Effect of Lactoferrin on Oxidative Stability of Corn Oil Emulsions and Liposomes

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Interest in using lactoferrin in foods for its antimicrobial activity inspired the present study of its antioxidant activity. Natural bovine lactoferrin inhibited oxidation in buffered corn oil emulsions and lecithin liposome systems at pH 6.6 and 50 °C. The antioxidant activity increased with lactoferrin concentration in both phosphate- and Tris-buffered emulsions, but not in both buffered liposome systems. A mixture of 1 μM lactoferrin and 0.5 μM ferrous ions was a significantly better antioxidant than 1 μM lactoferrin alone in Tris-buffered emulsions and in phosphate-buffered liposomes. Lactoferrin was a prooxidant at 1 μM in phosphate-buffered liposomes and at 15 and 20 μM in Tris-buffered liposomes. Copper was a stronger prooxidant than iron in both buffered emulsions. Lactoferrin decreased the prooxidant effect of iron, but not of copper, in emulsions. The antioxidant or prooxidant activities of lactoferrin depended on the lipid system, buffer, its concentration, the presence of metal ions, and oxidation time.

Keywords: *Lactoferrin; iron ions, copper ions; corn oil emulsions; liposomes; hydroperoxides; hexanal; antioxidant activity; prooxidant activity*

INTRODUCTION

Lactoferrin is a single-chain glycoprotein, belongs to the transferrin family, and mainly exists in mammalian milk. It has the ability to tightly bind two ferric ions together to two associated suitable anions, but reversibly (Anderson et al., 1989; Aisen, 1980; Groves, 1960; Penner et al., 1983; Rogers et al., 1977a). Lactoferrin binds ferric ions with an overall binding constant on the order of 10^{20} in cooperation with two hydrogen carbonate ions (Masson and Heremans, 1968). The iron saturation of human lactoferrin is naturally 1−4% at most in human milk (Fransson and Lönnerdal, 1980). The iron-binding stability of its amino-terminal iron-binding lobe is different from that of its carboxyl-terminal lobe, and primarily the carboxyl-terminal lobe drives cooperative interactions between its two domains (Ward et al., 1996). In addition to ferric ions, many other metal ions are able to bind at the iron sites, such as cupric, manganese(III), cobalt(III), aluminum(III), and lanthanum(IV) ions (Smith et al., 1994a,b; Baker et al., 1997).

Lactoferrin exerts a bacteriostatic effect in vitro by its ability to sequester free iron required for microbial growth (Oram and Reiter, 1968; Weinberg, 1978), and bactericidal activity by the interaction of its N-terminal basic peptide with bacterial phospholipid membranes (Bellamy et al., 1993; Kang et al., 1996; Longhi et al., 1994; Yamauchi et al., 1993). Both antibacterial activities of lactoferrin are decreased by iron ions (Bullen et al., 1972; Longhi et al., 1994) and calcium and magnesium ions (Bellamy et al., 1993; Longhi et al., 1994; Yamauchi et al., 1993).

Studies of the modification of transferrins showed that two or three tyrosines and two histidines are essential in binding metal ions and that one arginine may be a binding site for one anion that is bound to the metal ion (Azari and Feeney, 1961; Rogers et al., 1977b, 1978). However, crystallographic studies on human diferric lactoferrin (Anderson et al., 1989) and dicupric lactoferrin (Smith et al., 1992) demonstrated that both metal ions bind to the same four protein ligands and to a carbonate ion. The overall structure of the copper−lactoferrin complex is the same as that of the iron−lactoferrin complex (Smith et al., 1994a). The structure of the iron−transferrin complex was proposed to have a ferric ion octahedrally coordinated to three tyrosyl oxygen atoms, two nitrogen atoms, and one carbonate ion, whereas for the copper complex, a cupric ion was proposed to be bound to two tyrosyl oxygen atoms and two nitrogen atoms of the protein with square planar coordination (Windle et al., 1963). The copper-binding stability of conalbumin is lower than its iron-binding stability (Fraenkel-Conrat and Feeney, 1950). However, in the absence of synergistic anions, the metal binding sites of transferrins appear to have no affinity for metal ions and may even repel them (Bates and Schlabach, 1975). In addition, lactoferrin can bind iron at sites other than its chelating−binding sites (Nagasako et al., 1993).

As compared to the uncomplexed protein, the iron complex of conalbumin, human serum transferrin, and lactoferrin are generally more stable to physical and chemical treatments (Azari and Feeney, 1961; Kussendrager, 1994; Miller and Bezkorovainy, 1973; Sánchez et al., 1992). Hololactoferrin (100% iron saturation) is significantly less sensitive to heat-induced unfolding and aggregation than is apolactoferrin (Kussendrager, 1994). Differential scanning calorimetry of the naturally iron-saturated bovine lactoferrin at pH 6.5 shows two endothermic heat transitions with peak temperatures of ∼65 °C due to apolactoferrin and ∼90 °C due to hololactoferrin. The thermal stability of lactoferrin depends on its iron saturation. Both apolactoferrin and

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10.1021/jf9809939 CCC: $18.00 © 1999 American Chemical Society
Published on Web 04/01/1999

hololactoferrin are more heat-sensitive when heated in milk than in phosphate buffer (Sánchez et al., 1992).

Lactoferrin decreased the hydroxyl radical generation of phagocytes and inhibited iron-catalyzed autoxidation of the membrane of monocytes (Britigan et al., 1991). Lactoferrin and transferrin at 20% of iron saturation inhibited the iron ascorbate-catalyzed lipid peroxidation of bovine brain phospholipid liposomes in sodium chloride solution at pH 7.4, but saturation of both proteins with iron abolished their inhibitory effect (Gutteridge et al., 1981). Albumin with different levels of iron saturation did not inhibit lipid peroxidation in the same system. Bovine serum albumin was previously shown to greatly reduce the rate of oxidation of liposomes in succinate buffer at pH 4.7 (Heinonen et al., 1998). The insoluble protein and peptide complexes with cupric ion inhibited the catalytic autoxidation of linoleic acid by cupric ion in an aqueous ethanol solution (Yamashoji et al., 1979). Peptides of molecular weight of 200 formed the soluble complexes with cupric ion and promoted the catalytic autoxidation of linoleic acid by cupric ions.

Only limited information has been reported on the effect of lactoferrin on lipid oxidation in food systems. The effect of lactoferrin on the oxidative stability of lipids in different food model systems is of considerable interest to understanding and predicting its antioxidant actions and to preventing its prooxidant properties in the presence of metals. This study describes the effects of bovine lactoferrin on the oxidation of corn oil-in-water emulsions and soy lecithin liposomes in phosphate and Tris buffers at 50 °C. The effectiveness of lactoferrin as a metal sequestrant was evaluated at different stages of oxidation by measuring the formation of hydroperoxides (conjugated dienes) and the decomposition of hydroperoxides (hexanal).

MATERIALS AND METHODS

Materials. The corn oil triglycerides stripped of tocopherols used were those previously described (Huang et al., 1994). Soybean lecithin (~40% of L-α-phosphatidylcholine), ferrous sulfate heptahydrate, and tris(hydroxymethyl)aminomethane hydrochloride (Tris) were obtained from Sigma Chemical Co. (St. Louis, MO), and cupric sulfate, ethylenediaminetetraacetic acid (EDTA), potassium phosphate monobasic, and potassium phosphate dibasic heptahydrate were from Fisher Scientific (Fair Lawn, NJ). Bovine lactoferrin was isolated from cheese whey without any losses of iron-binding capacity, antigenic activity, and antibacterial activity (Abe et al., 1991) [~92.6% purity, 6% moisture, 0.5% ash, molecular mass of ~80000 Da, 22% iron saturation, and 70% iron-binding capacity (Kussendrager, 1994)] and was obtained from the DMV International (Veghel, The Netherlands).

Preparation of Emulsion and Liposome Samples. A mixture of corn oil triglycerides (10%) and soybean lecithin (1%) in 25 mM potassium phosphate or Tris buffer (pH 6.6) was first homogenized at 1800 rpm for 5 min, while the oil was added, and then at 2200 rpm for 2 min with an Ultraturrax T 25 homogenizer (IKA Works, Inc., Cincinnati, OH). The resulting emulsion was rehomogenized with a Rannie high-pressure laboratory homogenizer mini-lab type 8.30H (APV Homogenizer Group, Wilmington, MA) six times at 300 bar. The emulsion was filtered through a Whatman No. 4 paper filter and added to 50-mL Erlenmeyer flasks (30 mL) with or without bovine lactoferrin, ferrous sulfate, or cupric sulfate. The hydroperoxide content in the freshly prepared emulsion was 13 mmol/kg of oil in phosphate buffer and 11 mmol/kg of oil in Tris buffer. The metal-chelating effect of lactoferrin was compared to that of EDTA. The average particle size of fresh emulsions was 0.33−0.38 μm determined with a Microtrac ultrafine particle analyzer (Leeds & Northrup, North Wales, PA). Emulsions prepared in phosphate buffer contain 0.17 μg of Fe/30 mL. Phosphates are commonly present in food and biological systems. The iron-chelating activity of the phosphate buffer used in our experiments was corrected by using the same phosphate buffer in the controls without lactoferrin.

Liposomes containing 1% lecithin were prepared in 25 mM phosphate and Tris buffers at pH 6.6 as described previously (Huang et al., 1997). The hydroperoxide content in the freshly prepared phosphate- and Tris-buffered liposomes was 14 mmol/kg of lecithin. The procedures used for addition of the lactoferrin and metal ions were the same as those used for the emulsions. The particle size of liposome was between 0.03 and 0.1 μm. Liposomes prepared in phosphate buffer contain 0.19 μg of Fe/30 mL.

Oxidation. The oxidation of emulsion and liposome samples was carried out at 50 °C in a shaker oven (Lab-Line Instrument, Inc., Melrose Park, IL). Oxidation was catalyzed with either ferrous sulfate or cupric sulfate. These metal forms were chosen for solubility reasons, but both forms can act as oxidation catalysts by establishing a redox system so that lower and higher oxidation forms catalyze oxidation (Frankel, 1998). Oxidative stability of these samples was determined by measuring conjugated diene hydroperoxides spectrophotometrically and hexanal by headspace gas chromatography (GC).

Measurement of Conjugated Diene Hydroperoxides. Samples (0.1 mL) were dispersed in 5 mL of methanol and diluted with more methanol to obtain measurable absorbance. The absorbance was measured at 234 nm and calculated as millimoles of hydroperoxides per kilogram of oil as described previously (Frankel et al., 1994).

Measurement of Hexanal by Static Headspace GC. Hexanal is one of many important volatile products of lipid hydroperoxide decomposition and is a useful marker for the decomposition of $n-6$ PUFA (Frankel, 1982). The procedures used for hexanal measurements were described previously (Frankel et al., 1994), except that all samples were equilibrated at 60 °C for 15 min before GC analyses were carried out with an autosystem gas chromatograph equipped with an HS-40 headspace autosampler (Perkin-Elmer, Norwalk, CT). All determinations were done in duplicate, and the results were analyzed by one-way analysis of variance (Wagner, 1992).

RESULTS

The rate of oxidation of the corn oil emulsions and lecithin liposomes varied depending on experimental conditions. Oxidized samples were compared at the beginning of the propagation stage of the control (Figure 1, day 2). The extent of inhibition or prooxidant activity observed with lactoferrin, metal ions, and EDTA changed during the course of oxidation.

Effect of Bovine Lactoferrin. The effect of bovine lactoferrin on lipid oxidation was influenced by its concentration, the lipid system, the buffer, and oxidation time. Both hydroperoxide and hexanal formation was inhibited by bovine lactoferrin in corn oil emulsified with soybean lecithin in phosphate and Tris buffers at pH 6.6 and at 50 °C (Table 1). The inhibition of hydroperoxide formation by lactoferrin increased with increasing concentration, and the effect was larger in Tris than in phosphate buffer (Figure 2). The opposite trend was observed in the inhibition of hexanal. Lactoferrin was a less effective inhibitor of hydroperoxide formation than was EDTA at the same concentration in phosphate-buffered emulsions (Table 1). Inhibition by EDTA also increased with concentration.

In liposome systems, lactoferrin inhibited the formation of hydroperoxides at ≥5 μM in phosphate buffer (Table 2). Inhibition was similar between 10 and 20 μM lactoferrin during the overall oxidation. Oxidation was

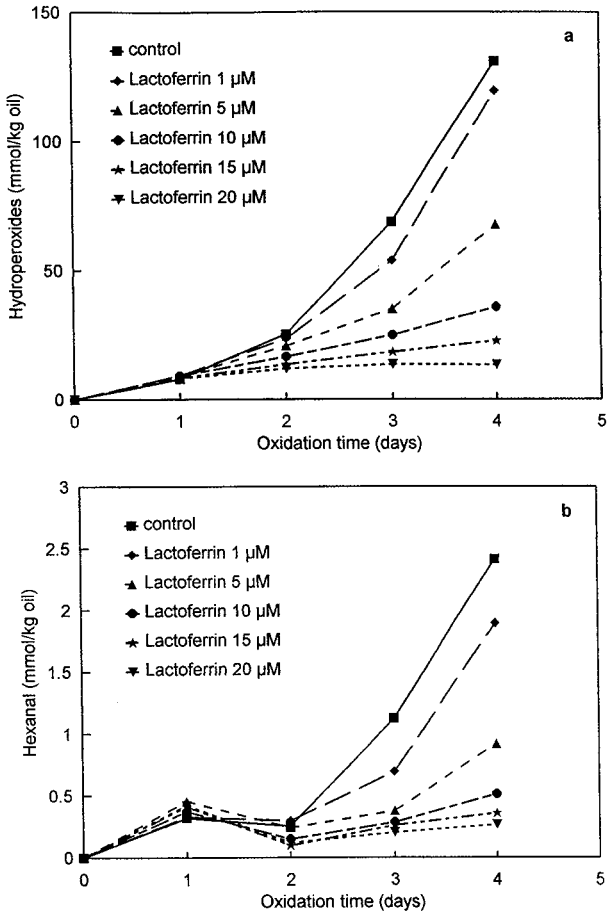

Figure 1. Effect of concentration of lactoferrin on the formation of (a) hydroperoxides and (b) hexanal in phosphate-buffered (pH 6.6) corn oil emulsions.

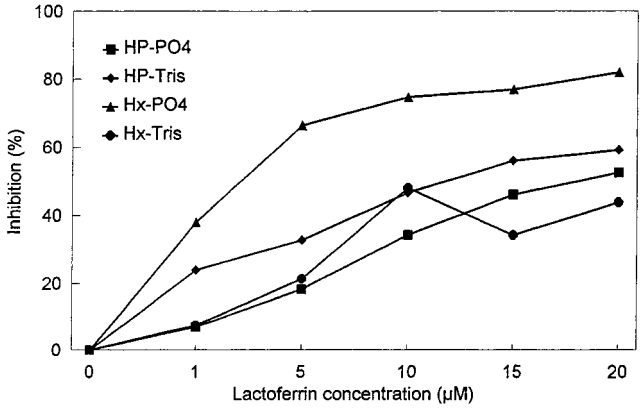

Figure 2. Effect of concentration of lactoferrin on the inhibition of hydroperoxide (HP, at day 2) and hexanal (Hx, at day 3) formation in corn oil emulsions in phosphate (PO_4) and Tris buffers.

relatively slower in Tris-buffered liposomes than in phosphate-buffered liposomes. Lactoferrin had the best antioxidant activity at 5 and 10 μM concentrations. In contrast to its inhibition of hydroperoxide formation in emulsions, the inhibitory activity of lactoferrin did not increase with concentration in liposomes in either phosphate or Tris buffers. Lactoferrin inhibited hydroperoxide formation to the greatest extent at 5 μM in Tris-buffered liposomes after 2 days of oxidation and at 5−10 μM after 6 days of oxidation. Lactoferrin at 1 μM promoted hydroperoxide formation on day 3, but not at the higher concentrations tested (Table 2). In Tris-buffered liposomes lactoferrin promoted hydroperoxide formation at 15 and 20 μM on day 2 and inhibited hydroperoxide formation after 6 days of oxidation at all of the concentrations tested.

Effects of Iron. Iron ions alone promoted hydroperoxide formation at 1−5 μM, but not at 0.5 μM, in both phosphate- and Tris-buffered emulsions (Table 3). This prooxidant effect of iron was not different between 1 and 2 μM but was significantly increased at 5 μM. Iron significantly decreased hexanal formation at 0.5 μM but not at 2 and 5 μM in phosphate buffer. Iron did not significantly affect hexanal formation between 0.5 and 2 μM in Tris buffer but promoted hexanal formation at 5 μM.

At 0.5 μM iron increased the inhibitory effect of 1 μM lactoferrin on hydroperoxide formation in Tris-buffered emulsions (Table 3). The inhibitory effect of 1 μM lactoferrin in both buffered emulsions was decreased in the presence of $\geq$1 μM iron. Lactoferrin thus decreased the catalytic activity of 1 and 5 μM iron in phosphate-buffered emulsions and the catalytic activity of $\geq$1 μM iron in Tris-buffered emulsions. In the presence of lactoferrin, iron was not a prooxidant at $\leq$5 μM in Tris-buffered emulsions. Lactoferrin was a significantly better inhibitor of hexanal formation in the presence of 0.5 or 1 μM iron in phosphate-buffered emulsions and of 0.5, 1, and 5 μM iron in Tris-buffered emulsions. Inhibition of hexanal formation by 1 μM lactoferrin was better in the presence of 0.5 or 1 μM iron than in the absence of added iron in Tris-buffered emulsions.

Table 1. Inhibition of Hydroperoxide and Hexanal Formation by Lactoferrin in Corn Oil Emulsified with Soybean Lecithin in 25 mM Phosphate and Tris Buffers at pH 6.6 and 50 °C (Percent Inhibition, Mean ± SD)[a,b]

	hydroperoxides		hexanal	
sample	phosphate (day 2)	Tris (day 2)	phosphate (day 3)	Tris (day 3)
control	0.0 ± 3.7^{f}	0.0 ± 4.1^{e}	0.0 ± 8.3^{d}	0.0 ± 0.1^{d}
+ 1 μM lactoferrin	7.0 ± 0.0^{e}	24.0 ± 1.6^{d}	38.1 ± 0.9^{c}	7.5 ± 2.5^{cd}
+ 5 μM lactoferrin	18.3 ± 1.1^{d}	32.9 ± 0.1^{c}	66.3 ± 1.4^{b}	21.3 ± 5.4^{c}
+ 10 μM lactoferrin	34.4 ± 0.8^{c}	46.6 ± 1.6^{b}	74.7 ± 6.4^{ab}	48.0 ± 2.1^{a}
+ 15 μM lactoferrin	46.0 ± 1.6^{b}	56.0 ± 3.3^{a}	76.9 ± 3.0^{ab}	34.3 ± 0.1^{b}
+ 20 μM lactoferrin	52.5 ± 1.3^{a}	59.2 ± 0.5^{a}	82.0 ± 1.6^{a}	43.8 ± 6.2^{ab}
control	0.0 ± 1.6^{e}		0.0 ± 0.9^{e}	
+ 1 μM lactoferrin	40.7 ± 0.5^{c}		60.0 ± 0.2^{b}	
+ 5 μM lactoferrin	51.5 ± 1.7^{b}		56.2 ± 0.5^{b}	
+ 0.5 μM EDTA	27.9 ± 0.8^{c}		43.9 ± 1.5d	
+ 1 μM EDTA	51.4 ± 0.8^{b}		57.6 ± 0.7^{bc}	
+ 2 μM EDTA	85.9 ± 0.7^{a}		68.6 ± 1.3^{a}	

[a] Percent inhibition = $[(C - S)/C] \times 100$, where C = hydroperoxides or hexanal formed in control and S = hydroperoxides or hexanal formed in sample. Negative values indicate prooxidant activity. $n = 2$. [b] Values within each column followed by the same superscript letter are not significantly different ($p < 0.05$).

Table 2. Inhibition of Hydroperoxide Formation by Lactoferrin in Liposomes−25 mM Phosphate and Tris Buffers at pH 6.6 and 50 °C (Percent Inhibition, Mean ± SD)[a,b]

	hydroperoxides		
sample	phosphate (day 3)	Tris (day 2)	Tris (day 6)
control	0.0 ± 0.8[d]	0.0 ± 0.1[b]	0.0 ± 3.5[c]
+ 1 μM lactoferrin	−19.1 ± 2.0[e]	4.6 ± 4.4[b]	21.6 ± 0.5[bc]
+ 5 μM lactoferrin	79.9 ± 0.1[c]	117 ± 2[a]	83.7 ± 3.5[a]
+ 10 μM lactoferrin	88.4 ± 1.8[ab]	0.0 ± 28.3[b]	78.4 ± 4.5[a]
+ 15 μM lactoferrin	90.3 ± 0.6[a]	−172 ± 1[c]	23.8 ± 14.0[bc]
+ 20 μM lactoferrin	83.8 ± 0.8[bc]	−249 ± 27[d]	31.6 ± 11.0[b]

[a,b] See footnotes in Table 1.

Table 3. Inhibition of Hydroperoxide and Hexanal Formation by Lactoferrin and Iron in Corn Oil Emulsified with Soybean Lecithin in 25 mM Phosphate and Tris Buffers at pH 6.6 and 50 °C (Percent Inhibition, Mean ± SD)[a,b]

	hydroperoxides		hexanal	
sample	phosphate (day 3)	Tris (day 2)	phosphate (day 4)	Tris (day 3)
control	0.0 ± 4.8[b]	0.0 ± 4.1[d]	0.0 ± 0.1[d]	0.0 ± 0.1[cd]
+ 1 μM lactoferrin	19.7 ± 2.1[a]	24.0 ± 1.6[b]	41.2 ± 5.6[a]	7.5 ± 2.6[c]
+ 0.5 μM Fe^{2+}	−4.1 ± 1.1[bc]	−0.6 ± 6.7[d]	16.7 ± 1.1[bc]	12.5 ± 9.8[c]
+ 1 μM Fe^{2+}	−15.0 ± 1.4[d]	−12.8 ± 5.7[e]	−21.6 ± 5.9[e]	−7.8 ± 3.4[d]
+ 2 μM Fe^{2+}	−11.6 ± 1.2[cd]	−13.2 ± 2.2[e]	5.8 ± 1.3[cd]	13.4 ± 3.6[bc]
+ 5 μM Fe^{2+}	−29.2 ± 4.5[e]	−32.8 ± 2.4[f]	2.3 ± 3.1[d]	−92.1 ± 14.6[e]
+ 0.5 μM Fe^{2+}−1 μM lactoferrin	20.8 ± 1.8[a]	38.9 ± 4.4[a]	49.0 ± 5.0[a]	37.9 ± 2.6[a]
+ 1 μM Fe^{2+}−1 μM lactoferrin	2.3 ± 2.6[b]	10.4 ± 0.9[c]	26.1 ± 3.7[b]	28.9 ± 1.6[ab]
+ 2 μM Fe^{2+}−1 μM lactoferrin	−18.3 ± 4.5[d]	1.7 ± 0.7[cd]	8.4 ± 3.7[cd]	14.5 ± 0.2[bc]
+ 5 μM Fe^{2+}−1 μM lactoferrin	−13.6 ± 5.5[cd]	−4.7 ± 4.3[de]	6.2 ± 8.5[cd]	3.9 ± 3.0[cd]

[a,b] See footnotes in Table 1.

Table 4. Inhibition of Hydroperoxide Formation by Lactoferrin and Iron in Liposomes−25 mM Phosphate and Tris Buffers at pH 6.6 and 50 °C (Percent Inhibition, Mean ± SD)[a,b]

	hydroperoxides	
sample	phosphate (day 3)	Tris (day 2)
control	0.0 ± 0.9[d]	0.0 ± 0.0[a]
+ 1 μM lactoferrin	−19.1 ± 2.0[e]	4.6 ± 4.4[a]
+ 0.5 μM Fe^{2+}	−47.4 ± 0.4[f]	−131 ± 9[b]
+ 1 μM Fe^{2+}	−105 ± 3[g]	−259 ± 11[c]
+ 2 μM Fe^{2+}	−242 ± 2[i]	−494 ± 4[d]
+ 5 μM Fe^{2+}	−241 ± 3[i]	−3977 ± 17[f]
+ 10 μM Fe^{2+}	−212 ± 1[h]	−4486 ± 13[g]
+ 0.5 μM Fe^{2+}−1 μM lactoferrin	84.4 ± 2.1[a]	−15.4 ± 28.3[a]
+ 1 μM Fe^{2+}−1 μM lactoferrin	54.7 ± 0.6[b]	−9.2 ± 4.4[a]
+ 2 μM Fe^{2+}−1 μM lactoferrin	18.2 ± 5.1[c]	−105 ± 7[b]
+ 5 μM Fe^{2+}−1 μM lactoferrin	−218 ± 6[h]	−391 ± 9[cd]
+ 10 μM Fe^{2+}−1 μM lactoferrin	−215 ± 1[h]	−1952 ± 15[e]

[a,b] See footnotes in Table 1.

In liposome systems, ≥0.5 μM iron promoted hydroperoxide formation in phosphate buffer (Table 4). The prooxidant activity of iron increased up to 2 μM and did not increase further at higher concentrations in both phosphate and Tris buffers. In phosphate-buffered liposomes, lactoferrin promoted hydroperoxide formation at 1 μM but inhibited hydroperoxide formation in the presence of 0.5−2 μM iron. Inhibition by the mixture of lactoferrin and iron decreased in the order ratio 2:1 > 1:1 > 1:2. Lactoferrin also decreased the prooxidant effect of 5 μM iron on hydroperoxide formation in phosphate buffer and of ≥0.5 μM iron in Tris buffer.

Iron had stronger prooxidant activity in liposomes than in emulsions, especially in Tris buffer (Tables 3 and 4). In phosphate buffer, the mixtures of lactoferrin and iron had a stronger synergistic effect in liposomes than in emulsions.

Effects of Copper. Copper promoted hydroperoxide formation more strongly than iron in emulsions (Tables 3 and 5). This prooxidant effect increased with copper concentration in both phosphate- and Tris-buffered emulsions (Table 5). Copper did not promote hydroperoxide formation and slightly inhibited hexanal formation at 0.5 μM in Tris-buffered emulsions. Hexanal formation was promoted by ≥0.5 μM copper in phosphate-buffered emulsions and by ≥2 μM copper in Tris-buffered emulsions. The prooxidant effect increased with copper concentration. Addition of 1 μM lactoferrin did not decrease the prooxidant effect of copper on hydroperoxide or hexanal formation in either phosphate- or Tris-buffered emulsions.

DISCUSSION

The commercial bovine lactoferrin used in this study, saturated with ∼22% iron, effectively inhibited hydroperoxide formation in phosphate-buffered corn oil emulsions at pH 6.6. The antioxidant effect of lactoferrin was less than that of EDTA, which combines metal ions in a 50% ratio regardless of the charge of the cation. The inhibition of oxidation by lactoferrin can probably be attributed to its metal-binding capacity. The lower antioxidant activity of lactoferrin than of EDTA may be explained by lactoferrin partial iron saturation and lower affinity for metal ions. Indeed, the formation constant for the ferric−EDTA complex is 1.3×10^{25} (Skoog and West, 1976), compared to $\sim 10^{20}$ for the ferric−lactoferrin complex (Masson and Heremans, 1968).

Ferrous and cupric ions accelerated lipid oxidation of phosphate- and Tris-buffered emulsions. Lactoferrin decreased iron-catalyzed but not copper-catalyzed autoxidation in buffered emulsions. The 1:2 mixture of ferrous ions and lactoferrin had better antioxidant activity than lactoferrin alone, whereas most mixtures of cupric ions and lactoferrin promoted lipid oxidation more than did cupric ions alone. Apparently, lactoferrin decreased the iron-catalyzed autoxidation by chelating ferrous ions. However, whether lactoferrin did not inhibit copper-catalyzed autoxidation because it did not chelate cupric ions or because its copper complexes

Table 5. Inhibition of Hydroperoxide and Hexanal Formation by Lactoferrin and Copper in Corn Oil Emulsified with Soybean Lecithin 25 mM Phosphate and Tris Buffers at pH 6.6 and 50 °C (Percent Inhibition, Mean ± SD)[a,b]

	hydroperoxides		hexanal	
sample	phosphate (day 2)	Tris (day 2)	phosphate (day 3)	Tris (day 3)
control	0.0 ± 5.1[a]	0.0 ± 4.1[b]	0.0 ± 0.1[a]	0.0 ± 0.1[b]
+ 1 μM lactoferrin	15.1 ± 0.6[a]	24.0 ± 1.7[a]	17.4 ± 2.7[a]	7.5 ± 2.6[a]
+ 0.5 μM Cu^{2+}	−54.1 ± 4.3[b]	−5.5 ± 2.2[b]	−44.4 ± 1.8[b]	14.0 ± 0.6[a]
+ 1 μM Cu^{2+}	−127 ± 2[c]	−50.9 ± 3.7[d]	−85.7 ± 19.8[c]	−3.6 ± 5.1[b]
+ 2 μM Cu^{2+}	−306 ± 21[e]	−148 ± 5[f]	−180 ± 6[d]	−46.3 ± 2.4[e]
+ 5 μM Cu^{2+}	−351 ± 12[f]	−164 ± 1[g]	−270 ± 1[f]	−96.8 ± 5.2[g]
+ 0.5 μM Cu^{2+}−1 μM lactoferrin	−60.0 ± 1.1[b]	−21.9 ± 1.3[c]	−51.1± 0.1[b]	−14.3 ± 0.9[c]
+ 1 μM Cu^{2+}−1 μM lactoferrin	−182 ± 3[d]	−94.7 ± 1.8[e]	−155 ± 6[c]	−31.2 ± 0.4[d]
+ 2 μM Cu^{2+}−1 μM lactoferrin	−308 ± 16[e]	−162 ± 3[g]	−205 ± 9[e]	−51.3 ± 1.5[e]
+ 5 μM Cu^{2+}−1 μM lactoferrin	−395 ± 21[g]	−159 ± 3[g]	−290 ± 12[g]	−67.3 ± 6.0[f]

[a,b] See footnotes in Table 1.

catalyzed oxidation is not clear. Although lactoferrin was demonstrated to bind cupric ions at the same sites for iron(III) ions (Smith et al., 1994a), the cupric complexes of the protein may be more labile than its iron complexes at 50 °C, according to the stability of the cupric−conalbumin complex (Fraenkel-Conrat and Feeney, 1950). Lactoferrin did not inhibit copper-catalyzed autoxidation in emulsions probably because of its lower affinity toward cupric ions and the poor stability and catalytic ability of the cupric−lactoferrin complexes.

Increasing the concentration of lactoferrin at >10 μM did not further enhance its antioxidant activity in phosphate-buffered liposomes without added metal ions. Therefore, the antioxidant effect of lactoferrin was not improved by using concentrations higher than that necessary to bind all chelatable metal ions in phosphate-buffered liposomes. It is unclear, however, why 1 μM lactoferrin did not inhibit lipid oxidation in phosphate-buffered liposomes.

According to Nagasako et al. (1993), ferrous iron is easily oxidized to the insoluble ferric state in phosphate buffer. If ferric iron was formed under the conditions used, lactoferrin could bind iron and stabilize it in solution at sites other than its chelate-binding sites. In addition, the inhibitory actions observed with the 2:1 mixture of iron and lactoferrin suggest that lactoferrin may be supersaturated with iron in phosphate-buffered liposomes. The strong antioxidant activity of the 1:2 mixture of iron and lactoferrin and the order of antioxidant activity of the iron−lactoferrin mixtures (1:2 > 1:1 > 2:1) in phosphate-buffered liposomes suggested that it was necessary for lactoferrin to chelate metal ion impurities as well as added iron ions to inhibit lipid oxidation. According to Ward et al. (1996), the unique binding stability of lactoferrin contributes to cooperative interactions between the amino- and carboxyl-terminal binding sites. Lactoferrin may bind iron ions primarily at the carboxyl-terminal binding site and assist the binding of other metal ions to or stabilize the amino-terminal binding site.

Lipid oxidation was slower and the concentration dependencies of iron and lactoferrin on lipid oxidation were greater in Tris- than in phosphate-buffered liposome systems. The higher concentration of hydroperoxide promoted by lactoferrin in Tris-buffered liposomes initially may be due to differences in the concentration of metal ion impurities and carbonate ions acting as synergistic anions. In fact, although carbonate ions needed for iron binding can be fixed in the lactoferrin molecule from atmospheric carbon dioxide, carbonate exchange proceeds at a low rate (Aisen et al., 1973). On the other hand, it is not easy to prepare transferrin that is free of bicarbonate (Harris and Aisen, 1989). The lack of added bicarbonate may pose a problem if the system contains a substance that competes with the carbonate ion for the binding site. Otherwise, ambient bicarbonate seems sufficient for the iron binding activity of lactoferrin (Feeney et al., 1983). Further studies are needed in different lipid systems to determine whether synergistic anions affect the antioxidant activity of lactoferrin, whether iron ions interact with other metal ions in binding with lactoferrin, and how oxidized lactoferrin affects lipid oxidation.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; GC, gas chromatography; PUFA, polyunsaturated fatty acids; SD standard deviation.

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Received for review September 8, 1998. Revised manuscript received February 17, 1999. Accepted February 19, 1999. This research was supported by the California Dairy Research Foundation, Davis, CA.

JF9809939