either from feeding or from external contamination of the milk have been proposed to be a major factor in the acceleration of ascorbate oxidation (*6*, *7*).

The present study was conducted to identify potential endogenous factors as pro-oxidative enzymes and transition metal ions of importance for the acceleration of ascorbate oxidation in milk in relation to the potential use of ascorbate as an early marker of spontaneous oxidation in raw milk.

MATERIALS AND METHODS

Chemicals. *m*-Phosphoric acid was purchased from Merck (Darmstadt, Germany), and L-ascorbic acid, dimethyl-*p*-phenyldiamine (DMPD), potassium iodide, iode, xanthine oxidase, hypoxanthine, catalase, lactoperoxidase, hydrogen peroxide, ferric chloride hexahydrate, and cupric sulfate were obtained from Sigma (St. Louis, MO).

Milk Samples and Initiation of Oxidation. The raw milk samples were obtained from our own production herd at

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Research Centre Foulum and used for oxidation studies 16 h after milking. Ultrahigh-temperature-treated (UHT) milk (141 °C for 4 s) was purchased from the local supermarket.

The reaction mixture was made from 60% milk and 40% 50 mM phosphate buffer, pH 6.6.

Ascorbate was added to the milk to mimic the concentration found in milk immediately after milking. Supposed initiators of ascorbic acid oxidation such as Fe(III), Cu(II), hypoxanthine, and hydrogen peroxide were dissolved in the buffer fraction and added to the milk at time zero. The oxidation of ascorbate was followed in milk stored at 25 °C for 120 min. All experiments were performed in at least duplicates. All data were evaluated by one-tailed analysis of variance.

Analysis of Ascorbate and Dehydroascorbate. The quantification of ascorbate was based on analysis of a fluorescence derivative of dehydroascorbate using HPLC separation and fluorescence detection (HP1100, Agilent Technologies, Palo Alto, CA) based on a modified method of Tessier et al. (*8*).

Ascorbate and dehydroascorbate were isolated from the raw milk by precipitation of the protein with *m*-phosphoric 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 the iodine solution with water. Subsequently, the solutions were derivatized in the presence of 150 μL of phosphate buffer, pH 5.4 (1.63 M), and 60 μL of 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 (1:1 v/v), pH 7.4, 80 mM as mobile phase (flow = 0.6 mL/min), and fluorescence detection (excitation, 360 nm; emission, 440 nm).

RESULTS AND DISCUSSION

Initiation and propagation of oxidative deterioration in milk have been suggested to be brought about by a complex series of reactions involving dissolved oxygen, transitions metal ions, ascorbic acid, lipids, enzymes, and possible other milk constituents (*7*, *9*−*12*). Moreover, interactions between the factors influencing oxidative deterioration in milk may take place, and it has been shown that the oxygen concentration and the presence of transition metal ions affect the rate of ascorbate oxidation in milk (*13*, *14*). More recently, Ford et al. (*6*) reported an increase of 58% in oxidized ascorbate during 2 days of storage at 4 °C in raw milk with high copper content (60 μg/L) compared with "normal" milk (35 μg/L). Pasteurization (72 °C for 15 s) of the milk rich in copper reduced the oxidation of ascorbate in milk to 17%, whereas pasteurization at 82 °C for 15 s completely eliminated the oxidation of ascorbate. Finally, the same study also reported that exposure of the raw milk to light caused rapid destruction of ascorbate irrespective of the pasteurization temperature. These results indicate in addition to copper that enzymatic and light-induced degradations of ascorbate most probably are important factors in the degradation of ascorbate in milk. An early study by King and Dunkley (*13*) stated that transition metal-mediated ascorbate oxidation was highly correlated to simultaneous lipid oxidation. Consequently, a more thorough understanding of enzymatic and light-induced mechanisms for ascorbate degradation is necessary if strategies for improving the oxidative stability of milk have to be established. In the present study we focus on the potential role of enzyme-induced oxidation of ascorbate.

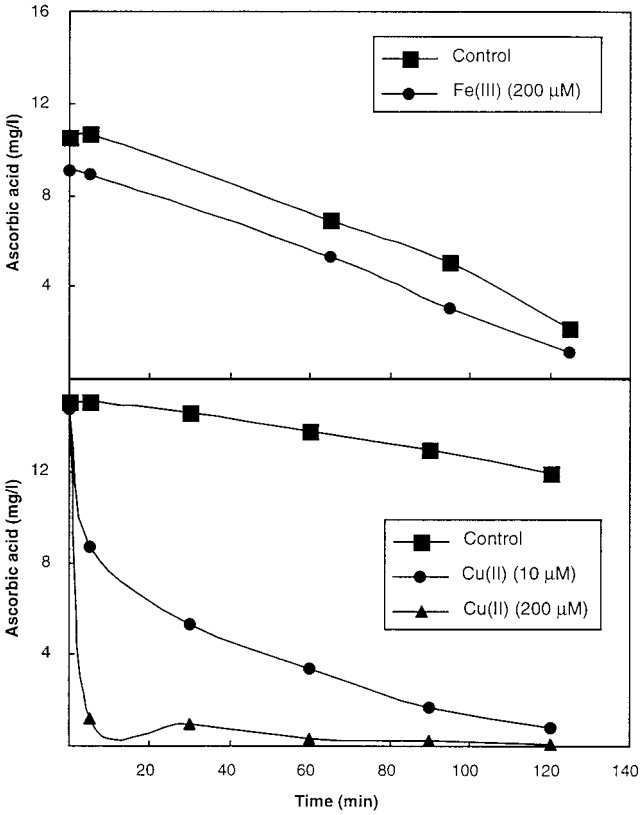

Figure 1. (A, top) Spontaneous oxidation of ascorbate and the influence of 200 μM Fe(III) addition on ascorbate oxidation in raw milk spiked with ascorbate (10 mg/L) during incubation for 120 min at 25 °C ($p > 0.05$). (B, bottom) Spontaneous oxidation of ascorbate and the influence 10 and 200 μM Cu(II) addition on ascorbate oxidation in raw milk spiked with ascorbate (10 mg/L) during incubation for 120 min at 25 °C ($p < 0.05$).

Figure 1 shows spontaneous oxidation of ascorbate in raw milk spiked with ascorbate to reflect the levels found immediately after milking and the influence of the addition of Fe(III) (200 μM) and Cu(II) (10 and 200 μM) on transition metal ion-mediated ascorbate oxidation in raw milk. Besides a small reduction in ascorbate immediately after the addition of 200 μM Fe(III), the addition of free iron ions was not able to initiate notable oxidation of ascorbate in raw milk. In contrast, the addition of both an unnaturally high concentration of Cu(II) (200 μM) and a concentration normally found in milk [10 μM Cu(II)] immediately accelerated oxidation of ascorbate compared with the spontaneous oxidation of ascorbate. This support earlier studies, which also found that copper in concentrations relevant to milk accelerates the degradation of ascorbate. In contrast, we could not confirm previous data on ferric iron-mediated ascorbate degradation (*13*) as we did not measure any pro-oxidative activity even at 200 mM Fe(III), which is ∼10 times the iron concentration normally reported in raw milk (*15*). This might be explained by excess iron-chelating capacity in the raw milk, for example, by proteins and citrate, which prevents redox cycling of iron through reaction with ascorbate and thereby potential formation of reactive oxygen species.

To investigate the potential pro-oxidative activity of the membrane-associated enzyme xanthine oxidase in the oxidation of ascorbate, a xanthine oxidase specific substrate, hypoxanthine, was added to raw milk spiked with ascorbate to a level normally found in raw milk

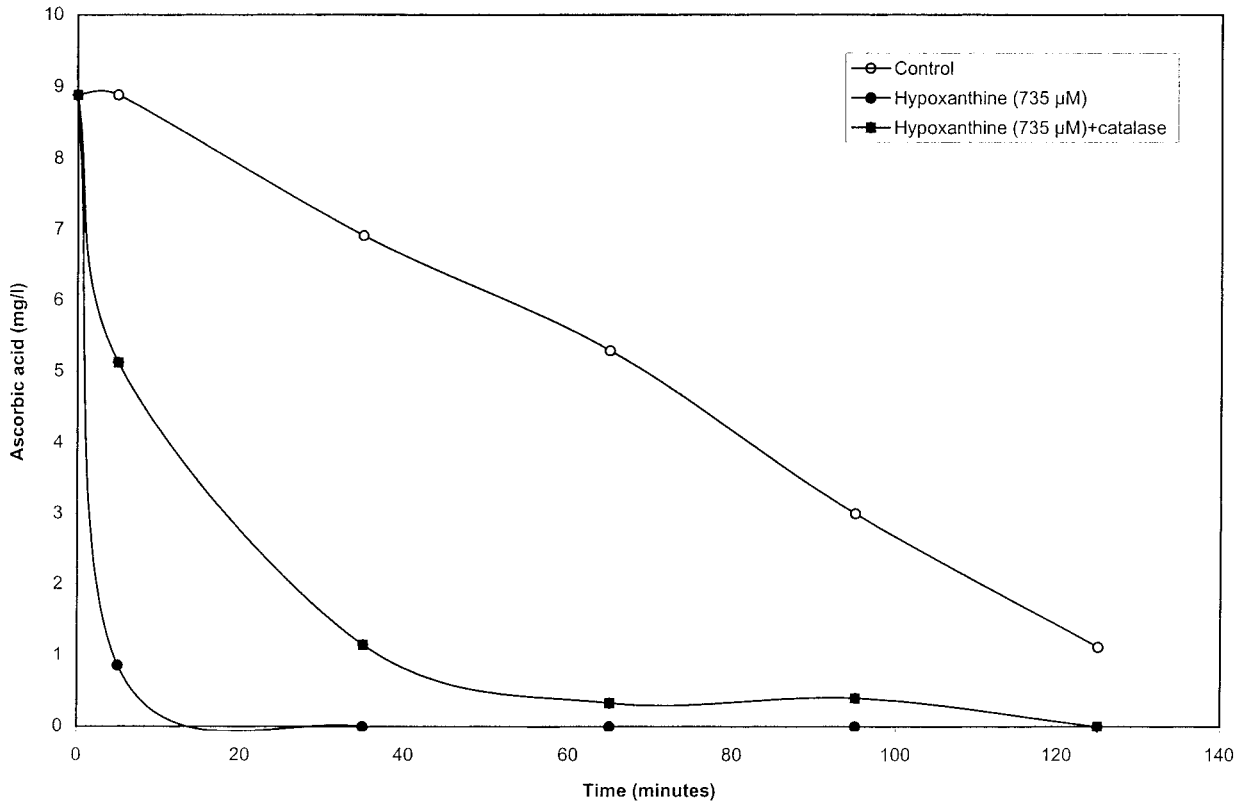

Figure 2. Spontaneous oxidation of ascorbate and the influence of addition of either hypoxanthine (733 μM) ($p < 0.05$) or hypoxanthine (733 μM) in combination with catalase (530 IU/mL) ($p > 0.05$) on ascorbate oxidation in raw milk spiked with ascorbate (10 mg/L) during incubation for 120 min at 25 °C.

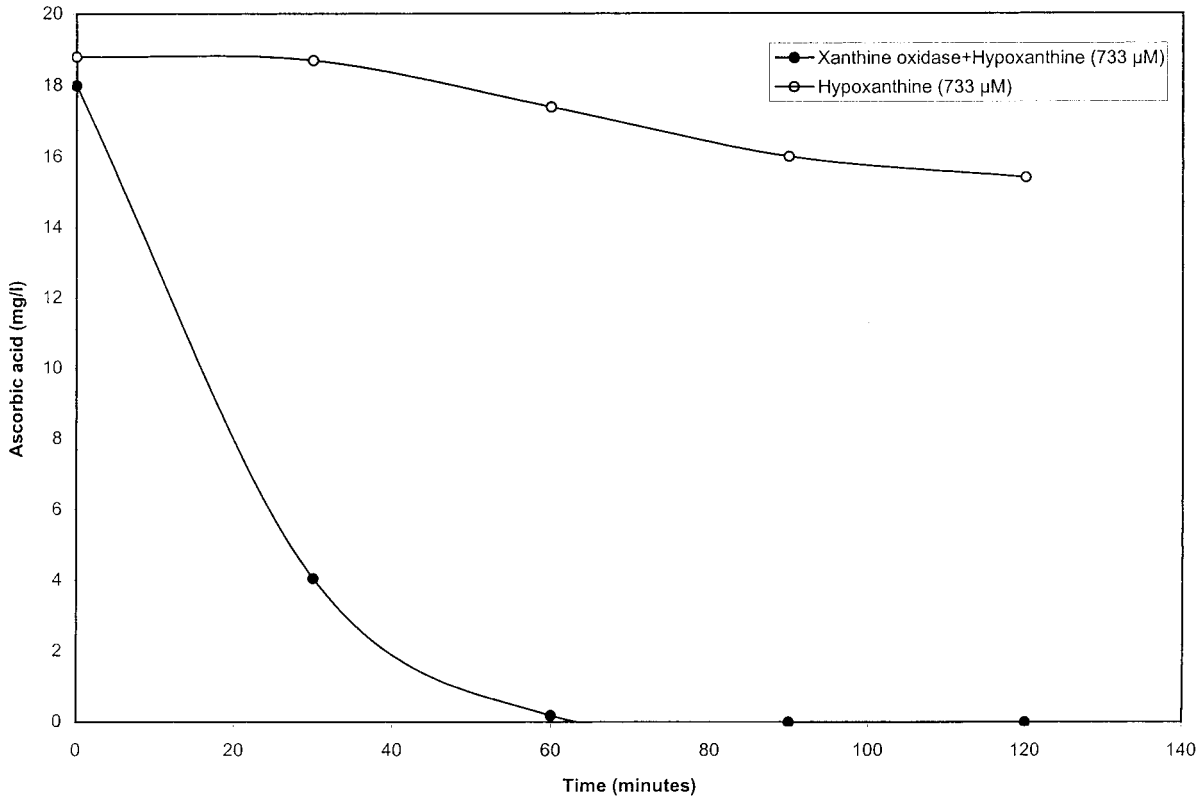

Figure 3. Spontaneous oxidation of ascorbate and the influence of addition of either hypoxanthine (733 μM) or hypoxanthine (733 μM) in combination with xanthine oxidase (0.28 IU/mL) on ascorbate oxidation in UHT milk spiked with ascorbate (10 mg/L) during incubation for 120 min at 25 °C ($p < 0.05$).

immediately after milking. Figure 2 compares spontaneous oxidation of ascorbate with the addition of hypoxanthine alone and a simultaneous addition of hypoxanthine and catalase in raw milk. Addition of 735 μM hypoxanthine initiated instant oxidation of ascorbate, resulting in nearly 100% depletion within ~20

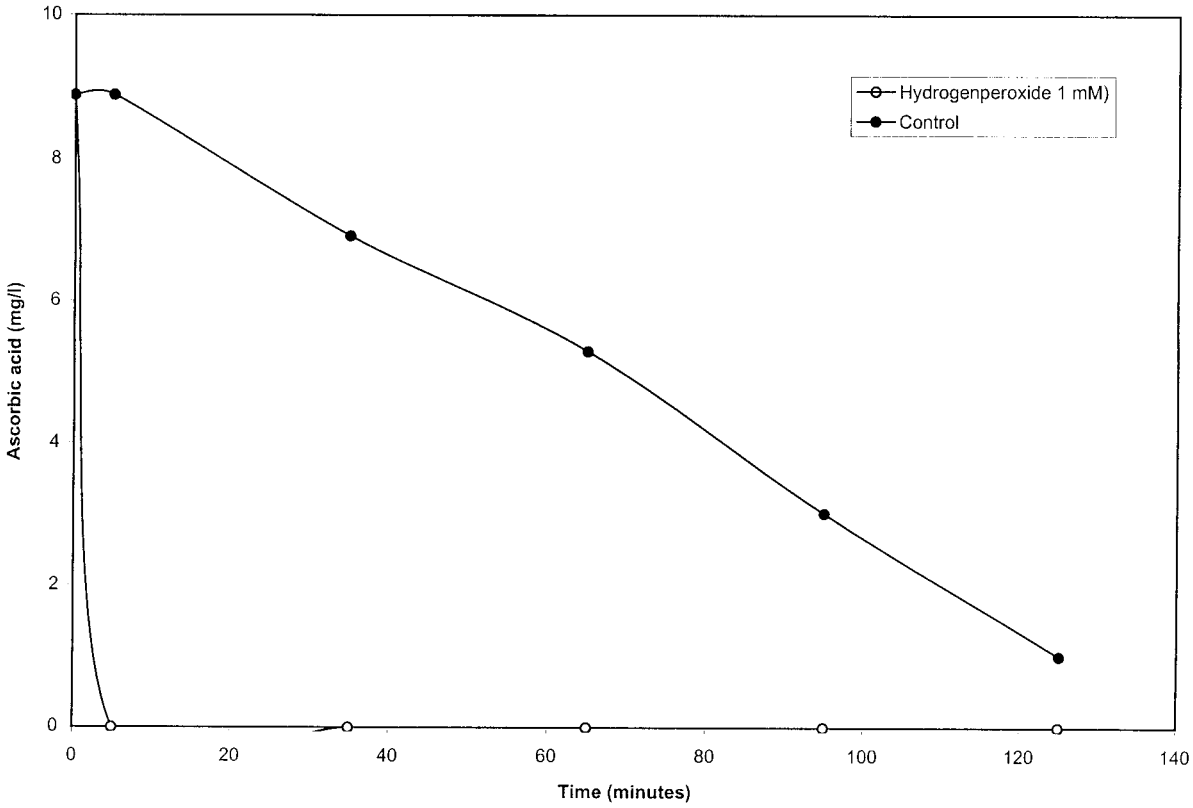

Figure 4. Spontaneous oxidation of ascorbate and the influence of addition of hydrogen peroxide (1 mM) on ascorbate oxidation in raw milk spiked with ascorbate (10 mg/L) during incubation for 120 min at 25 °C ($p < 0.05$).

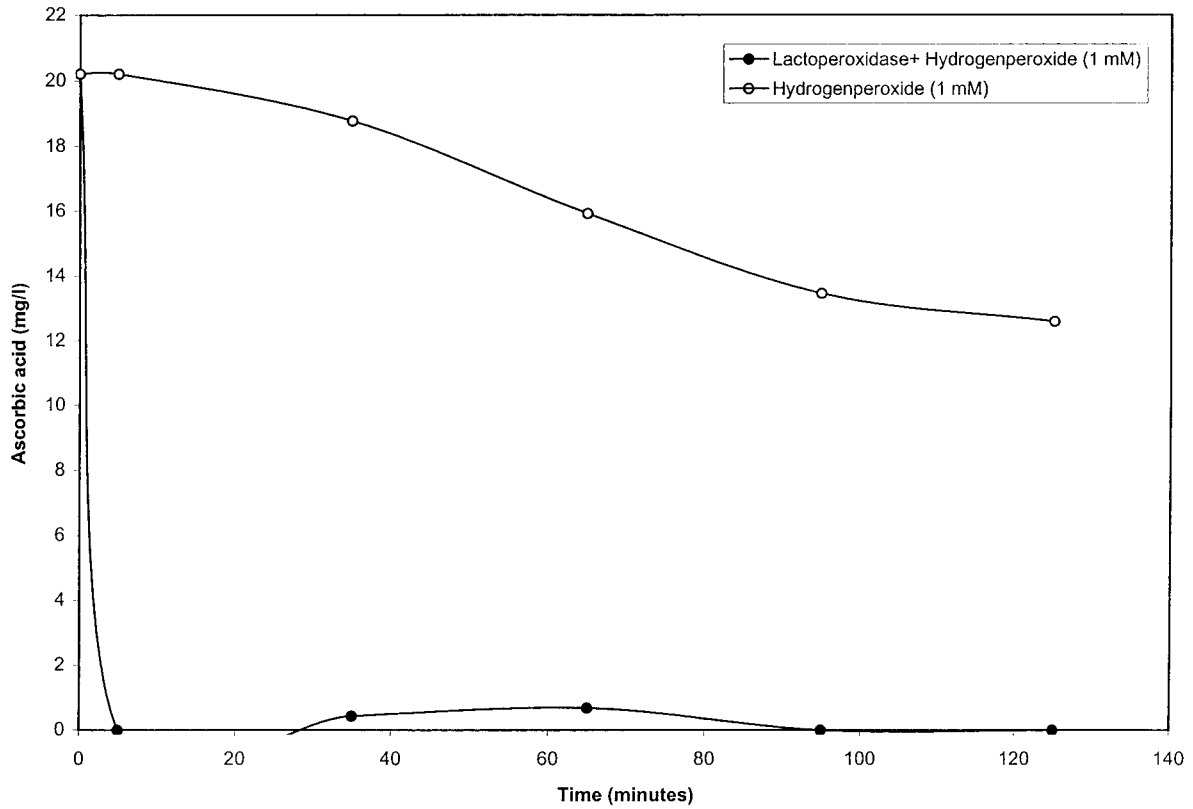

Figure 5. Spontaneous oxidation of ascorbate and the influence of addition of either hydrogen peroxide (1 mM) or hydrogen peroxide (1 mM) in combination with lactoperoxidase (10 IU/mL) on ascorbate oxidation in UHT milk spiked with ascorbate (10 mg/L) during incubation for 120 min at 25 °C ($p < 0.05$).

min, whereas >120 min was necessary to reach the same degree of spontaneous oxidation. Simultaneous addition of hypoxanthine (735 μM) and catalase (530 IU/mL) also strongly accelerated the oxidation of ascorbate compared with spontaneous oxidation, however, not to the same degree as hypoxanthine alone. Obtained

data show that the presence of a specific substrate for xanthine oxidase efficiently stimulates ascorbate degradation in raw milk (Figure 2). This may proceed through direct reaction between formed superoxide and ascorbate, resulting in the ascorbyl radical, which is a well-described reaction. Alternatively, superoxide may dismutate to hydrogen peroxide, which subsequently reacts with transition metals or peroxidases giving rise to compound **I** species (eq 1) (*16*), compound **II** species

$$\text{compound } \mathbf{I} + \text{ascorbate} \rightarrow \rightarrow \text{ascorbyl radical} \xrightarrow{-e^-} \rightarrow \text{dehydroascorbate} \quad (1)$$

$$\text{compound } \mathbf{II} + \text{ascorbate} \rightarrow \rightarrow \text{ascorbyl radical} \xrightarrow{-e^-} \text{dehydroascorbate} \quad (2)$$

$$\text{(peroxidase) protein radical} + \text{ascorbate} \rightarrow \rightarrow \text{ascorbyl radical} \xrightarrow{-e^-} \text{dehydroascorbate} \quad (3)$$

(eq 2) (*17*, *18*), or protein radical species (eq 3) (*19*), which all can react with ascorbate and form the ascorbyl radical recently reported to be present in bovine milk (*20*).

Simultaneous addition of hypoxanthine and catalase to the raw milk diminished ascorbate degradation considerably (Figure 2). Catalase catalyzes the degradation of hydrogen peroxide to water and oxygen, which indicates that hydrogen peroxide formed by dismutation of superoxide is a crucial mediator in the degradation of ascorbate in raw milk in addition to the direct degradation of ascorbate through reaction with superoxide.

To verify whether xanthine oxidase mediated the observed oxidation in raw milk, hypoxanthine was added to ascorbate-spiked UHT milk, in which no xanthine oxidase activity can be measured. Figure 3 shows that hypoxanthine (735 μM) did not initiate ascorbate oxidation in UHT milk unless xanthine oxidase (0.28 IU/mL) was added simultaneously. This clearly supports the fact that either xanthine oxidase activity, the presence of hydrogen peroxide formed by dismutation of superoxide, or a mutual combination of these is a crucial factor in the oxidation of ascorbate in raw milk.

Likewise, hypoxanthine, hydrogen peroxide (1 mM) was added to raw milk spiked with ascorbate to the level found immediately after milking to evaluate potential peroxidase activity as a mechanistic mediator of ascorbate oxidation. Figure 4 shows that the addition of hydrogen peroxide (1 mM) immediately oxidized the present ascorbate. To verify whether peroxidase activity—here represented by the major peroxidase present in milk, lactoperoxidase—mediated the observed oxidation of ascorbate in raw milk, hydrogen peroxide was added to ascorbate-spiked UHT milk, in which no peroxidase activity can be measured. Figure 5 shows that ascorbate oxidation in UHT milk upon direct addition of hydrogen peroxide (1 mM) proceeded slowly unless lactoperoxidase (10 IU/mL) was added simultaneously. This states that Fenton chemistry-mediated degradation of ascorbate seems to be less likely in milk. In contrast, it can be concluded that enzymatically mediated ascorbate degradation in raw milk, either by xanthine oxidase resulting in superoxide or by present peroxidases after reaction with hydrogen peroxide (eqs 1−3), are the most probable mechanisms behind the rapid oxidation of ascorbate. Data moreover support the suggested role of lactoperoxidase activity as a crucial factor in ascorbate degradation in milk (*9*).

The present data cannot establish the relative importance of the different enzymatic pathways involved in the oxidation of ascorbic acid in raw milk. However, it seems to be reasonable to suggest that intrinsic enzyme activities in raw milk, especially those of xanthine oxidase and lactoperoxidase, are major candidates in the oxidation of ascorbate in raw milk. It may be suggested that fresh milk, with respect to optimal oxidative stability, should be pasteurized to such an extent that the milk is peroxidase-negative in order to minimize the spontaneous oxidation in the milk. Moreover, the comparative part of the present study indicates that the pro-oxidative effect of transition metals in noncontaminated raw milk described earlier (*6*, *7*, *15*) seems to be somehow overestimated. The complex and multiple pathways leading to oxidation of ascorbate in raw milk show that the concentration of this species would be an extremely susceptible indicator of oxidative stress, which thereby makes the rate of ascorbate oxidation a potential, sensitive marker of oxidative stress in raw milk.

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