Lactoperoxidase-Induced Protein Oxidation in Milk

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The reaction between lactoperoxidase (LPO) and H_2O_2 in the presence of bovine serum albumin (BSA), β-lactoglobulin, or casein was investigated for the formation of protein radicals by freeze-quench electron spin resonance (ESR) and by the formation of the protein oxidation product, dityrosine. The presence of BSA resulted in a dramatic change after 1 min of reaction in the obtained ESR spectrum compared with the spectrum obtained for LPO and H_2O_2 alone. Furthermore, experiments employing BSA or β-lactoglobulin resulted in the formation of long-lived protein radicals detectable 10 min after initiation of the reaction. The presence of casein resulted in a minor change in the fine structure of the ESR spectrum after 1 min of reaction compared with LPO and H_2O_2 alone, but no difference between the two reaction mixtures could be observed after 10 min of reaction. The formation of dityrosine could be detected in reaction mixtures containing LPO and H_2O_2 after 1 and 10 min of incubation at 25 °C both in the absence and in the presence of BSA, β-lactoglobulin, or casein. The presence of casein resulted in an increased dityrosine concentration compared with the reaction with LPO and H_2O_2 alone. Endogenous LPO in unpasteurized milk was activated at 25 °C by adding 1 mM H_2O_2. Radical species could be detected directly in the milk by freeze-quench ESR during the initial phase of the reaction, and dityrosine could be measured after 4 h of incubation. The role of LPO activity in the formation of ESR detectable radical species and dityrosine in milk was further verified in ultrahigh temperature (UHT) milk with no endogenous enzyme activity, as the formation of ESR detectable radical species and dityrosine took place in UHT milk only upon the addition of both H_2O_2 and exogenous LPO.

Keywords: *Lactoperoxidase; milk; electron spin resonance; protein radicals; dityrosine*

INTRODUCTION

Traditionally, oxidation in milk has focused on lipid oxidation; however, proteins should also be considered as targets for free radical attacks [reviewed by Davies and Dean (1997)]. The interest of oxidative deterioration of lipids is based on the production of off-flavors formed as a consequence of the oxidative reactions. Oxidative changes of proteins are not believed to have significant influence on flavor but may influence rheological parameters (Liu and Xiong, 1996) due to protein cross-linkage, protein backbone breakdown, and conformational changes [reviewed by Dean et al. (1997)]. Furthermore, reactive intermediates formed during the oxidation of proteins may induce oxidation of other components such as low molecular antioxidants (Østdal et al., 1997) and possibly lipids. Finally, transfer of free radicals to lipids will render protein as an inducer of lipid oxidation.

Lactoperoxidase (LPO), an enzyme present in milk, is supposed to be an important component in the defense against microbial activity in raw milk. LPO is a heme protein that prevents the growth of bacteria by catalyzing the oxidation of thiocyanate to hypothiocyanite using H_2O_2 as the electron acceptor: $H_2O_2 + SCN^- \rightarrow H_2O + OSCN^-$ (Aune and Thomas, 1977). This reaction is believed to protect against microbial activity, with hypothiocyanite being the active component that reacts with free SH groups in the bacterial enzymes (Thomas and Aune, 1978). LPO is highly abundant in bovine milk, but the concentrations of H_2O_2 and thiocyanate in fresh milk are not sufficient to provide an effective defense against microorganisms (Björck et al., 1979). Therefore, it is likely that the antimicrobial activity of LPO is experienced only during the feeding of calves, when thiocyanate is provided both through the milk and by secretion in the abomasum of the calf and H_2O_2 is formed by microbial activity at the latter location (Reiter et al., 1980). Several studies have been performed to use endogenous LPO to preserve unpasteurized milk in countries where cold storage is unavailable. Addition of small quantities of H_2O_2 and thiocyanate ($\geq$0.25 mM of each) can retard the growth of undesired bacteria in unpasteurized milk at room temperature (Björck and Rosén, 1976; Björck et al., 1979).

LPO has, however, also been proposed as a possible candidate for initiating oxidation in milk. It has been reported that addition of LPO in milk-related model systems resulted in increased lipid oxidation (Allen and Wrieden, 1982). Addition of LPO during the production of yogurt can suppress acid production in the product during refrigerated storage (Nakada et al., 1996) but may change the rheological properties of the yogurt (Hirano et al., 1998a). This was ascribed to changes in the protein fraction caused by $OSCN^-$ (Hirano et al.,

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1998b), whereas a potential interaction between the LPO/H_2O_2 system and the milk proteins has never been discussed. The reaction between LPO and H_2O_2 is very similar to that of horseradish peroxidase (HRP). HRP and metmyoglobin, a pseudo-peroxidase, will in the presence of H_2O_2 and other proteins form long-lived protein radicals (half-life > 13−15 min) by an intermolecular radical transfer from the heme protein and onto the other protein (Østdal et al., 1997, 1999). A similar radical transfer has been reported for cytochrome *c* after activation with H_2O_2 (Deterding et al., 1998). Dityrosine, a typical marker for protein oxidation, is formed during these reactions (Østdal et al., 1997). These long-lived protein radicals are still active toward low molecular weight scavengers such as ascorbic acid, tyrosine, cysteine, and glutathione (Østdal et al., 1997). We here present data showing that radicals formed on LPO during its reaction with H_2O_2 can be transferred onto other milk-related proteins such as β-lactoglobulin, casein, and bovine serum albumin (BSA). Furthermore, dityrosine was detected in unpasteurized milk after the addition of H_2O_2, indicating that protein radical transfer reactions can proceed in milk.

MATERIALS AND METHODS

Materials. LPO, BSA, casein, and chelating resin (Chelex 100) were purchased from Sigma Chemical Co. (St. Louis, MO). β-Lactoglobulin (A-form) was purified from samples of β-lactoglobulin (A- and B-forms) donated by Arla Foods Ingredients, Videbæk, Denmark. The separation was performed on a Fractogel DEAE-650 (S) column (Bio-Rad Laboratories, Hercules, CA), and the protein was eluted with a gradient A (0.02 M Tris-HCl, pH 7.0) and B (0.02 M Tris-HCl, 0.35 M NaCl, pH 7.0). Fresh unpasteurized milk was obtained from our own production herd at Research Centre Foulum (64 Danish Holstein cows, milk was used within 16 h of milking), whereas ultrahigh temperature (UHT) milk was obtained from the local supermarket. All other chemicals were of analytical grade, and double-deionized water was used throughout.

Freeze-Quench ESR on the Model System. The reaction between LPO, H_2O_2, and BSA, casein, or β-lactoglobulin was investigated using freeze-quench ESR. The reaction mixtures contained 0.1 mM LPO, 0.1 mM H_2O_2, and 0.2 mM protein (BSA, casein, or β-lactoglobulin) in 50 mM phosphate buffer (pH 7.4; $I = 0.16$). All buffers and protein solutions were treated with chelating resin to remove interference from free transition metal ions. The reaction was initiated by the addition of LPO. After 1 and 10 min of reaction at 25 °C, samples were withdrawn, transferred to ESR tubes (Wilmad LabGlass, Buena, NJ), and frozen in liquid nitrogen. Each sample was subsequently mounted within an ER 200E ESR X-band spectrometer (Bruker Analytische Messtechnik, Rheinstetten, Germany) equipped with a capillary Dewar. The sample temperature was kept at 150 ± 1 K with a temperature-controlled N_2 gas flow directly onto the quartz Dewar. The magnetic field was modulated with a frequency of 25 kHz using a 5 G field modulation amplitude, 2 mW of microwave power, and a receiver gain of 2.5×10^5, and spectra were accumulated through eight scans. All ESR measurements were performed twice. To determine *g* values of the obtained radical species, a pitch standard was used ($g = 2.0028$).

Dityrosine Formation in the Model System. Dityrosine was detected using acid hydrolysis of the proteins and subsequent HPLC separation as described by Daneshvar et al. (1997). LPO (final concentration = 0.1 mM) was added to a solution of H_2O_2 (0.1 mM) and casein, β-lactoglobulin, or BSA (0.2 mM) in 50 mM phosphate buffer (pH 7.4; $I = 0.16$). Protein solutions and buffer were all treated with Chelex 100 to remove free transition metal ions. After 1 and 10 min of incubation at 25 °C, 100 μL samples were withdrawn and mixed with 400 μL of H_2O and 500 μL of 37% HCl. Control samples were prepared as described above, but without LPO or either of the other three proteins used. Subsequently, samples were flushed with argon and hydrolyzed overnight (105 °C). Samples were neutralized with 6 M NaOH. Twenty microliters of the hydrolyzed sample was injected onto an HPLC column (Microsorb 100-5 C-18, 250 × 4.6, Varian, Walnut Creek, CA), which was equilibrated with 4% acetonitrile in aqueous 0.10 M citric acid (pH 2.55), with a flow of 1 mL/L. Chromatographic separation was performed on an HPLC system consisting of a Varian 9012 HPLC pump, connected to a Varian 9100 autosampler and a Varian 9075 fluorescence detector (ex = 283 nm; em = 410 nm) (Varian Chromatographic Systems, Walnut Creek, CA). Samples were spiked with a dityrosine standard for identification and quantified by the use of a standard curve made from the same standard. The dityrosine standard was prepared according to the method of Nomura et al. (1990).

Freeze-Quench ESR on Milk with Endogenous LPO Activity. Unpasteurized milk was incubated with or without 1 mM H_2O_2 at 25 °C. Samples were withdrawn after 20 s and 1, 10, 30, and 90 min, transferred to ESR tubes, and frozen in liquid N_2. ESR measurements were performed as described for the model systems.

Freeze-Quench ESR on Milk with Exogenous LPO Activity. UHT milk with added LPO in 50 mM phosphate buffer (pH 7.4; $I = 0.16$) to a concentration of 30 mg/L was incubated at 25 °C with or without 1 mM H_2O_2. Samples were withdrawn and frozen in liquid N_2 as described for unpasteurized milk. Control samples were made with and without the addition of heat-denatured LPO (100 °C, 2 min). The enzyme solutions were treated with Chelex to remove free transition metal ions.

Dityrosine Formation in Milk with Endogenous LPO Activity. Unpasteurized milk was incubated with or without 1 mM H_2O_2 at 25 °C. After 30 min and 4 h of incubation, 3 mL samples were withdrawn and mixed with 3 mL of 0.1 M H_2SO_4 in 1 M NaCl and 12 mL of 2-propanol. The samples were shaken for 3 min, 9 mL of hexane was added, and the samples were shaken for an additional 3 min. Samples were precipitated by centrifugation (12000*g* for 5 min). The supernatant was discharged, and the extraction procedure was repeated. The pellet was redissolved in 5 mL of 50 mM phosphate buffer (pH 7.4; $I = 0.16$), and the protein was precipitated by adding trichloroacetic acid to a final concentration of 10%. The samples were allowed to stand in the hood for 10 min before centrifugation (12000*g* for 10 min). The pellet was washed with 4 mL of 6 M HCl before an additional centrifugation (12000*g* for 10 min). The pellet was mixed with 1 mL of 6 M HCl. Samples were flushed with argon and hydrolyzed overnight, and dityrosine was measured as described above.

Dityrosine Formation in Milk with Exogenous LPO Activity. LPO in 50 mM phosphate buffer (pH 7.4; $I = 0.16$) was added to UHT milk to a concentration of 30 mg/L and incubated at 25 °C with or without 1 mM H_2O_2. Control samples were made by heat deactivating (100 °C, 2 min) the LPO solution prior to the addition to the milk. The enzyme solutions were treated with Chelex to remove free transition metal ions. After 30 min and 4 h of incubation, 3 mL samples were withdrawn, and the extraction protocol and dityrosine detection were performed as described above.

RESULTS

The reaction between H_2O_2-activated LPO and other proteins was investigated in a simple model system and in milk using either freeze-quench ESR or the formation of dityrosine. Figure 1 shows the typical ESR-active species in the model system used: LPO activated by stoichiometric concentrations of H_2O_2 in the presence or absence of either BSA, β-lactoglobulin, or casein. The figure includes measurements at both 1 and 10 min after initiation of the reaction. Changes in the spectrum of H_2O_2-activated LPO (Figure 1A) by the presence of

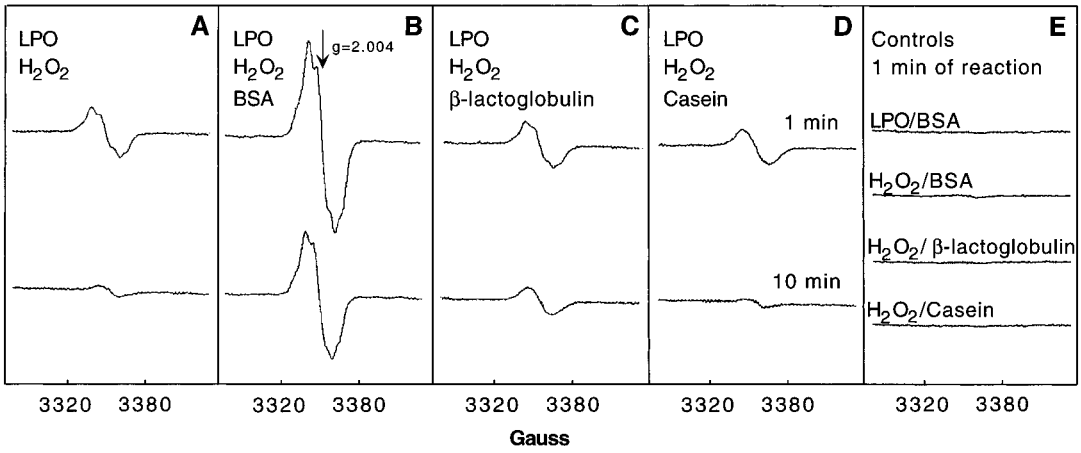

Figure 1. ESR spectra detected in the reaction of 0.1 mM LPO with 0.1 mM H_2O_2 (A) in the presence of 0.2 mM BSA (B), β-lactoglobulin (C), or casein (D). Control samples were prepared without either LPO or H_2O_2 (E). All reaction mixtures were incubated at room temperature in 50 mM phosphate buffer (pH 7.4; $I = 0.16$) for the indicated periods (1 and 10 min) before freeze-quenching in liquid N_2 and examination by ESR at 150 ± 1 K.

another protein were used as an indication of reaction between the two proteins present. Furthermore, radical species detected after 10 min of reaction were used as an indication of the formation of long-lived protein radicals as previously described by Østdal et al. (1997, 1999). The presence of BSA during the activation of LPO with H_2O_2 (Figure 1B) resulted in dramatically altered ESR signals after both 1 and 10 min of reaction compared with LPO and H_2O_2 in the absence of BSA (Figure 1A). No difference could be detected after 1 min of reaction with or without the presence of β-lactoglobulin in the reaction mixture, but after 10 min of reaction the presence of this protein resulted in a more intense ESR signal compared with LPO and H_2O_2 alone (Figure 1A,C). Substitution of β-lactoglobulin with casein showed a very minor change in the fine structure of the spectrum after 1 min, but the signal after 10 min of reaction was no different from that observed for LPO and H_2O_2 alone (Figure 1A,D). All ESR-active species measured were accessed to the H_2O_2 activation of LPO as no ESR signals could be detected in the absence of either H_2O_2 or LPO (Figure 1E).

The formation of protein oxidation products in the model system was evaluated by measuring dityrosine in the reaction mixtures. Dityrosine is a typical protein oxidation product, which is often used to study protein oxidation (Daneshvar et al., 1997; Davies, 1987; Kikugawa et al., 1994; Vissers and Winterbourne, 1991; Østdal et al., 1996, 1997). Table 1 shows that dityrosine is formed during H_2O_2 activation of LPO both in the absence and in the presence of BSA, β-lactoglobulin, or casein. However, the concentration of dityrosine formed in the presence of casein was more than twice that formed in the absence of casein, indicating that H_2O_2-activated LPO induces oxidation of casein, which can be monitored by measuring dityrosine. The presence of BSA or β-lactoglobulin resulted in only minor differences in the dityrosine concentration compared with LPO and H_2O_2 alone.

To investigate whether LPO-induced protein oxidation can proceed in milk, we incubated unpasteurized milk with H_2O_2. It can be seen from Figure 2 that despite relatively high noise to signal in the ESR spectra, radical species were registered in unpasteurized milk with added H_2O_2. The signals were most pronounced at the beginning of the reaction, whereas only small differences existed between the H_2O_2-added

Table 1. Formation of Dityrosine in the Reaction between LPO (0.1 mM), H_2O_2 (0.1 mM), and Casein, β-Lactoglobulin, or BSA (0.2 mM) in 50 mM Phosphate Buffer (pH 7.4; $I = 0.16$) at 25 °C[a]

	dityrosine concn (μM)	
sample	1 min	10 min
LPO/H_2O_2/casein	15.21 ± 0.24^{a}	18.52 ± 0.47^{b}
LPO/H_2O_2/β-lactoglobulin	6.68 ± 0.29^{c}	7.39 ± 0.60^{ce}
LPO/H_2O_2/BSA	4.12 ± 0.69^{df}	6.23 ± 0.53^{ce}
LPO/H_2O_2	6.16 ± 0.68^{cd}	5.82 ± 0.87^{cef}
H_2O_2/casein	<0.025	<0.025
H_2O_2/β-lactoglobulin	<0.025	<0.025
H_2O_2/BSA	<0.025	<0.025

[a] No dityrosine could be detected in either of the native proteins. All values are the average of three independent measurements. Dityrosine concentrations in the different reaction mixtures were compared using the paired t test (different superscripts indicate $p < 0.05$).

samples and the controls after 10, 30, and 90 min of reaction. The role of LPO in the present reactions was further verified in an experiment using UHT milk in which no residual activity of LPO exists due to the heat treatment. No ESR signals could be detected after the addition of H_2O_2 to UHT milk, whereas UHT milk with added LPO and H_2O_2 resulted in ESR signals similar to, but more intense than, those detected in the unpasteurized milk with added H_2O_2 (see Figure 3). It was subsequently found that addition of heat-denatured LPO to UHT milk did not give rise to ESR signals upon addition of H_2O_2 (data not shown), stressing the importance of the enzymatic activity of LPO in the measured ESR spectra. Furthermore, the addition of H_2O_2 to unpasteurized milk and UHT milk with added LPO led to the formation of dityrosine after 4 h of incubation as shown in Table 2. This formation of dityrosine was influenced by a lag phase as no dityrosine was detected after 30 min of incubation. The concentration of dityrosine was ~10 times higher in the unpasteurized milk compared with UHT milk with added LPO. No dityrosine (<0.025 μM) could be detected after 4 h of incubation in the absence of LPO activity (UHT milk with added heat-denatured LPO and H_2O_2).

DISCUSSION

Previous studies have suggested that LPO may be a potential inducer of lipid oxidation in milk and dairy

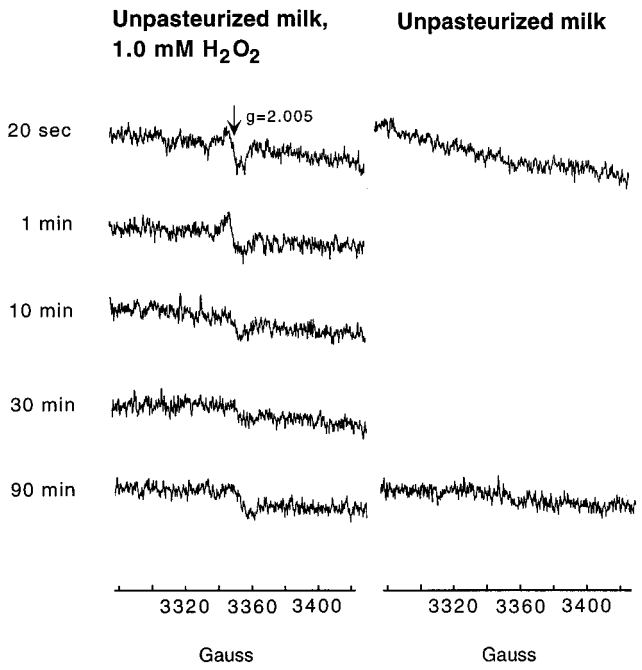

Figure 2. ESR spectra of unpasteurized milk with added 1.0 mM H_2O_2. Control samples were made without H_2O_2 addition. All samples were incubated at 25 °C (see Materials and Methods) for the indicated periods prior to freeze-quenching in liquid N_2 and examination by ESR spectroscopy at 150 ± 1 K.

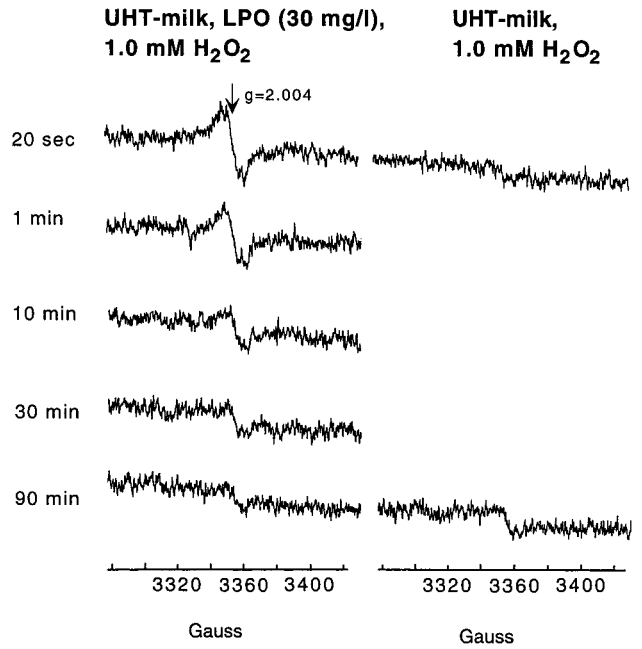

Figure 3. ESR spectra of UHT milk with added LPO (30 mg/L) and 1.0 mM H_2O_2. Control samples were made without LPO addition. All samples were incubated at 25 °C (see Materials and Methods) for the indicated periods prior to freeze-quenching in liquid N_2 and examination by ESR spectroscopy at 150 ± 1 K.

products (Allen and Wrieden, 1982). The present study shows that milk proteins must also be considered as substrates for H_2O_2-activated LPO. Moreover, the experiments show that these changes can occur through a direct protein−protein interaction and thereby not necessarily promoted by hypothiocyanite as proposed previously (Hirano et al., 1998b).

The reaction between the LPO/H_2O_2 system and other proteins was investigated by freeze-quench ESR spectroscopy and by the formation of dityrosine in the samples. The obtained ESR spectra of LPO, H_2O_2, and BSA after 1 and 10 min of reaction show that this system gives rise to long-lived protein radical species similar to what has been observed for the pseudoperoxidase metmyoglobin (Østdal et al., 1997) and HRP (Østdal et al., 1999) when these heme proteins were activated by H_2O_2 in the presence of BSA. The ESR signal measured when HRP was used as a radical generator has been ascribed to a tyrosyl radical (Østdal et al., 1999). The high resemblance between the ESR signal measured for HRP/H_2O_2/BSA and the ESR signals currently obtained highly suggests that a tyrosyl radical is the ESR active species in the present study. Furthermore, the *g* value calculated for the model system is similar to the *g* value for the tyrosyl radical free in solution (2.0046) (Sealy et al., 1985). In addition, a tyrosyl radical is formed during H_2O_2 activation of LPO (Lardinois et al., 1999) and explains the high resemblance between the spectra measured in the model system. Likewise, the LPO/H_2O_2 system also gave rise to long-lived protein radical species when BSA was exchanged with β-lactoglobulin. The difference in ESR signal intensity between samples containing either BSA or β-lactoglobulin has also been observed in samples in which H_2O_2-activated metmyoglobin was used as a protein radical generator (Østdal et al., 1997). However, in the present study BSA, β-lactoglobulin, and casein were used on an equal molar basis. The molecular weight of BSA is ∼3.7 times greater than that of β-lactoglobulin, making the number of amino acids available for radical transfer substantially higher. This may influence the ESR signal intensity, and the aspect of concentration is currently under investigation in our laboratory. The reaction between LPO, H_2O_2, and casein only indicates a weak interaction after 1 min of incubation and no formation of long-lived protein radicals, as the signal after 10 min of incubation was no different from the signal of LPO and H_2O_2 alone. However, the formation of dityrosine in the reaction between H_2O_2-activated LPO and casein supports the assumption that LPO/H_2O_2 can induce oxidative modifications of casein. In contrast, the presence of either BSA or β-lactoglobulin with the LPO/H_2O_2 system did not result in increased formation of dityrosine compared with LPO and H_2O_2 alone. This is notable as the formation of dityrosine has previously been shown to proceed simultaneously with the formation of long-lived protein radicals (Østdal et al., 1997), and tyrosine residues in BSA have been reported to be crucial for the formation of long-lived protein radicals (Irwin et al., 1999; Østdal et al., 1999). However, the relationship between long-lived protein radicals and dityrosine formation is yet to be estab-

Table 2. Formation of Dityrosine in Unpasteurized Milk and UHT Milk after Activation of LPO with 1.0 mM H_2O_2[a]

sample	dityrosine concn (μM) after incubation at 25 °C for 30 min	4 h
unpasteurized milk with 1 mM H_2O_2	<0.025	2.10 ± 0.08
UHT milk with heat-denatured LPO and 1 mM H_2O_2	<0.025	<0.025
UHT milk with LPO (30 mg/L) and 1 mM H_2O_2	<0.025	0.21 ± 0.05

[a] All values are the average of three independent measurements. No dityrosine was detected in the absence of H_2O_2 for either sample.

lished. The present results indicate that the formation of long-lived protein radicals and the production of dityrosine originate from two independent reactions when H_2O_2-activated LPO is used as a free radical generator. Thus, proteins such as BSA and casein have different pathways of dealing with an unpaired electron. The tyrosine content in casein (5−8%) is approximately twice as high as the tyrosine content in BSA (3%) and β-lactoglobulin (2.5%). This supports the ability of casein to form dityrosine compared with BSA and β-lactoglobulin. However, the fact that the presence of BSA or β-lactoglobulin did not increase dityrosine formation compared with LPO and H_2O_2 alone must be explained by the influence of protein structure. More work is needed to understand the relationship between the presence of long-lived radicals and dityrosine formation in relation to the heme protein used, protein structure, and tyrosine content of the proteins present. Formation of dityrosine after H_2O_2 activation of LPO in the absence of other proteins is probably due to the fact that LPO self-oxidizes in the presence of H_2O_2 under the formation of dityrosine (Lardinois et al., 1999). Such reactions have also been reported for the reaction between metmyoglobin and H_2O_2 (Østdal et al., 1997).

The dityrosine formed in unpasteurized milk after treatment with H_2O_2 indicates that the reactions observed in the LPO/H_2O_2/casein model system are likely to occur in milk. LPO is a rather heat-stable enzyme (Barrett et al., 1999), which is why UHT milk was used as a milk source with no LPO activity. The formation of dityrosine in unpasteurized milk with added H_2O_2 and the absence of dityrosine in UHT milk with added H_2O_2 indicate that the present reaction is enzyme related. The formation of dityrosine in UHT milk after the addition of LPO further indicates that the activity originated from LPO. The concentration of dityrosine measured was ∼10 times higher for unpasteurized milk compared with UHT milk with added LPO. The actual activity of LPO in the unpasteurized milk was not measured, but 30 mg/L LPO was added to the UHT milk, which is the concentration of LPO normally reported for bovine milk (Ye and Yoshida, 1995; Yoshida and Ye, 1991). Therefore, the decreased formation of dityrosine in UHT milk with added LPO compared with the unpasteurized milk was not ascribed to the activity of LPO but more as a consequence of changes in the structure of the other milk proteins induced by the UHT treatment. Potential antioxidative protein-bound thiol groups in, for example, β-lactoglobulin (two disulfide bonds and one free cysteine) are known to be more exposed to the solvent after denaturation (Møller et al., 1998). Furthermore, protein structure is also known to be important in relation to their reaction with H_2O_2-activated heme proteins (Østdal et al., 1996, 1999). The effect of heat treatment of LPO has also been reported in relation to initiation of lipid oxidation in model systems (Allen and Wrieden, 1982), whereas others have reported an increased catalytic activity on lipid oxidation caused by heat denaturation of LPO in a lipid model system (Eriksson, 1970). Similar pro-oxidative effects have been reported for mildly heat-denatured metmyoglobin, whereas more severe heat treatment resulted in a less pro-oxidative protein compared with native metmyoglobin (Kristensen and Andersen, 1997). In milk, increased pasteurization temperatures have been reported to increase the oxidative stability (Ford et al., 1986). This increased oxidative stability may be ascribed to exposure of antioxidative protein-bound thiols, whereas the effect of LPO is probably influenced by the degree of heat treatment used.

In the present study, the formation of dityrosine in milk was influenced by incubation time. The model system containing LPO, H_2O_2, and casein gave instant formation of dityrosine, whereas raw milk with added H_2O_2 had to be incubated for between 30 min and 4 h before dityrosine could be detected. The difference is probably due to the fact that milk contains a wide variety of components which can act as antioxidants such as ascorbic acid, uric acid, and protein-bound thiol groups. The formation of dityrosine may therefore be retarded until the antioxidative capacity of the milk is diminished. The role of ascorbic acid and other low molecular weight antioxidants in relation to LPO-induced protein oxidation in milk is currently under investigation in our laboratory.

Despite the proposed role of LPO in protein radical formation and protein oxidation, the nature of the radical species formed in milk after LPO activation is not known at present. The ESR spectra of the milk samples resemble organic radicals with *g* values similar or close to the one calculated for the protein radical measured in the LPO/H_2O_2/BSA system (compare Figures 1, 2, and 3). However, the complex composition of milk makes it difficult to give an absolute characterization of the relatively weak signals identified in the milk samples as these likewise could originate from lipids, carbohydrates, or low molecular weight compounds in the milk. More experiments are needed to investigate the actual nature of the measured radicals in milk, but it must be concluded from the present experiments that activation of LPO in milk initiates free radical reactions which lead to oxidative modification of other milk components including proteins.

In conclusion, the activity of LPO may lead to oxidative changes in the protein fraction in milk by a direct protein−protein reaction. The significance of such reactions in milk and dairy products is unknown at present. However, if the protein radicals formed in the present system can trigger lipid oxidation, this reaction might play a role in the spontanous oxidation of milk, the mechanism of which is yet to be established (Granelli et al., 1998). Moreover, activation of LPO may cause structural changes in the milk proteins, which again may affect the acceptance of dairy products such as yogurt, as indicated by the data of Hirano et al. (1998a). Finally, the activity of LPO/H_2O_2 toward milk proteins observed in the present study should be kept in mind in relation to the potential use of the LPO/H_2O_2 system as an antimicrobial agent (Atamer et al., 1999).

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