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Role of Ferritin as a Lipid Oxidation Catalyst in Muscle Food†

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Iron was released from ferritin by both cysteine and ascorbate at the pH found in muscle foods (5.5–6.9). The rate of iron release from ferritin was influenced by temperature and ferritin and reducing agent concentrations. Storing beef muscle at 4 °C for 11 days resulted in a decrease in the concentration of ferritin antibody precipitatable iron, suggesting that iron is released from ferritin in situ. Physiological concentrations of ferritin catalyzed lipid oxidation in vitro, and heating ferritin increased the rate of lipid oxidation. These data suggest that ferritin could be involved in the development of off-flavors in both cooked and uncooked muscle foods.

Lipid oxidation is one of the major causes of the chemical deterioration of muscle foods. The primary catalysts of lipid oxidation in skeletal muscle have been suggested to be hemoproteins and ferrous iron (Igene et al., 1979; Pearson et al., 1977). Soluble low molecular weight chelates of iron are thought to be the form of iron responsible for enzymic and nonenzymic lipid oxidation in vivo (Halliwell and Gutteridge, 1986). Hazell (1982) and Decker et al. (1989) reported that 4–14% of the soluble iron in beef, lamb, chicken, pork, and flounder have molecular weights of less than 12 000.

Muscle foods are subjected to several processes that may increase the low molecular weight or "catalytic" iron. Storing muscle at refrigerator temperatures increases low molecular weight (<10 000) iron concentrations in mackerel ordinary muscle 1.4-fold in 7 days (Decker and Hultin, 1989). Kanner and co-workers (1988) also reported "catalytic" iron, as measured by the bleomycin method, to increase over 3-fold in turkey muscle stored at 4 °C for 7 days.

Cooking affects the distribution of iron in muscle foods. Schricker and Miller (1983) observed increases in nonheme iron in baked and microwaved ground beef. They also reported that addition of ascorbic acid and hydrogen peroxide to ground beef resulted in increased nonheme iron concentrations. Although storage, cooking, and chemicals change the iron distribution of muscle, little direct evidence is available to identify the source of iron affected by these treatments.

Ferritin, a soluble iron storage protein found in liver, spleen, and skeletal muscle, has a molecular mass of 450 000 Da and contains 4500 iron molecules when fully loaded (LaCross and Linder, 1980). Ferritin releases iron in the presence of reducing agents such as superoxide anion, ascorbate, and thiols (Boyer and McCleary, 1987). Iron released from ferritin by ascorbate (Gutteridge et al., 1983) and superoxide anion (Thomas et al., 1985) catalyzes lipid oxidation in vitro. These data suggest that ferritin could be partially responsible for the observed changes in iron distribution and catalysis of lipid oxidation in stored and processed muscle foods.

The objectives of this research were to (1) determine whether iron is released from ferritin by ascorbate and cysteine at the pH and temperatures expected in muscle foods, (2) determine whether iron is released from ferritin by heat, and (3) determine whether physiological concentrations of ferritin in beef muscle could catalyze lipid oxidation in vitro.

MATERIALS AND METHODS

Materials. Ascorbate, cysteine, Ferrozine [4,4′-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl]bisbenzenesulfonic acid], horse ferritin serum antibodies, soybean phosphatidylcholine, and 2,2′-dipyridyl were purchased from Sigma Chemical Co. Ferritin (horse spleen) was obtained from Boehringer Mannheim Biochemicals. All other chemicals were reagent grade or purer.

Methods. Ferritin was separated from loosely bound and unbound iron by dialysis against Chelax resin for 12 h. The iron concentration of dialyzed ferritin was determined by using dipyridyl as described by Drysdale and Munro (1965). The concentration of iron bound to ferritin in beef psoas major muscle was determined by antibody precipitation of ferritin according to the method of Linder and Munro (1972). The psoas major muscle used in these experiments was removed from the animals within 2 h of slaughter, chopped in a food processor, and stored at 4 °C for the indicated time. A 10% muscle homogenate was prepared by blending coarsely chopped muscle for two, 1-min bursts in 0.015 M NaCl. The extract was then heated in a water bath (70 °C) for 10 min, cooled on ice for 15 min, and centrifuged at 14000*g* for 20 min. The supernatant was collected and used for the determination of ferritin-bound iron concentration using antibody precipitation as described by Linder and Munro (1972). Although this method can result in proteins other than ferritin being precipitated by the ferritin antibodies, over 95% of the iron precipitated by the antibodies is bound to ferritin (Linder and Munro, 1972). Existence of fer-

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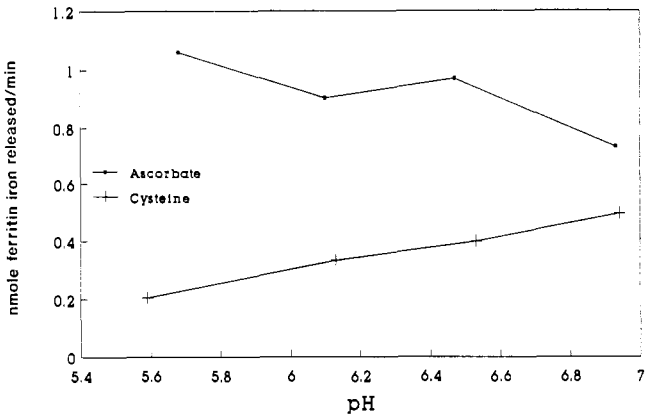

Figure 1. Effect of pH on the release of iron from ferritin by cysteine and ascorbate. All assays contained 5 mM histidine buffer, 0.12 M KCl, 1 mM Ferrozine, 15 μM ferritin, and 1 mM of the indicated reducing agent.

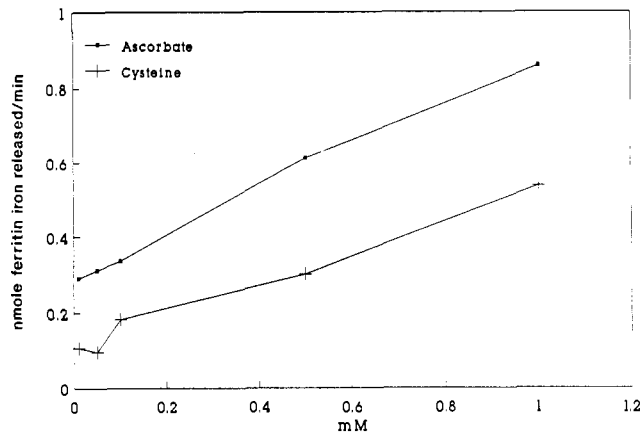

Figure 2. Effect of reducing agent concentration on the release of iron from ferritin. All assays contained 5 mM histidine buffer (pH 5.5), 0.12 M KCl, 1 mM Ferrozine, and 15 μM ferritin.

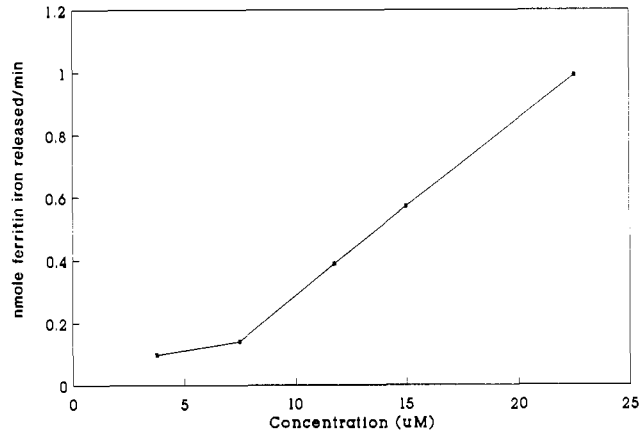

Figure 3. Effect of ferritin concentration on the release of iron from ferritin by ascorbate. All assays contained 5 mM histidine buffer (pH 5.5), 0.12 M KCl, 1 mM Ferrozine, and 1 mM ascorbate.

ritin in the heated muscle extract was verified by SDS discontinuous gel electrophoresis as described by Laemmli (1970).

Iron mobilization from ferritin was determined by using Ferrozine as described by Boyer and McCleary (1987). Release of ferritin iron was monitored by measuring the formation of a ferrous iron–Ferrozine complex at 562 nm. Rates of iron mobilization were determined by using the molar extinction coefficient of 27 900 M^{-1} cm^{-1} (Stookey, 1970). Initial rates of iron release (0–10 min) were obtained every 2 min with a Milton-Roy 1201 spectrophotometer. Assays contained 5 mM histidine (pH 5.5) and 0.12 M KCl unless otherwise indicated. Release of ferritin-bound iron by heat was determined by heating a solution of ferritin in a boiling-water bath for 15 min. The heated ferritin was then centrifuged at 2500*g* for 10 min in Amicon Centricon ultrafiltration cells using 30 000 molecular weight cutoff membranes. The iron concentration in the <30 000-Da fraction was determined with a Varian AA-10/20 atomic absorption spectrophotometer with a graphite furnace accessory (Decker et al., 1989). Atomic absorption was used to detect the iron in the <30 000-Da fraction because the concentrations were too low to measure colorimetrically.

Ferritin-catalyzed lipid oxidation was monitored in a model system (5 mL) containing 0.12 M KCl, 5 mM histidine buffer (pH 5.5), 0.1 mg of phosphatidylcholine liposomes, and 50 μM ascorbate. Liposomes were prepared by sonication and quantitated by measuring phosphate (Decker and Hultin, 1989). The extent of lipid oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) at 532 nm as described by McDonald and Hultin (1987). TBARS concentrations were calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

All assays were performed in triplicate on at least two different experiments.

RESULTS

Characterization of Iron Release from Ferritin. The effect of pH on the rate of iron release from ferritin by 1 mM ascorbate and cysteine is shown in Figure 1. The rate of iron release decreased with increasing pH for ascorbate but increased with increasing pH for cysteine. Ascorbate caused a higher rate of iron release from ferritin than cysteine over a pH range of 5.5–6.9 although the rate of iron released from ferritin by cysteine and ascorbate approached each other at pH 6.9. Boyer and McCleary (1987) reported an optimal release of iron from ferritin by ascorbate at pH 7 when initial rates were measured over a pH range of 6–8. The differences observed in our study and the work of Boyer and McCleary could be due to differences in the buffer systems used (MOPS vs histidine buffer) since histidine has been reported form chelates with iron (Uchida and Kawakishi, 1989).

The effect of ascorbate and cysteine concentration on the release of iron from ferritin at pH 5.5 is shown in Figure 2. Iron release from ferritin could be measured at ascorbate and cysteine concentrations as low as 0.01 mM. Ascorbate stimulated a greater rate of iron release from ferritin than cysteine at all concentrations tested (0.01–1.0 mM). The rate of ascorbate-stimulated release of iron from ferritin increased 3-fold as ascorbate concentrations were increased with 0.01 to 1.0 mM. The rate of cysteine-stimulated iron release from ferritin increased 5-fold over the same concentration range.

Figure 3 shows the effect of ferritin concentration on the rate of iron release in the presence of 1 mM ascorbate. As ferritin concentrations were increased from 3.75 to 22.5 μM, iron release increased 10-fold.

The effect of incubation temperature on the release of iron from ferritin in the presence of ascorbate and cysteine is shown in Table I. Temperature had a marked effect on the release of iron from ferritin, with ascorbate-released iron increasing 3.3-fold and cysteine-released iron increasing 5-fold as the temperature was increased from 2 to 37 °C. Measurable iron release from ferritin occurred for both ascorbate and cysteine at refrigerator temperature (2 °C).

The concentration of iron bound to ferritin was determined in beef psoas major muscle stored at 4 °C (Figure 4). Muscle obtained from two separate animals was used in this study. The concentration of iron bound to ferritin was determined by isolating ferritin serum anti-

Table I. Effect of Incubation Temperature on the Release of Iron from Ferritin in the Presence of either Ascorbate or Cysteine

	rate of iron release,[a] nM iron/min	
temp, °C	ascorbate (1 mM)	cysteine (1 mM)
2	0.15	0.05
22	0.32	0.20
37	0.50	0.22

[a] Iron release was determined by measuring the formation of a Ferrozine–iron complex at 562 nm. All assays contained 15 μM ferritin, 1 mM Ferrozine, 5 mM histidine buffer (pH 5.5), 0.12 M KCl, and the indicated reducing agent.

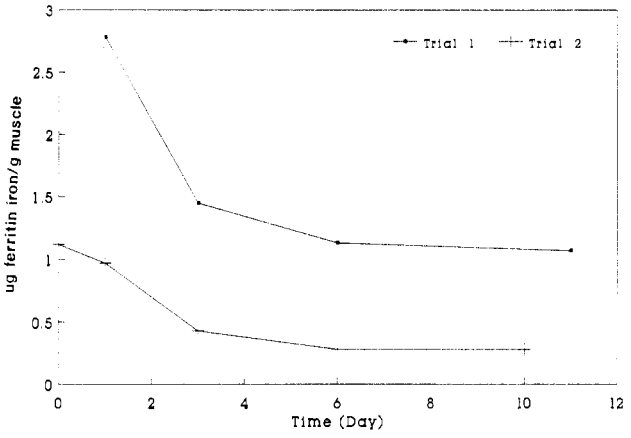

Figure 4. Effect of storage time at 4 °C on the concentration of ferritin serum antibody precipitatable iron in two trials using beef psoas major muscle.

body precipitated (FSAP) iron in a heated muscle extract. Linder and Munro (1972) have reported that heating a tissue extract (70 °C for 10 min) allows the removal of many of the soluble proteins in the extract without affecting the ferritin. Horse ferritin serum antibodies were used in this study because they were readily available and have been reported to react with ferritin from different mammalian species (Munro and Linder, 1978). The antibody concentration used in this study was determined by adding increasing concentrations of antibody to the heated muscle extract until no more iron could be precipitated. Linder and Munro (1972) have reported that over 95% of the iron bound to ferritin is precipitated by this method in heated rat liver extracts. We verified the existence of ferritin in the heated extract with SDS discontinuous gel electrophoresis using a ferritin standard.

Initial FSAP iron concentration was 1.6-fold higher in the muscle used in animal 1 compared to animal 2 (Figure 4). The observed differences in initial FSAP iron concentrations could be due to the diet of the animals prior to slaughter but no information was available on the diet of the animals used in this study. However, Linder and Munro (1972) reported that injection of iron into rats resulted in increased concentrations of iron bound to ferritin in liver and kidney, suggesting that the availability of iron to the animal could influence the concentration of ferritin-bound iron in tissue. Storing beef muscle at 4 °C decreased FSAP iron concentration in the heated muscle extract 62% in 11 days in animal 1 and 75% after 10 days in animal 2. The observed decrease in FSAP iron in muscle suggests that the iron is being released from ferritin during refrigerator storage. The decrease in FSAP iron concentrations could also be due to conformation changes in the ferritin during storage which affects its solubility (extractibility) or ability to interact with serum ferritin antibodies.

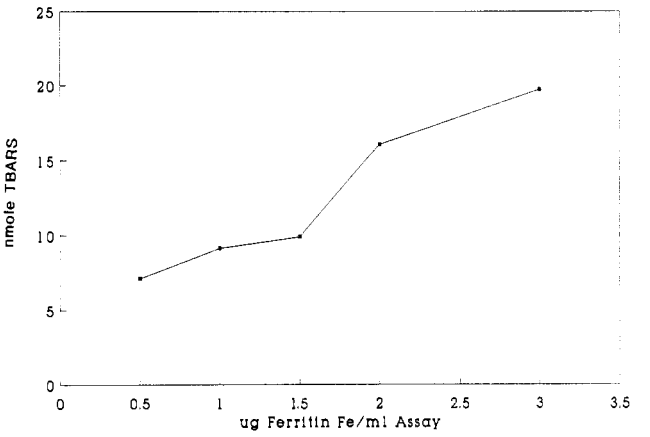

Figure 5. Effect of physiological ferritin-bound iron concentrations on the production of thiobarbituric acid reactive substances (TBARS). All assays (5 mL) contained 5 mM histidine buffer (pH 5.5), 0.12 M KCl, 50 μM ascorbate, 0.1 mg of phosphatidylcholine liposomes, and the indicated amount of ferritin iron. TBARS were determined after 30 min of incubation at 37 °C.

Table II. Effect of Heat on Iron Release from Ferritin and Ferritin-Catalyzed Lipid Oxidation

treatment	iron released[a] ± SD, μg/mL	TBARS[b,c] ± SD
unheated	0.13 ± 0.05	7.4 ± 0.8
heated[d]	0.18 ± 0.04	12.9 ± 2.9

[a] Iron released was determined by measuring ultrafiltratable (<30 kDa) iron with an atomic absorption spectrophotometer with a graphite furnace accessory. Initial ferritin iron concentration was 3.6 mg/mL of 5 mM histidine buffer (pH 5.5) and 0.12 M KCl. [b] TBARS (thiobarbituric acid reactive substances) were determined in a model system (5 mL) containing 5 mM histidine buffer (pH 5.5), 0.12 M KCl, 0.1 mg of phosphatidylcholine liposomes, 50 μM ascorbate, and 7.5 μg of ferritin iron after 30 min at 37 °C. [c] TBARS expressed as nmol/0.1 mg phosphatidylcholine liposomes. [d] Ferritin was heated for 15 min at 100 °C.

Catalysis of Lipid Oxidation by Ferritin. Addition of physiological concentrations of ferritin (0.5–3.0 μg of ferritin-bound iron/mL) to a model system containing phosphatidylcholine liposomes and 50 μM ascorbate resulted in the production of TBARS (Figure 5). Catalysis of lipid oxidation increased 2.7-fold as ferritin-bound iron concentration was increased from 0.5 to 3.0 μg of ferritin-bound iron/mL of assay. TBARS production was not observed in the absence of ascorbate during the time period examined. Gutteridge et al. (1983) reported that ferritin catalyzed the oxidation of brain phospholipid liposomes in the presence of ascorbate. The majority of the observed oxidation was reported to be due to the release of iron from ferritin since the oxidation was inhibited by metal chelators. Gutteridge and co-workers (1983) did observe ferritin-catalyzed lipid oxidation in the absence of ascorbate but their experiments were performed for 2 h whereas ours were only run for 30 min.

Table II shows the effect of heating ferritin on the concentration of ultrafiltratable iron and the catalysis of lipid oxidation. Heating ferritin at 100 °C for 15 min had little effect on the <30 000-Da iron concentration. However, heated ferritin catalyzed 1.6 times more TBARS production than unheated samples in the presence of 50 μM ascorbate (Table II). No lipid oxidation was observed in the absence of ascorbate. The observed increase in TBARS production by heated ferritin could be due to conformational changes in the ferritin molecule which may increase the accessibility of ascorbate to interact with and stimulate the release of iron bound to ferritin.

DISCUSSION

Experiments measuring the release of iron from ferritin using Ferrozine indicate that iron is released from ferritin in the presence of either ascorbate or cysteine at the pH and temperatures commonly found in muscle foods (Figure 1 and Table I). Ascorbate and cysteine are capable of releasing iron from ferritin in vitro at concentrations as low as 10 μM (Figure 2). Decreased FSAP iron concentrations in stored beef muscle (Figure 4) suggest that iron is being released from ferritin in situ. The distribution of free and bound iron has been reported to change during the refrigerator storage of mackerel ordinary muscle (Decker and Hultin, 1989) and turkey muscle (Kanner et al., 1988). The observed decrease in FSAP iron in this study suggests that iron released from ferritin could be at least partially responsible for the observed increase in "catalytic" iron and low molecular weight iron in stored mackerel and turkey muscle. Besides ascorbate and cysteine, superoxide anion and reduced flavins could cause the release of iron from ferritin in muscle tissue (Munro and Linder, 1978).

Temperature and the concentration of ferritin-bound iron had a marked effect on the rate of iron mobilization from ferritin (Table I and Figure 3). At bovine body temperature (38.5 °C) the rate of iron released from ferritin was 3–5-fold greater in the presence of 1 mM ascorbate or cysteine than at 4 °C. This suggests that rapidly decreasing carcass temperature could decrease the rate of release of iron from ferritin in muscle foods. Increasing ferritin-bound iron concentration increased the rate of iron release from ferritin at a constant ascorbate concentration (Figure 3). Since ferritin functions as an iron storage protein, this suggests that manipulation of diet to decrease the concentrations of iron bound to ferritin in muscle would decrease ferritin-catalyzed lipid oxidation in muscle foods.

Physiological concentrations of ferritin-bound iron found in beef muscle (Figure 4) catalyzed lipid oxidation in vitro, and heating (100 °C for 15 min) increased the ability of ferritin to promote lipid oxidation. Lipid oxidation increases in cooked muscle foods, resulting in the formation of warmed-over flavors (Igene et al., 1979; Pearson et al., 1977). The ability of physiological concentrations of heated and unheated ferritin to catalyze lipid oxidation in the presence of ascorbate or cysteine suggests that iron released from ferritin is involved in the development of rancidity and warmed-over flavors in stored beef products.

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