Preparation of Apolactoferrin with a Very Low Iron Saturation

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ABSTRACT

Iron(III) removal from lactoferrin by an iron(III)-chelating resin with immobilized 3-hydroxy-2-methyl-4(*1H*)-pyridinone ligands was studied at physiological pH in the presence of citrate. The resin had a marked effect on the extent of iron removal. By using the iron(III)-chelating resin, removal of iron from lactoferrin was nearly complete in <24 h. Apolactoferrin with 4% iron saturation could be prepared conveniently from 100% or from 18% iron-saturated lactoferrin under mild conditions without affecting the iron-binding capacity of the protein. The iron saturation of the obtained apolactoferrin was much lower than that of the apolactoferrin prepared by reported methods.

(**Key words**: lactoferrin, apolactoferrin, iron removal, chelating resin)

Abbreviation key: **AHMP** = 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(*1H*)-pyridinone, **apoLf** = apolactoferrin, **Lf** = lactoferrin.

INTRODUCTION

The use of lactoferrin (**Lf**) as a natural antimicrobial agent in dairy products has been suggested recently (23). Lactoferrin is partially saturated with iron(III) (20), and data from the literature (4, 16, 23) show that the antimicrobial activity of Lf depends on its iron saturation. Batish et al. (3) reported that the antibacterial activity of apolactoferrin (**apoLf**; iron-free Lf) is greater than that of Lf. Therefore, as part of our investigations on iron chelation, we decided to study the preparation of apoLf by iron removal from Lf.

Mobilization of iron(III) from Lf is possible by using water-soluble iron chelators (6, 8, 13, 18, 19). However, complete removal of iron is difficult, and the iron saturation of apoLf was always >10% (3, 4, 6, 23). Kontoghiorghes (15) found that complete mobilization of iron from Lf by a wide variety of soluble chelators at physiological pH was not possible because of the high affinity of Lf for iron(III) (1, 6, 17). Insoluble chelating resins have also been used at a pH <4 to prepare apoLf, but the apoLf still had an iron saturation of 15% (6, 13, 23). In addition, because the iron removal processes were usually performed at a pH <4.0, development of turbidity in the solutions because of protein precipitation (6) and modification of the conformation of the protein (19) were sometimes observed.

To prepare apoLf with a very low iron saturation, we used an insoluble iron-chelating resin that had already been shown to be effective in detoxification of human plasma that had been poisoned with iron (11) and in reducing the iron content in media for the inhibition of bacterial growth (10, 12). The resin is a crosslinked copolymer of 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(*1H*)-pyridinone (**AHMP**) and N,N-dimethyl-acrylamide (9, 12).

MATERIALS AND METHODS

Materials

The iron(III)-chelating resin was prepared by the polymerization of AHMP and N,N-dimethyl-acrylamide with N,N′-bis(acrylyl)-1,2-diaminoethane as a crosslinking agent (9). The resin had an iron(III)-chelating capacity of 89 μmol/g at pH 6.8 (9). Bovine Lf was provided by Sigma Chemical Co. (St. Louis, MO). The iron content of the protein was 260 μg/g, which was determined with atomic absorption spectrophotometry. Compared with

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the theoretical saturation (1450 μg of iron/g) (5), Lf was approximately 18% saturated with iron. Other reagents, such as $FeCl_3 \cdot 6H_2O$, sodium citrate$\cdot 2H_2O$, citric acid$\cdot H_2O$, and sodium acetate, were from Merck (Darmstadt, Germany) or Aldrich (Steinheim, Germany) and were used as received.

The spectra under UV-visible light were recorded with a Uvikon 930 spectrophotometer (Kontron Instruments, Munich, Germany), which was used for the determination of the iron concentration of Lf solutions. Iron contents in solid Lf were determined with an atomic absorption spectrophotometer (Perkin-Elmer Zeeman 5000; Perkin-Elmer, Norwalk, CT).

Buffers with different pH were prepared by mixing acetic acid solution (.1 *M*) and sodium acetate solution (.1 *M*) or Tris solution (.1 *M*) and Tris·HCl solution (.1 *M*) at various ratios.

Stock Lf Solution (100% Iron Saturation)

Lactoferrin (1500 mg; 18% iron saturation) was dissolved in about 35 ml of .1 *M* sodium bicarbonate (pH 8.2) and transferred to a 50-ml volumetric flask. The Lf was completely saturated with iron(III) by addition of an appropriate amount of a freshly prepared $FeCl_3$ solution (12.5 m*M*; pH 2.3) and diluted to the mark (50 ml) with .1 *M* sodium bicarbonate. The Lf solution (pH 6.8) had an iron(III) concentration of .78 m*M* and was stored at 4°C. The Lf concentration of the solution was .39 m*M*. The molar absorbance coefficient at 462 nm of the iron-Lf complex at pH 6.4 was 1740/*M*cm based on its iron concentration.

Iron Release from Lf

To a tube containing 1 ml of the 100% iron-saturated Lf solution were added 3 ml of the acetate or Tris buffer of various pH and 1 ml of a sodium citrate solution (generally .1 *M*). The tube was rotated at room temperature (20°C) for 24 h unless otherwise indicated. The absorbance at 462 nm and the pH of the solution were measured, and the iron release from Lf was calculated from the residual iron bound to Lf by using the calibration curve of the iron-Lf complex.

Iron Removal from Lf in the Presence of the Iron(III)-Chelating Resin

A mixture of 1 ml of the 100% iron-saturated Lf solution, 3 ml of the acetate or Tris buffer, 1 ml of a sodium citrate solution (generally .1 *M*), and a known amount of the resin was rotated at room temperature (20°C) for 24 h unless otherwise indicated. The rotation was stopped, and the resin was allowed to precipitate. The absorbance at 462 nm and the pH of the supernatant were measured, and the iron removal from Lf was calculated as indicated in the previous section.

Preparation of ApoLf from 100% Iron-Saturated Lf

A mixture of 2 ml of the 100% iron-saturated Lf solution, 6 ml of .1 *M* acetate buffer, 2 ml of .1 *M* sodium citrate, and 50 mg of the resin was rotated at room temperature (20°C) for 24 h and centrifuged for 5 min at 3000 rpm. The supernatant was separated from the resin, dialyzed against distilled water (3 × 100 ml) for 3 d, and then lyophilized. The iron content of the apoLf was then determined by atomic absorption spectrophotometry. To examine the effectiveness of the resin, a control without the resin was carried out at the same conditions.

Preparation of ApoLf from 18% Iron-Saturated Lf

Two hundred milligrams of Lf (18% iron saturated) were dissolved in 10 ml of citrate buffer (.5 or .1 *M*; pH 6.0 or 6.4), and then 40 mg (or 20 mg) of the resin were added to the solution. The mixture was rotated at room temperature (20°C) for 24 or 48 h and centrifuged for 5 min at 3000 rpm. The supernatant was separated from the resin, dialyzed, and lyophilized as described. The iron content of the apoLf prepared was determined by atomic absorption spectrophotometry. A control experiment without the resin was carried out at the same conditions.

Resaturation of ApoLf with Iron(III)

Resaturation of the apoLf obtained was performed as in the preparation of the 100% iron-saturated Lf. The iron bound by the Lf was

determined by measuring the absorbance at 462 nm of the iron-Lf complex at pH 6.4.

RESULTS AND DISCUSSION

Iron Release from Lf

In a preliminary study (7), we found that the presence of citrate was necessary for iron removal from Lf by an insoluble iron-chelating resin. Therefore, the effect of citrate on the release of iron from Lf (in the absence of the resin) was studied in more detail. Various amounts of citrate were added to 100% iron-saturated Lf solutions. Results in Table 1 show that the percentage of iron released increased as citrate concentration increased. For the same citrate concentration, iron release also increased as pH declined in the range of 6.6 to 6.2.

These results indicate that citrate acts as a mediator in the transport of iron, an effect that was obviously not realized by Mazurier and Spik (19) and Kontoghiorghes (15). They concluded that iron release from Lf was due to the action of sodium phosphate as an iron-chelating agent, but they used citrate solution as a buffer and added ferric citrate, respectively. We found that iron release by sodium phosphate hardly occurred from 100% iron-saturated Lf. The mobilization of iron by citrate might also explain the reduced antibacterial activity of Lf by addition of citrate, as observed by Bishop et al. (4).

The effect of the pH on the iron release from Lf was also investigated. Iron(III) release from Lf by citrate (20 m*M*) was studied between pH 5.0 and 9.4 in acetate or Tris buffer (Figure 1). No release occurred at a pH >7, and the amount of iron released increased from pH 7 to 5.5, but most of the iron was released from Lf at a pH <5.5.

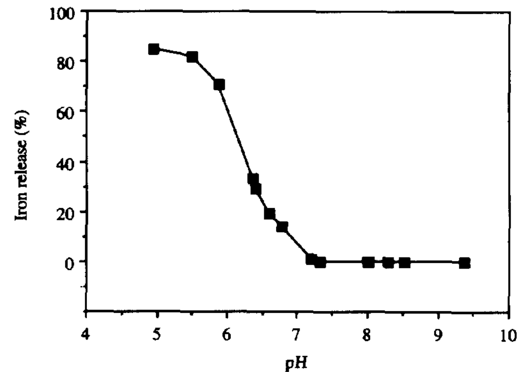


Figure 1. Iron release from 100% iron-saturated lactoferrin solutions versus pH in the presence of citrate (20 m*M*). Values are the means of three measurements.

TABLE 1. Effect of citrate concentration on iron release from 100% iron-saturated lactoferrin in acetate buffer.

Citrate	pH	Iron release
(m*M*)		(%)
0	6.2	0
2.0	6.3	2
4.0	6.3	4
10.0	6.4	11
16.0	6.5	14
20.0	6.5	18
40.0	6.6	24

The pH dependence of iron release from Lf (Figure 1) appeared to be different from the results reported by Mazurier and Spik (19). Their results suggest that from 100% iron-saturated human Lf the acid-labile iron and the acid-stable iron were released in the pH region of 6.9 to 6.0 and 6.0 to 4.0, respectively. However, those researchers did not realize the role of citrate as a mediator and assumed that iron release resulted from the action of sodium phosphate (19). In our case, iron release by citrate occurred at a higher pH. In addition, the two iron-binding sites appeared to be affected simultaneously as observed by Chung and Raymond (6) in iron removal from Lf with other chelators.

Iron release by citrate from Lf as a function of time was also monitored spectrophotometrically in a 100% iron-saturated Lf solution containing 20 m*M* citrate in an acetate buffer at pH 6.8, as presented in Figure 2. The release of iron was fast and almost complete within 1 h. After about 1 h, the release did not increase, indicating equilibrium in the distribution of iron between Lf and citrate.

Iron Removal from Lf

Comparative studies on iron removal from 100% iron-saturated Lf by the iron-chelating resin in the presence and absence of citrate as a

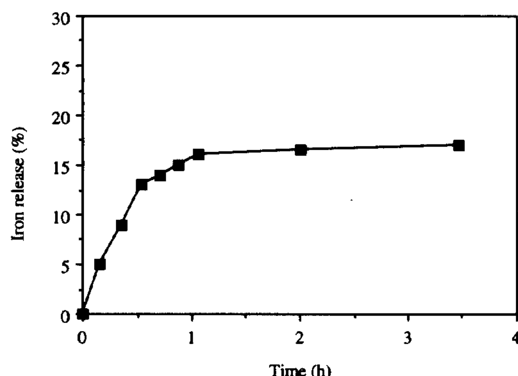


Figure 2. Iron release from 100% iron-saturated lactoferrin in the presence of citrate (20 m*M*) (pH 6.8). Values are the means of two measurements.

Iron release and removal from 100% iron-saturated Lf as a function of time were studied with various amounts of the resin at pH 6.4 and 6.7 (Figure 4). In the absence of the resin, <30% of iron originally bound to Lf was released from Lf by citrate (curve a), but >80% of iron was removed in the presence of the resin (curves b and c, Figure 4). Figure 4 also illustrates that about 20% of the iron was released by citrate without resin (curve d), but >70% of the iron was removed in the presence of the resin (curves f and g). Curve e shows that the ratio for capacity to initial amount of iron was <1, resulting in iron removal of about 40%. Obviously, more iron could be removed from Lf because, by increasing the ratio to 1.1

mediator were performed at pH 4.2 to 9.4 (Figure 3). Iron removal by the resin in the presence of citrate was also dependent on pH as found for the iron release from Lf (Figure 1). However, iron was released between pH 6.9 and 5.5 (Figure 1), but, as in the presence of the resin, most of the iron was removed from Lf in the pH region of 7.5 to 6.3 (Figure 3). The results represented in Figure 3 show that citrate plays a key role in the iron removal from Lf by the iron-chelating resin because, in the absence of citrate, the resin could not remove iron from Lf.

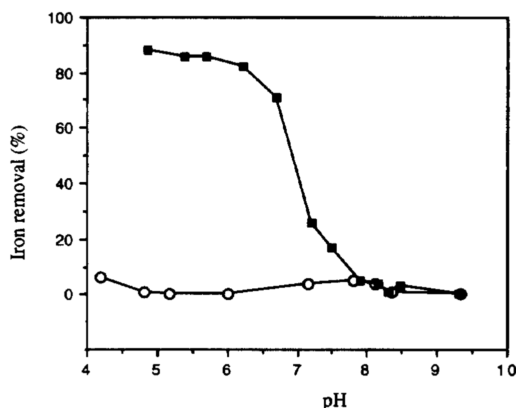
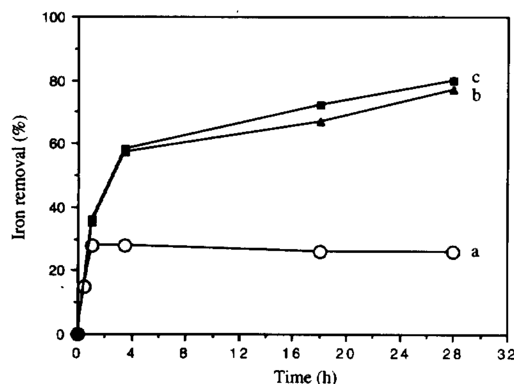


Figure 3. Iron removal by the iron-chelating resin (20 mg) from 100% iron-saturated lactoferrin in acetate or Tris buffer in the presence (■) or absence (O) of citrate (20 m*M*). Values are the means of three measurements.



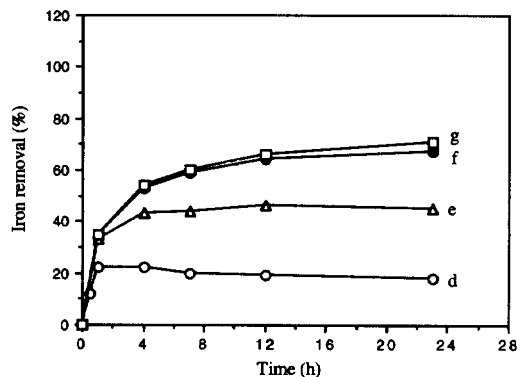


Figure 4. Top: Iron release in the absence of resin (O) and iron removal by 40 (▲) or 80 mg (■) of resin from 100% iron-saturated lactoferrin in the presence of citrate (20 m*M*) at pH 6.4. Bottom: Iron release in the absence of resin (O) and iron removal by 10 (Δ), 20 (●), or 50 mg (□) of resin from 100% iron-saturated lactoferrin in the presence of citrate (20 m*M*) at pH 6.7. Values are the means of two measurements.

TABLE 2. Preparation of apolactoferrin.

Experiment	Iron saturation of original Lf[1]	Conditions of iron removal				Iron saturation of obtained Lf
		pH	Citrate	Resin	Time	
	(%)		(mM)	(mg)	(h)	(%)
1	18	6.5	20	0	48	18
2	18	6.0	100	0	24	10
3	100	6.3	20	0	48	75
4	18	6.5	20	20	48	4
5	18	6.0	100	40	24	4
6	100	6.3	20	50	48	4

[1]Lactoferrin.

(curve f), iron removal was increased. However, addition of more resin (curve g) did not lead to an increase of iron removal, probably indicating that, after about 70% iron removal, an equilibrium existed in the distribution of iron between Lf and the resin.

As shown in Figure 4, varying the amount of the resin did not affect the initial rate of iron removal from Lf. In addition, the initial rate of iron release by citrate was about the same as the initial rate of iron removal when the resin was present.

One molecule of Lf binds two high spin iron(III) ions with very high affinity, and the iron-Lf complexes are stable at a pH >4.0 in the absence of chelators (13). The iron-Lf complex favors the closed conformation, in which the iron atoms are quite deeply buried in Lf, at around 1 nm below the protein surface (2). As observed by us (7), the key requirement for the iron removal from Lf by insoluble resins appeared to be the presence of a mediator, because the deep site of iron in Lf and the network nature of the resin did not allow iron removal. Others (7, 14, 15) have reported that iron uptake and removal from transferrin, ferritin, and Lf at physiological pH depend on thermodynamic and kinetic factors. For example, although desferrioxamine is thermodynamically effective in removing iron from transferrin, constraints imposed by the structure of the reactants slow the reaction considerably (21, 24, 25).

The structural barrier to iron removal from Lf by the resin could be overcome by using citrate as a mediator. Iron was released from Lf, and the released iron was brought to the iron-chelating resin. Iron removal in the presence of the resin was much higher than iron release in the absence of the resin because of the irreversible removal of iron by the resin, resulting in an increase of the releasing action of citrate.

Preparation of ApoLf

Table 2 shows that, in the absence of the resin, only a small part of the iron was released from 18% and from 100% iron-saturated Lf by the action of citrate (Experiments 1 to 3). However, with the resin, Lf with only 4% iron saturation could be obtained (Experiments 4 to 6).

The apoLf (sample 6 in Table 2) was resaturated, and the iron-binding capacity of the apoLf was assayed by measuring the absorbance at 462 nm of the resaturated iron-Lf complex. The iron-binding capacity of the apoLf was 1489 μg of iron/g, which was comparable with the theoretical value (1450 μg of iron/g) (5). This observation indicates that, in the preparation of apoLf in the presence of the resin, no conformational changes of Lf had taken place.

Compared with iron removal from Lf by the water-soluble chelators such as AHMP (8), more iron could be removed by using the iron(III)-chelating resin in the presence of citrate. The extent of iron removal with the resin was also higher than that with the other chelating resins such as desferrioxamine-Sepharose gel and 3-hydroxy-2-methyl-4(*1H*)-pyridinone-Sepharose gel we studied earlier (7). Furthermore, Lf was not precipitated, and the apoLf prepared had not lost its iron-binding capacity. Because of the mild conditions for iron removal and the possibility of reusing the resin

(9), application of the resin offers a suitable procedure for the preparation of apoLf with a very low iron saturation.

Iron removal is of great importance in the inhibition of bacterial growth; in vitro studies on antibacterial activity have shown that apoLf inhibited the growth of a wide variety of microbial species by depriving bacteria of essential iron (3, 4, 22, 23). As shown, the iron(III)-chelating resin could remove almost all of the iron from Lf at physiological pH and room temperature (20°C) in the presence of citrate.

CONCLUSIONS

Iron could be removed from Lf by an iron(III)-chelating resin with AHMP ligands in the presence of citrate as a mediator. The removal of iron from Lf by the resin was related to the pH and the concentration of the mediator. Apolactoferrin with a very low iron saturation (4%) could be prepared conveniently, and the apoLf had the same iron-binding capacity as the original Lf, indicating no conformational change of the protein.

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