Lactoferrin in Infant Formulas: Effect on Oxidation

M. Teresa Satué-Gracia,† Edwin N. Frankel,*,† Nagendra Rangavajhyala,‡ and J. Bruce German†

Department of Food Science and Technology, University of California, Davis, California 95616, and Nestlé R&D Center, Inc., New Milford, Connecticut 06776

Lactoferrin is an iron transport protein present in human milk at an average concentration of 1.4 mg/mL. Commercially modified infant formulas based on cow's milk contain much lower amounts of lactoferrin (0.1 mg/mL lactoferrin) and soy based formulas have none. In addition to its role in iron transport, lactoferrin has bacteriostatic and bactericidal activities. Infant formulas are supplemented with relatively large amounts of iron (up to 12 mg/L). The effect of various concentrations of added lactoferrin and supplemental iron on lipid oxidation was tested in two different infant formulas. The extent of oxidation in the formulas as a function of time was determined by formation of hydroperoxides, production of hexanal, and fluorescence. On the basis of all three of these determinations, lactoferrin acted as an antioxidant in the absence and presence of different concentrations of supplemented iron. Lactoferrin inhibited oxidation in a concentration-dependent manner even at concentrations beyond its capacity to bind iron at its two high affinity binding sites. Lactoferrin can be used, therefore, as a dual purpose additive in infant formulas and similar food products for its antioxidant and its antimicrobial properties.

Keywords: *Lactoferrin; oxidation; infant formula; iron supplementation; antioxidant activity; iron-binding activity*

INTRODUCTION

Lactoferrin is a non-heme iron-binding glycoprotein found in many biological secretions from various species. The protein structure, affinity for iron, and isoelectric point are similar among species (Brock, 1997). The amino acid composition of bovine lactoferrin is similar to that of human lactoferrin (69% sequence homology) (Pierce at al., 1991), but the iron saturation and concentration in milk vary considerably. Bovine milk contains approximately 0.1 mg/mL of lactoferrin that is 22% saturated with iron, compared to an average of 1.4 mg/mL of lactoferrin (10−20% total protein) that is only 4% iron-saturated in mature human milk (Lönnerdal et al., 1976). Lactoferrin concentrations in human milk increase in response to iron supplementation and increasing iron status in human mothers (Zapata et al., 1994). The role of lactoferrin as an iron carrier is unclear. Despite the low concentration of iron in human milk, iron deficiency is uncommon in breast-fed infants under 6 months of age. However, attempts to relate the high bioavailability of iron in human milk to lactoferrin have thus far been unsuccessful. Commercial infant formulas are supplemented with iron, generally in the form of ferrous sulfate, to meet the nutritional requirements of infants. This iron supplementation decreases the oxidative stability and creates a shelf life problem for the product. The usual level of iron fortification in the U.S. is about 220 μM (11 mg/L), compared to 4−7 μM (0.2−0.4 mg/L) iron in mother's milk. According to Lönnerdal and Hernell (1994), 72 μM (4 mg/L) iron as ferrous sulfate is adequate for infants up to 6 months of age.

In addition to its role in iron transport, lactoferrin has bacteriostatic (Oram and Reiter, 1968) and bactericidal (Arnold et al., 1977) and fungistatic (Kuipers et al., 1999) activities. The bacteriostatic activity is thought to be in part due to iron deprivation of bacteria (Sánchez et al., 1992), but the bactericidal activity is independent of iron withholding. This activity is mediated by basic regions near the N-terminal region of lactoferrin (Bellamy et al., 1992). Furthermore, the peptide obtained by partial proteolysis of lactoferrin, termed lactoferricin, is more active than intact lactoferrin (Facon et al., 1996). The mechanism of the bactericidal effects of lactoferrin peptide implicates a strong synergism with lysozyme, another protein constituent of milk (Leitch and Willcox, 1999).

The antimicrobial activities of lactoferrin make it an attractive supplement for value-added foods, and infant formula constitutes an obvious choice. Roberts et al. (1992) compared the development of faecal flora in newborns fed breast milk, adapted formula, and lactoferrin supplemented (1 mg/mL) adapted formula and found that lactoferrin stimulated a Bifidobacterial flora typical of breast milk-fed infants. This beneficial shift in infant microflora was associated with a preferential interaction between lactoferrin and Bifidobacterium (Petschow et al., 1999), possibly though not exclusively through the selective provision of iron to Bifidobacterial species (Miller-Catchpole et al., 1997).

The effects of lactoferrin on the oxidation of a corn oil emulsion and a liposome model system was previously reported (Huang et al., 1999). The antioxidant or prooxidant activity of lactoferrin depended on the lipid system, buffer, lactoferrin concentration, the concentration of metal ions and oxidation time.

Proteins were reported to have antioxidant effects in different test systems. Bovine serum albumin reduced the rate of oxidation in liposomes (Dean et al., 1991;

* To whom correspondence should be addressed.
† University of California, Davis.
‡ Nestlé.

10.1021/jf0002490 CCC: $19.00
Published on Web 08/25/2000

Table 1. Identification and Metal Concentration of the Infant Formula Samples

formulas	iron[a] (μM)	copper[a] (μM)
whey-based formula		
no iron	0	8.5
WF-88	88	8.5
WF-172	172	8.5
WF-220	220	8.5
casein-predominant formula		
CF-17	17	8.2
CF-113	113	8.2
CF-171	171	8.2
CF-216	216	8.2

[a] Iron and copper were analyzed by the method of Suddendorf and Cook (1984).

Heinonen et al., 1998). The presence of 20% bovine serum albumin, influenced the activity of various phenolic antioxidants (Heinonen et al., 1998). Various proteins showed antioxidant properties in fatty acid and oil systems (Yamashoji et al., 1979), in emulsions (Lin et al., 1993) and in lipoproteins (Kunitake et al., 1992). The proposed mechanism for this antioxidant action generally involves the capacity of proteins to bind or chelate metal ions. At high relative metal concentrations, however, these complexes can be active oxidation catalysts. Whether or not lactoferrin promotes or reduces the rates of oxidation of unsaturated lipids in complex emulsion systems such as infant formulas has not yet been established. The present study was aimed at determining the effect of lactoferrin on oxidative stability of infant formulas supplemented with different concentrations of iron.

MATERIALS AND METHODS

Materials. A whey-based liquid infant formula (whey formula, WF) and a casein-predominant formula (CF) were prepared on a pilot plant scale. The whey-based formula (WF) contained water, enzymatically hydrolyzed reduced minerals whey protein concentrate (from cow's milk), vegetable oils (5.1 g/100 Kcal), lactose, corn maltodextrin, minerals, vitamins, taurine, nucleotides, and L-carnitine. The casein-predominant formula (CF) contained nonfat milk, corn syrup solids, vegetable oils (4.1 g/100 kcal), corn maltodextrin, minerals, soy lecithin, and vitamins. The two formulas were supplemented with four different concentrations of iron (Table 1). The highest iron concentration for both types of formulas is that most used commercially. Some infant formula products contain low concentrations of iron, which correspond to the two intermediate concentrations in the formulas tested. Iron and copper contents in the formulas are shown in Table 1. Bovine lactoferrin isolated from cheese whey (92.6% purity, 22% iron saturation) was donated by DMV International (Fraser, NY), bovine serum albumin, BSA fraction V (min 96%), was obtained from Sigma Chemical Co. (St. Louis, MO). Dextrose tryptone agar and bacto-peptone were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals and solvents used were of ACS quality and were obtained from commercial sources.

Metal Analysis. Iron and copper were analyzed by the method of Suddendorf and Cook (1984).

Preparation of Formulas and Oxidation. Different amounts of lactoferrin or bovine serum albumin were directly added to the formulas and dissolved by gentle shaking. Tables 2 and 3 show the molar ratios of lactoferrin to iron in the formulas tested. The oxidation of the formulas was carried out in 50-mL Erlenmeyer flasks with screw caps at 50 °C in a shaker oven (Lab-Line Instrument, Inc., Melrose Park, IL). Oxidative stability of the samples was determined by measuring conjugated dienes spectrophotometrically, hexanal by headspace gas chromatography (GC), and protein−lipid oxidation interactions fluorometrically. All experiments and analyses were carried out in duplicate, and the results were analyzed by one-way analysis of variance (ANOVA) (Wagner, 1992).

Microbiological Test. To ensure that there was no microbial growth during oxidation, four formulas (lowest and highest iron content for both formulas) were tested for thermophiles before and after incubation at 50 °C for 9 days. Each sample was tested at 10°, 10^1, and 10^2 dilutions that were made with 0.1% bacto-peptone in water. Samples were inoculated by the pour-plate technique in plates containing dextrose tryptone agar medium and incubated for 48 h at 55 °C. No colonies were observed in any plate.

Lipid Extraction. A solvent extraction method was used for recovering lipids from the infant formulas. Briefly, 0.25 mL of liquid formula was mixed with 2.5 mL each of ethyl acetate, ethanol, hexane, and water. After centrifugation, the aqueous phase was removed and used to measure fluorescence. The organic phase was washed with 2.5 mL of 1% saline solution, separated by centrifugation and evaporated under a nitrogen flow.

Measurement of Conjugated Diene Hydroperoxides. The organic phase from the lipid extraction was diluted with isooctane to obtain measurable absorbance at 234 nm. The results were expressed as millimoles of hydroperoxides per kilogram of fat using an extinction coefficient of 26 000 for methyl linoleate hydroperoxides (Chan and Levett, 1977).

Measurement of Protein-Oxidized Lipid Interactions. The aqueous phase from the lipid extraction was used to measure fluorescence (Fletcher et al., 1973) with a fluorometer (Turner Digital Fluorometer, Model 450) standardized with quinine sulfate, gain set at 5, excitation at 360 nm and emission at 415 nm.

Measurement of Hexanal by Static Headspace GC. A method was developed to determine hexanal directly in the infant formula samples without lipid extraction. The main polyunsaturated fatty acid in the infant formulas is linoleic acid. Hexanal is a volatile product formed in the decomposition of hydroperoxides and is a useful marker of *n*-6 polyunsaturated fatty acid oxidation (Frankel, 1982). One milliliter aliquots were placed in 22-mL headspace vials, sealed, and equilibrated at 60 °C for 15 min in an HS-40 headspace autosampler. An aliquot of the headspace was then injected in an autosystem gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a DB-1701 column, 30 m long, 0.32 mm i.d., and 1 μm film thickness (J&W Scientific, Folsom, CA). The injector and detector temperatures were 180 and 200 °C, respectively. The oven temperature was programmed at 30 °C for 4 min, followed by an increase to 80 °C at a rate of 10 °C/min. Hexanal was identified by comparison of its retention time with that of a hexanal standard.

RESULTS

Effect of Iron on Oxidative Stability of Infant Formula. The oxidative stability of infant formulas at 50 °C was followed using several indices of oxidation. The oxidation experiments were terminated when the

Table 2. Percentage Inhibition by Lactoferrin (LF) of the Formation of Hydroperoxides, Hexanal, and Fluorescence in a Whey-Based Formula (WF) Containing Different Molar Ratios Iron:Lactoferrin

formulas (μM iron)	LF (μM)	% inhibition[e]			Fe:lactoferrin molar ratio
		hydroperoxides	hexanal	fluorescence	
WF (0)[a]		0.0[c]	0.0[c]	0.0[b]	
+ LF	12.5	61.9[b]	72.0[b]	14.2[ab]	0.0:12.5
+ LF	25.0	92.1[a]	95.2[a]	39.1[a]	0.0:25.0
WF (88)[b]		0.0[d]	0.0[d]	0.0[b]	
+ LF	12.5	18.7[c]	27.8[c]	2.4[b]	7.0:1.0
+ LF	25.0	65.4[b]	77.7[b]	9.0[b]	3.5:1.0
+ LF	44.0	94.7[a]	95.8[a]	24.3[a]	2.0:1.0
WF (172)[c]		0.0[d]	0.0[d]	0.0[b]	
+ LF	12.5	17.5[c]	33.9[c]	8.0[b]	13.8:1.0
+ LF	25.0	48.0[b]	76.8[b]	51.9[a]	6.9:1.0
+ LF	86.0	94.4[a]	92.5[a]	69.0[a]	2.0:1.0
WF (220)[d]		0.0[d]	0.0[d]	0.0[c]	
+ LF	12.5	23.2[c]	65.9[c]	1.7[bc]	17.6:1.0
+ LF	25.0	64.8[b]	87.9[b]	16.4[b]	8.8:1.0
+ LF	37.5	92.9[a]	95.6[a]	32.2[ab]	5.9:1.0
+ LF	110.0	101.2[a]	97.8[a]	47.6[a]	2.0:1.0

[a] Data calculated at day 20 of oxidation. [b] Data calculated at day 14 of oxidation. [c] Data calculated at day 12 of oxidation. [d] Data calculated at day 11 of oxidation. [e] Values in each column for samples with the same amount of iron and with the same superscript letter are not significantly different ($p < 0.05$).

Table 3. Percentage Inhibition by Lactoferrin (LF) of the Formation of Hydroperoxides and Hexanal in a Casein-Predominant Formula (CF) Containing Different Molar Ratios Iron:Lactoferrin

formulas (μM iron)	LF (μM)	% inhibition[e]		Fe:lactoferrin molar ratio
		hydroperoxides	hexanal	
CF (17)[a]		0.0[c]	0.0[c]	
+ LF	12.5	26.3[b]	15.9[b]	1.4:1.0
+ LF	25.0	63.9[a]	63.3[a]	0.7:1.0
CF (117)[b]		0.0[d]	0.0[d]	
+ LF	12.5	31.2[c]	40.3[c]	9:1.0
+ LF	25.0	55.9[b]	60.9[b]	4.5:1.0
+ LF	58.5	83.7[a]	75.4[a]	2.0:1.0
CF (171)[c]		0.0[e]	0.0[e]	
+ LF	12.5	7.4[d]	22.9[d]	13.7:1.0
+ LF	25.0	29.3[c]	37.5[c]	6.8:1.0
+ LF	37.5	46.2[b]	62.2[b]	4.6:1.0
+ LF	85.5	92.7[a]	89.7[a]	2.0:1.0
CF (216)[d]		0.0[d]	0.0[e]	
+ LF	12.5	−2.0[d]	21.9[d]	17.3:1.0
+ LF	25.0	44.0[c]	45.3[c]	8.6:1.0
+ LF	37.5	77.1[b]	62.0[b]	5.8:1.0
+ LF	108.0	95.8[a]	89.5[a]	2.0:1.0

[a] Data calculated at day 7 (hydroperoxides) and day 8 (hexanal) of oxidation. [b] Data calculated at day 6 (hydroperoxides) and day 7 (hexanal) of oxidation. [c] Data calculated at day 7 (hydroperoxides) and day 8 (hexanal) of oxidation. [d] Data calculated at day 7 (hydroperoxides) and day 8 (hexanal) of oxidation. [e] Values in each column for samples with the same amount of iron and with the same superscript letter are not significantly different ($p < 0.05$).

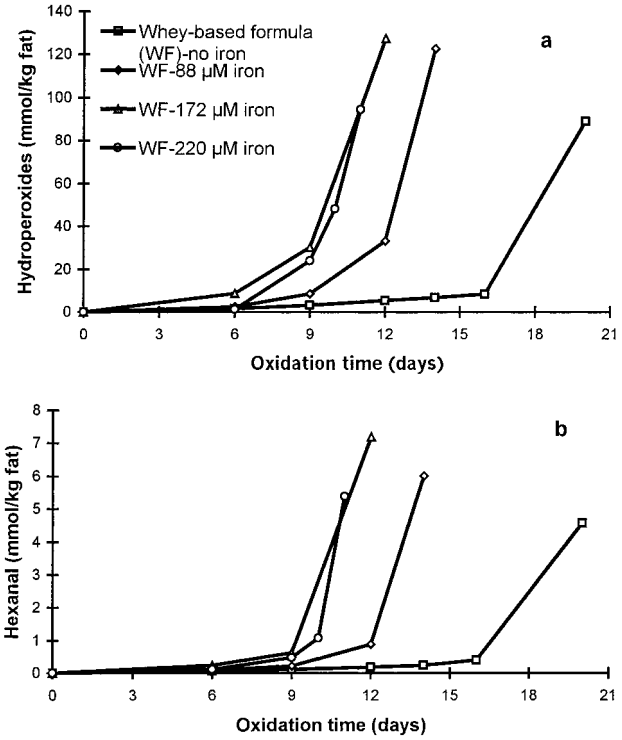

Figure 1. Effect of iron concentration on the formation of (a) hydroperoxides and (b) hexanal in a whey-based formula (WF).

control samples reached a maximum level of hydroperoxides. The effect of lactoferrin on oxidation was tested on a whey-based (WF) and a casein-predominant (CF) ready-to-feed infant formula prepared with four different concentrations of iron. With both formulas, oxidation rates increased with increasing iron concentration (Figures 1 and 2), but the casein-predominant formula oxidized faster than the whey-based formula, despite a similar initial level of oxidation (11−12 mmol hydroperoxides/kg oil).

In the presence of increasing concentrations of lactoferrin, the induction periods of both formulas increased and the rates of oxidation decreased (Figures 3 and 4). Percentage inhibitions were calculated during the last stages of the propagation phase (following the induction period) relative to the control sample. Tables 2 and 3 show the percentage inhibition of hydroperoxides and hexanal formation for all formulas tested. In all formulas, lactoferrin showed antioxidant activity, which increased with increasing concentration, in both the absence and presence of iron. Figure 5 shows the correlation of the iron:lactoferrin molar ratio with the percent inhibition of hydroperoxide and hexanal formation in the formulas containing amounts of iron similar to those found in commercial formulations. High correlations were obtained between inhibition of oxidation and iron:lactoferrin molar ratio in all the samples tested. In the whey-based formulas, correlation coefficients were higher than 0.97 for hydroperoxide, 0.98 for hexanal, and 0.89 for fluorescence inhibition. In the casein-predominant formulas, the correlation coefficients were higher than 0.90 for hydroperoxide and 0.91 for hexanal inhibition.

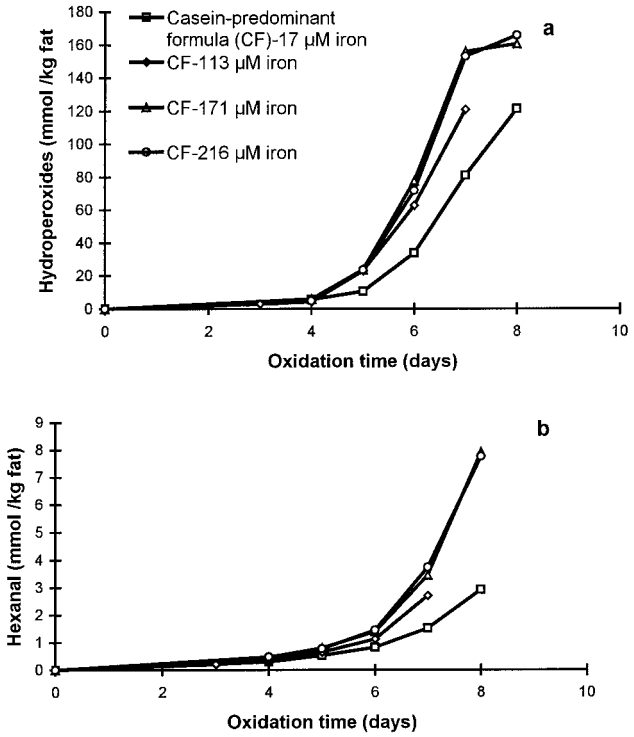

Figure 2. Effect of iron concentration on the formation of (a) hydroperoxides and (b) hexanal in a casein-predominant formula (CF).

Oxidation of Whey-Based Formula. In the absence of iron, the whey-based formula showed a lag period of 16 days for both hydroperoxide and hexanal formation (Figure 1). Bovine lactoferrin inhibited oxidation of the whey-based formula without and with iron. With increasing content of iron, the lag period decreased to 9 days for the formula containing 88 μM iron, and to 6 days for the formulas containing 172 and 220 μM iron. The inhibition of the formation of hydroperoxides, hexanal, and fluorescent products increased with increasing concentrations of lactoferrin. Figure 3 shows the oxidation of a whey-based formula fortified with 220 μM iron. Lactoferrin showed the most antioxidant activity at a lactoferrin:iron molar ratio of 1:2. Lactoferrin decreased oxidation at all the concentrations tested, and the order of activity increased with decreasing iron:lactoferrin molar ratio: 2:1 > 6:1 > 9:1 > 18:1. When lactoferrin was tested in the absence of iron, at a concentration in the range of that of human milk (1 mg/mL, 12.5 μM), the rate of oxidation decreased, and after 20 days, when oxidation of control reached a maximum, lactoferrin inhibited hydroperoxide formation 62% (1 mg/mL, 12.5 μM) and 92% (2 mg/mL, 25 μM) and hexanal formation 72% and 95% (Table 2).

Lactoferrin at 1 mg/mL (12.5 μM) inhibited hydroperoxide formation 19, 18, and 23% in the formulas containing, respectively, 88, 172, and 220 μM iron. Inhibition of hexanal formation was 28, 34, and 66% for the formulas containing, respectively, 88, 172, and 220 μM iron. Lactoferrin at 2 mg/mL (25 μM) decreased oxidation at least 48% as measured by hydroperoxide formation and at least 77% as measured by hexanal formation. At a molar ratio lactoferrin:iron of 1:2, formation of both hydroperoxides and hexanal was inhibited >90% in all the samples (Table 2).

Oxidation of Casein-Predominant Formula. The casein-predominant formula oxidized faster than the whey-based formula. The formula had a lag period of 5 days for hydroperoxide formation in the presence of 17 μM iron and 4 days in the presence of 113, 171, and 216 μM iron (Figure 2). The rate of oxidation increased at the higher concentrations of 113 and 171 μM iron, but the rate did not increase further at 216 μM iron (Figure 2). The order of antioxidant activity of lactoferrin decreased with increasing iron:lactoferrin molar ratio: 2:1 > 6:1 > 9:1 > 17:1. Figure 4 shows the development of oxidation of casein-predominant formula containing 216 μM iron.

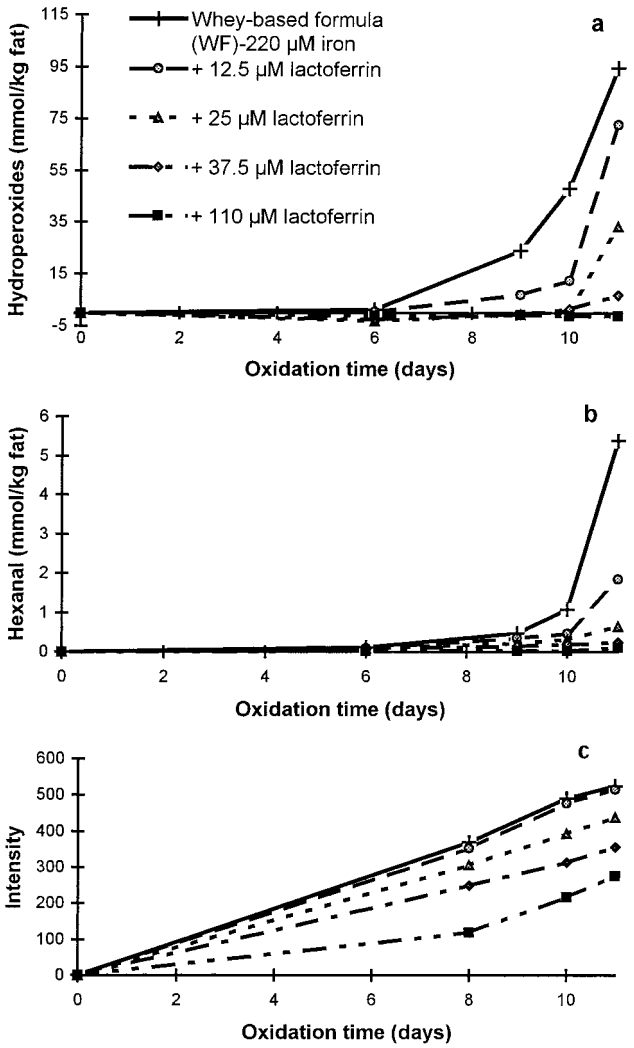

Figure 3. Effect of lactoferrin concentration on the formation of (a) hydroperoxides, (b) hexanal, and (c) fluorescent products in a whey-based formula fortified with 220 μM iron (WF-220).

As in the whey-based formula, lactoferrin was an antioxidant at all the concentrations tested except in the formula containing 216 μM iron at 1 mg lactoferrin/mL. With the formula containing traces of iron (17 μM), the addition of lactoferrin at 1 mg/mL (12.5 μM) decreased the formation of hydroperoxides by 26% and the formation of hexanal by 16%. At 2 mg/mL (25 μM), lactoferrin inhibited the formation of hydroperoxides 64% and the formation of hexanal 63% (Table 3). With the formula containing 216 μM iron, hexanal formation was inhibited 22%, although lactoferrin at a concentration of 12.5 μM (1 mg/mL) did not affect hydroperoxide formation. At an iron:lactoferrin molar ratio of 2:1 lactoferrin inhibited the formation of hydroperoxides 84% and the formation of hexanal 75% (Table 3).

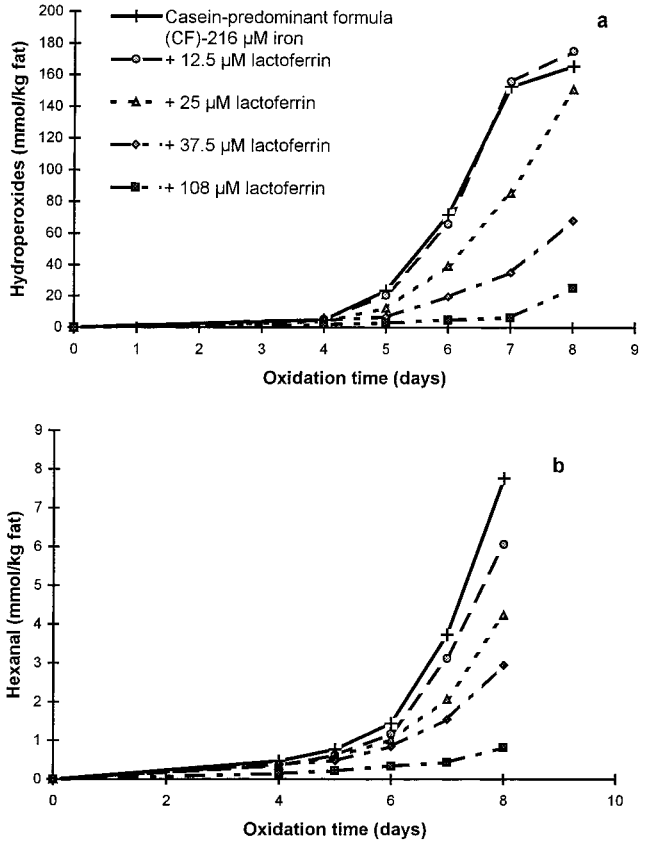

Figure 4. Effect of lactoferrin concentration on the formation of (a) hydroperoxides and (b) hexanal in a casein-predominant formula fortified with 216 μM iron (CF-216).

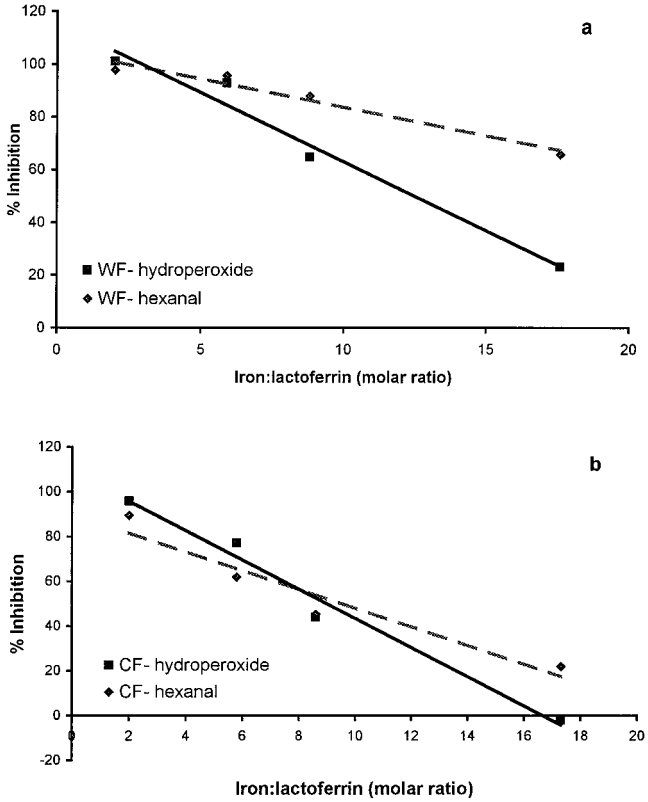

Figure 5. Correlations between percent inhibition of hydroperoxide formation (solid lines) and hexanal formation (dashed lines) and iron:lactoferrin molar ratio for (a) whey-based formula (WF) and (b) casein-predominant formula (CF).

Oxidation of Infant Formula in the Presence of Bovine Serum Albumin (BSA). To compare the activity of lactoferrin with other proteins in affecting oxidation, an oxidation stability experiment was carried out with bovine serum albumin (Table 4). This protein was added to infant formula at an iron:BSA molar ratio of 0:25 and 2:1. The samples containing BSA oxidized similarly to their corresponding controls. After 12 days of oxidation, in the absence of added iron, hydroperoxide and hexanal formation were slightly higher in the sample containing BSA compared to the control without added protein. However, these differences were significant ($p < 0.05$) only for the hydroperoxides. In the presence of 220 μM iron, both hydroperoxides (72.6 vs 65.1 mmol/kg) and hexanal (3.5 vs 3.1 mmol/kg) were significantly ($p < 0.05$) higher in the sample containing BSA.

Table 4. Effect of Bovine Serum Albumin (BSA) on Hydroperoxide and Hexanal Content in a Whey-Based Formula (WF) in the Absence and Presence of Iron

formula (μM iron)	BSA (μM)	hydroperoxides (mmol/kg)[a]	hexanal (mmol/kg)[a]	Fe:BSA molar ratio
WF (0)		14.0[a]	0.1[a]	
+ BSA	25.0	14.5[b]	0.2[a]	0:25
WF (220)		65.1[a]	3.1[a]	
+ BSA	110.0	72.6[b]	3.5[b]	2:1

[a] Data calculated at day 12 of oxidation. Values in each column for samples with the same amount of iron and with the same superscript letter are not significantly different ($p < 0.05$).

DISCUSSION

Lipid oxidation is known to be strongly catalyzed by trace amounts of transition metals such as iron and copper. The rate of oxidation increases with increasing amounts of metals. Metals promote the decomposition of hydroperoxides, favoring both the initiation of free radical propagation reactions as well as the formation of volatile break down products. High concentrations of metals are also known to promote chain termination (Frankel, 1998). This deteriorative action of transition metals poses a significant problem for foods such as infant formula that must meet complete nutritional requirements. Iron in particular is necessary as a nutritional component of the food, and yet its presence limits the shelf life of the product.

The differences in the rates of oxidation observed between the whey-based and casein-predominant formulas may be explained by differences in composition. The casein-predominant formula contained soy lecithin (not present in the whey-based formula) though less total fat than the whey-based formula (4.1 g vs 5.1 g/100 kcal). The more rapid oxidation of the casein-predominant formula may well be caused by the presence of soy lecithin, which is known to oxidize readily (Porter et al., 1980) and, as a good emulsifier, it may facilitate the reaction of oxygen with catalytic metals. Another compositional difference between the two formulas is the type of protein used. Milk whey protein, but not caseins, contain disulfide bonds (S−S) and sulfhydryl (−SH) groups (Brunner, 1977), which are reducing agents capable of scavenging free radicals.

Lactoferrin is an iron transport protein that binds iron with high affinity in two distinct iron-binding domains (Smith et al., 1994). These domains apparently bind the iron with sufficient coordination that these atoms appear to be no longer active in the redox cycling and hydroperoxide decomposition reactions that promote the

oxidation of polyunsaturated lipids. This ability of lactoferrin to avidly bind two atoms of metal per molecule is consistent with the high antioxidant effect observed at iron/lactoferrin molar ratios of 2:1. Lactoferrin previously was shown to have an antioxidant effect on a corn oil emulsion and on a lecithin liposome system (Huang et al., 1999). However, Gutteridge et al. (1981) reported that iron-saturated lactoferrin was unable to inhibit oxidation in a liposome system. Baldwin et al. (1984) suggested that lactoferrin promoted formation of hydroxyl radicals when the concentration of iron exceeded lactoferrin's chelating ability.

Bovine lactoferrin functioned as an antioxidant in all experiments with both whey-based and casein-predominant infant formulas, even when saturated with iron. These results are consistent with the report of Matsue et al. (1994) that lactoferrin could suppress malonaldehyde formation beyond the iron-binding capacity. Nonspecific binding of metal could account for the antioxidant effect observed at iron/lactoferrin molar ratios higher than 2:1. Nagasako et al. (1993) also reported evidence suggesting that bovine lactoferrin can bind iron at sites other than the two high affinity-binding sites. On the other hand, other proteins, like casein hydrolysate and bovine serum albumin, did not effectively stabilize iron in solution. In the present study, bovine serum albumin was not an antioxidant when tested under the same conditions as lactoferrin (Table 4).

The antioxidant activity of lactoferrin in inhibiting oxidation of an infant formula that contained no added iron could be explained by chelation of copper, which is present in all the formulas. Lactoferrin binds metals in the presence of a suitable anion, usually bicarbonate (Masson and Heremans, 1968). The addition of bicarbonate to the infant formula did not affect the antioxidant activity of lactoferrin (data not shown). This observation is consistent with the report of Aruoma and Halliwell (1987) that the presence of bicarbonate did not modify the inhibitory effects of apo-lactoferrin on hydroxyl radical generation at pH 7.4. At the physiological pH of 7 observed for the infant formulas, lactoferrin has high affinity for iron (binding constant $\approx 10^{20}$). Moreover, according to Feeney et al. (1983), ambient bicarbonate seems sufficient for the iron-binding activity of lactoferrin in the absence of competing substances.

In summary, lactoferrin has been proposed as a multifaceted ingredient for functional foods (Steijns, 1996). Its value to foods in providing iron binding and antimicrobial properties has been established. The present paper demonstrates the additional ability of lactoferrin to inhibit oxidation, which is one of the main causes of food spoilage. This beneficial effect, in addition to other proposed functions, makes lactoferrin a very useful ingredient for foods, such as infant formula, that are relatively rich in fat and especially those for which the addition of catalytic iron is mandated for nutritional requirements.

ACKNOWLEDGMENT

The authors thank DMV for providing lactoferrin for the experiments, Dr. Linda Harris for helping with the microbiological tests and Steve Potts for the use of the fluorometer.

LITERATURE CITED

Arnold, R. R.; Cole, M. F.; McGhee, J. R. A bactericidal effect for human lactoferrin. *Science* **1977**, *197*, 263−265.

Aruoma, O. I.; Halliwell, B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. *Biochem J.* **1987**, *241*, 273−278.

Baldwin, D. A.; Jenny E. R.; Aisen, P. The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. *J. Biol. Chem.* **1984**, *259*, 13391−13394.

Bellamy, W.; Takase, M.; Yamauchi, K.; Wakabayashi, H.; Kawase, K.; Tomita, M. Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta* **1992**, *1121*, 130−136.

Brock, J. H. Lactoferrin structure−function relationships. In *Lactoferrin-Interactions and biological functions*; Hutchens, T. W.; Lönnerdal, B., Eds.; Humana Press: Totowa, NJ, 1997.

Brunner, J. R. Milk proteins. In *Food Proteins*; Whitaker, J. R.; Tannenbaum, S. R., Eds.; The Avi Publishing Company, Inc.: Westport, CT, 1977.

Chan H. W.-S.; Levett, G. Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and methyl hydroxylinoleates. *Lipids* **1977**, *12*, 99−104.

Dean, R. T.; Hunt, J. V.; Grant, A. J.; Yamamoto, Y.; Niki, E. Free radical damage to proteins: the influence of the relative localization of radical generation, antioxidants and target proteins. *Free Radical Biol. Med.* **1991**, *11*, 161−168.

Facon, M. J.; Skura, B. J. Antibacterial activity of lactoferricin, lysozyme and EDTA against Salmonella enteritidis. *Int. Dairy J.* **1996**, *6*, 303−313.

Feeney, R. E.; Osuga, D. T.; Meares, C. F.; Babin, D. R.; Penner, M. H. Studies on iron binding sites of transferrin by chemical modification. In *Structure and Function of Iron Storage and Transport Proteins*; Urushizaki, I., et al., Eds.; Elsevier Science Publishers: New York, 1983.

Fletcher, B. L.; Dillard, C. J.; Tappel, A. L. Measurement of fluorescent lipid peroxidation products in biological systems and tissues. *Anal. Biochem.* **1973**, *52*, 1−9.

Frankel, E. N. Volatile lipid oxidation products. *Prog. Lipid Res.* **1982**, *22*, 1−33.

Frankel, E. N. *Lipid oxidation*; The Oily Press: Dundee, 1998.

Gutteridge, J. M. C.; Paterson, S. K.; Segal, A. W.; Halliwell, B. Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. *Biochem. J.* **1981**, *199*, 259−261.

Heinonen, M.; Rein, D.; Satué-Gracia, M. T.; Huang, S.-W.; German, J. B.; Frankel, E. N. Effect of protein on the antioxidant activity of phenolic compounds in a lecithin-liposome oxidation system. *J. Agric. Food Chem.* **1998**, *46*, 917−922.

Huang, S.-W.; Satué-Gracia, M. T.; Frankel, E. N.; German, J. B. Effect of lactoferrin on oxidative stability of corn-oil emulsions and liposomes. *J. Agric. Food Chem.* **1999**, *47*, 1356−1361.

Kuipers, M. E.; de Vries, H. G.; Eikelboom, M. C.; Meijer, D. K. F.; Swart, P. J. Synergistic fungistatic effects of lactoferrin in combination with antifungal drugs against clinical Candida isolates. *Antimicrob. Agents Chemother.* **1999**, *43*, 2635−2641.

Kunitake, S. T.; Jarvis, M. R.; Hamilton, R. L.; Kane, J. P. Binding of transition metals by apolipoprotein A-1-containing plasma lipoproteins: inhibition of oxidation of low-density lipoproteins. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6993−6997.

Leitch, E. C.; Willcox, M. D. P. Elucidation of the antistaphylococcal action of lactoferrin and lysozyme. *J. Med. Microb.* **1999**, *48*, 867−871.

Lin, C.-C.; Fujimoto, K.; Hwang, L. S. The antioxidative effect of protein on the hemoglobin-catalyzed oxidation of sardine oil in an emulsion system. *Nippon Shokuhin Gakkaishi* **1993**, *40*, 602−608.

Lönnerdal, B.; Forsum, E.; Hambraeus, L. A longitudinal study of the protein, nitrogen and lactose contents of human milk from Swedish well-nourished mothers. *Am. J. Clin. Nutr.* **1976**, *29*, 1127−1133.

Lönnerdal, B.; Hernell, O. Iron, zinc, copper and selenium status of breast-fed infants and infants fed trace element fortified milk-based infant formula. *Acta Paediatr.* **1994**, *83*, 367−373.

Masson, P. L.; Heremans, J. F. Metal-combining properties of human lactoferrin (red milk protein). I. The involvement of bicarbonate in the reaction. *Eur. J. Biochem.* **1968**, *6*, 579−584.

Matsue, M.; Tomita, S.; Nyui, S.; Matuyama, J.; Kiyosawa, I. Suppressive effects of lactoferrin on bleomycin-dependent DNA damage by the iron ion and ascorbate. *Biosci. Biotech. Biochem.* **1994**, *58*, 67−71.

Miller-Catchpole, R.; Kot, E.; Haloftis, G.; Furmanov, S.; Bezkorovainy, A.. Lactoferrin can supply iron for the growth of Bifidobacterium breve. *Nutr. Res.* **1997**, *17*, 205−213.

Nagasako, Y.; Saito, H.; Tamura Y.; Shimamura, S.; Tomita, M. Iron-binding properties of bovine lactoferrin in iron-rich solution. *J. Dairy Sci.* **1993**, *76*, 1876−1881.

Oram, J. B.; Reiter, B. Inhibition of bacteria by lactoferrin and other iron-chelating agents. *Biochim. Biophys. Acta* **1968**, *170*, 351−365.

Petschow, B. W.; Talbott, R. D.; Batema, R. P. Ability of lactoferrin to promote the growth of Bifidobacterium spp. in vitro is independent of receptor binding capacity and iron saturation level. *J. Med. Microb.* **1999**, *48*, 541−549.

Porter, N. A.; Wolf, R. A.; Weenen, H. The free radical oxidation of polyunsaturated lecithins. *Lipids* **1980**, *15*, 163−167.

Pierce, A.; Colavizza, D.; Benaissa, M.; Tartar, A.; Montreuil, J.; Spik, G. Molecular cloning and sequence analysis of bovine lactotransferrin. *Eur. J. Biochem.* **1991**, *196*, 177−184.

Roberts, A. K.; Chierici, R.; Sawatzki, G.; Hill, M. J.; Volpato, S.; Vigi, V. Supplementation of an adapted formula with bovine lactoferrin: 1. Effect on the infant faecal flora. *Acta Paediatr.* **1992**, *81*, 119−124.

Sánchez, L.; Calvo, M.; Brock, J. H. Biological role of lactoferrin. *Arch. Dis. Child* **1992**, *67*, 657−661.

Smith, C. A.; Anderson, B. F.; Baker, H. M.; Baker, E. N. Structure of copper- and oxalate-substituted human lactoferrin at 2.0 Å resolution. *Acta Crystallogr., Sect. D* **1994**, *50*, 302−316.

Steijns, J. M. *Lactoferrin: getting the best out of iron to keep up performance*; Food Ingredients Europe FIE: Paris, 1996.

Suddendorf, R. F., and Cook, K. K. Inductively coupled plasma emission spectroscopic determination of nine elements in infant formula: collaborative study. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 985−992.

Wagner, S. F. Analysis of variance. *Introduction to Statistics*; Harper Perennial: New York, 1992; Chapter 11.

Yamashoji, S.; Yoshida, H.; Kajimoto, G. Studies on metal-protein complex. XII. Effects of proteins and peptides on catalytic autoxidation of linoleic acid by Cu (II) in an aqueous ethanol solution. *Oil Chem. (Jpn)* **1979**, *28*, 421−423.

Zapata, C. V.; Donangelo, C. M.; Trugo, N. M. F. Effect of iron supplementation during lactation on human milk composition. *J. Nutr. Biochem.* **1994**, *5*, 331−337.

Received for review February 25, 2000. Revised manuscript received July 13, 2000. Accepted July 19, 2000. This research was supported by the California Dairy Foundation, Davis, CA.

JF0002490