Iron Removal from Milk and Other Nutrient Media with a Chelating Resin

M. FENG, L. VAN DER DOES,[1] and A. BANTJES
Department of Chemical Technology
Biomaterials Section
University of Twente
PO Box 217
7500 AE Enschede, The Netherlands

J.M.F.H. DE GROOTE
Mona R&D/QA
Campina Melkunie B. V.
PO Box 222
3440 AE Woerden, The Netherlands

ABSTRACT

A water-insoluble iron(III)-chelating resin was used to study iron removal from milk and other nutrient media. Seventy to 85% of the iron could be removed from wine and beer with the resin, which was a crosslinked copolymer of 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(1H)- pyridinone and N,N-dimethylacrylamide. Iron removal from milk was dependent on the pH of milk and on the concentration of soluble chelators added. Under the same conditions as used for the removal of iron from wine and beer, only 11 to 19% of the iron could be removed from milk. However, in combination with water-soluble chelators, the resin removed 60 to 75% of the iron from the milk. Preliminary results showed that the growth of spores of *Clostridium tyrobutyricum* in the treated milk was reduced. Moreover, addition of the resin and sodium bicarbonate to the milk completely inhibited the growth of the spores.

(**Key words**: chelating resins, iron removal, milk, wine)

Abbreviation key: **AHMP** = 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(1H)-pyridinone, **DMAA** = N,N-dimethylacrylamide, **EHMP** = 1-ethyl-3-hydroxy-2-methyl-4(1H)-pyridinone, **Lf** = lactoferrin.

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[1]To whom correspondence should be addressed.

INTRODUCTION

A method to prolong keeping quality and storage of raw milk will provide greater flexibility in the utilization of milk and will ease the economic pressures on the dairy industry (28). Because iron is an essential element for the growth of almost all microorganisms (8, 31, 32, 33), removal of the available iron with iron chelators results in an inhibition of bacterial growth. One advocated method to remove iron is the use of apolactoferrin, which inhibits the growth of a wide range of bacteria (5, 6, 25). Lactoferrin (**Lf**) is an iron-binding protein that is present in milk (15), and its antibacterial activity is dependent on the degree of iron saturation (26, 27). Because Lf is partly saturated with iron, the antibacterial activity of Lf in milk is limited (23, 24). Therefore, we decided to investigate the removal of iron from milk with a resin to decrease the iron saturation of the Lf present and to enhance its antibacterial activity. The removal of iron from milk to inhibit the bacterial growth will not lead to iron deficiency because milk contains only .12 to .20 ppm of iron. Important sources of iron in the human diet are meat and fish (400 to 600 ppm) (1), egg white, bread and grain products, potatoes, vegetables, and fruit (21).

In addition, iron can also lead to cloudiness and undesired oxidation reactions in some nutrient media because of its action as a catalyst, and excess iron must be removed from nutrient media such as beverages (22) and wine (16, 17). To reduce iron and copper concentrations in beverages, chelating resins have been used (22). Recently, Kern et al. (16, 17) also reported the use of various chelating agents for

the removal of heavy metals, including iron, from wine to improve the taste and prolong the storage of wine. However, these chelators were nonselective and resulted in only low reductions in iron contents, and other physiologically important metal ions were removed.

Iron removal from milk is more difficult than from aqueous solutions because the iron in milk is considered to be tightly bound by several substances (21). For removal of iron from milk, a selective iron(III)-chelating resin, AHMP-DMAA resin, a crosslinked copolymer of 1-(β-acrylamidoethyl)-3-hydroxyl-2-methyl-4(1H)-pyridinone (**AHMP**) and N,N-dimethylacrylamide (**DMAA**), was used in this study. The AHMP-DMAA resin reduces iron(III) concentrations from aqueous solutions to a very low level at a physiological pH (13) and can also remove iron from Lf (our unpublished results).

In this paper, results are reported on the iron removal from wine, beer, and milk. Iron removal from milk was studied in more detail, and the effects of pH and of mediatory, water-soluble iron(III) chelators and citrate were investigated. Also given are preliminary results of the effect of iron(III) chelators on the growth of spores of *Clostridium tyrobutyricum* in milk; these bacteria pose a particular problem for certain varieties of cheese.

MATERIALS AND METHODS

Materials

Fresh, pasteurized whole milk, wine, and beer were purchased from a supermarket. For the preparation of skim milk, whole milk was centrifuged for 20 min at 3000 rpm, the fat layer was discarded, and the skim milk was removed. The AHMP-DMAA resin was synthesized by copolymerization of AHMP and DMAA in the presence of a crosslinking agent (13). For the iron removal experiments a resin with a HMP ligand density of 343 μmol/g was used. A soluble iron(III) chelator, 1-ethyl-3-hydroxy-2-methyl-4(1H)-pyridinone (**EHMP**) (molecular weight, 153 g/mol) was prepared according to a reported method (19). The structure of the copolymer of AHMP and DMAA as well as the structure of EHMP are shown in Figure 1. Lactoferrin with 18% iron saturation was provided by Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade and used as received.

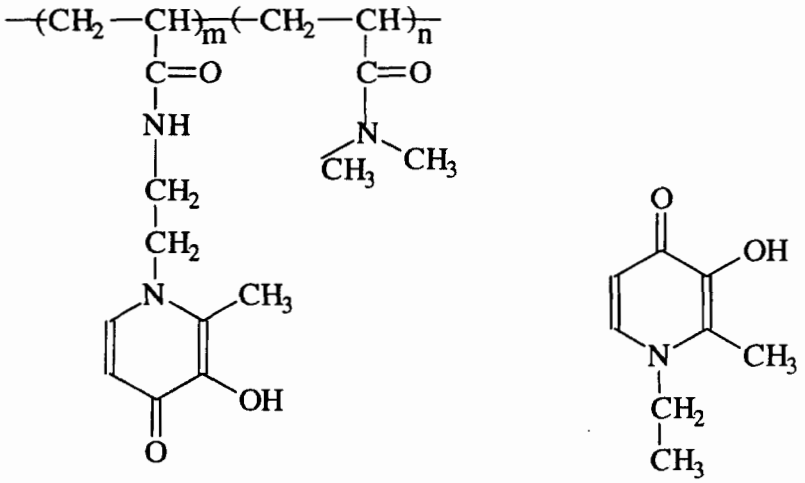

Figure 1. Structure of the AHMP-DMAA copolymer and of EHMP. AHMP = 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(1H)-pyridinone, DMAA = N,N-dimethylacrylamide, and EHMP = 1-ethyl-3-hydroxy-2-methyl-4(1H)-pyridinone.

Iron Removal from Milk or Other Media

The AHMP-DMAA resin was added to a medium, and the mixture was stirred. The iron content in the supernatant was determined by atomic absorption spectrophotometry. A blank control was also carried out and analyzed at the same conditions.

Iron Removal from Milk in the Presence of a Mediator

A solution of a mediator (EHMP or sodium citrate) in PBS (pH 7.4), adjusted to pH 6.7, was added to the milk and stirred at 20°C for 1 h. The milk was treated with the AHMP-DMAA resin as described.

Iron Removal from Acidified Milk

Milk was acidified with 1 *M* HCl to various pH; 50 mg of the resin were added to 40 ml of the acidified milk and stirred at 20°C for 24 h. The iron concentration in the milk was determined by atomic absorption spectrophotometry.

Evaluation of Growth of Spores of *Clostridium tyrobutyricum* in Milk

A mixture of spores from three cultures of *C. tyrobutyricum*, isolated from cheese, was used (from the Netherlands Institute for Dairying Research, Ede, The Netherlands; amounts 6×10^4, 8×10^7 and 6×10^5, respectively). Before inoculation, a heat treatment of 5 min at 80°C was given to the spore mixture. This mixture was added to pasteurized milk at the low level of 100 spores/ml and incubated at 15°C for several days. Viable counts were determined after 3 d of incubation on reinforced clostridial agar (Oxoid CM 149; London, England) under anaerobic conditions at 37°C.

RESULTS AND DISCUSSION

Iron removal from various nutrient media was studied in the presence of the AHMP-DMAA resin. As reported in our previous papers (11, 13), the AHMP-DMAA resin showed a very high affinity and specificity for iron(III) and appeared to be stable under conditions of the iron removal.

Data for the iron removal from wine, beer, and milk are given in Table 1. In all cases, the capacity of the added resin was high enough to bind all the iron present in the medium. The initial iron concentration and the pH of the various nutrient media differed markedly. The resin was able to remove iron from all the media, and the pH of the media remained the same. Iron in wine and beer could easily be removed, but removal of iron from milk was difficult. When twice the amount of the resin was used, as shown in Table 1, no increase of iron removal occurred. The difference in the iron removal from wine and beer compared with that of milk indicates that the type of iron binding is not the same in the various media.

Iron in milk is mainly held by lipids, Lf, and other low molecular weight substances (14); however, the distribution of iron in these substances is still unclear (7, 14). Nevertheless, the lipids and low molecular weight substances in milk should possess an affinity for iron that is comparable with that of Lf because they can

TABLE 1. Iron removal from various nutrient media with the AHMP-DMAA resin.[1]

Name	pH	Resin added	Iron content		Iron removal
			Initial	Treated	
		(mg)	(ppm)		(%)
Red wine	3.6	200	5.68	.96	83
White wine	3.3	200	5.21	1.35	74
Beer	4.5	50	.11	.032	71
Whole milk	6.7	50	.148	.120	19
Whole milk	6.7	100	.148	.117	19
Skim milk	6.7	50	.140	.125	11
Skim milk	6.7	100	.140	.125	11

[1]Mixtures of the resin and 40 ml of the medium were stirred at 20°C for 24 h. AHMP = 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(1H)-pyridinone; DMAA = N,N-dimethylacrylamide.

bind iron in the presence of not fully iron-saturated Lf. However, the lipids in milk seem to bind only a small proportion of the iron present in milk because whole milk had an iron content of .148 ppm and skim milk an iron content of .140 ppm (Table 1). Lactoferrin is a strong iron chelator, and the stability constant is 10^{36} (6). In an earlier study (1994, unpublished results), we found that the AHMP-DMAA resin could remove a large part of the iron(III) from Lf at a physiological pH in the presence of a mediator, such as citrate, and the extent of iron removal increased as citrate concentration increased. Table 1 shows that a small part of the iron could be removed from milk by the resin, probably by the action of citrate as a mediator, because bovine milk contains 4 to 8 m*M* citrate (23). This observation is in agreement with our previous results with other iron(III)-chelating systems (9, 10). The difficulty of complete removal of iron from milk may result from the high affinity of the substances holding the iron in milk or from the lack of sufficient amounts of mediators.

The EHMP has an iron(III) affinity [stability constant $10^{36.7}$ (20)] that is comparable with that of Lf [stability constant 10^{36} (6)], and their affinity for iron(III) is much higher than that of desferrioxamine B, a siderophore produced by bacteria [stability constant 10^{31} (30)]. Because the soluble iron(III) chelator, EHMP, was able to mobilize iron(III) effectively from Lf (12), iron removal from milk in the presence of the resin and EHMP was studied (Table 2). It is obvious that more iron could be removed from both whole and skim milk by addition of EHMP and that the iron removal increased significantly as concentrations of EHMP increased.

Citrate can act effectively as a mediator in iron removal from Lf with the AHMP-DMAA resin (our unpublished results), and the effect of citrate on the iron removal from milk was also explored. Figure 2 shows that iron removal with added citrate was similar to that using EHMP.

Iron removal from Lf was dependent on pH; therefore, the effect of the pH on the iron removal from milk was studied (Figure 3). When the pH of the milk was changed from pH 6.7 to 6.5, more than 65% of the iron could be removed by the resin; changing the pH from 6.5 to 5.9 caused no additional effect.

TABLE 2. Effect of a soluble iron(III) chelator (EHMP)[1] on the iron removal from milk in the presence of the resin.[2]

	Whole milk[3]		Skim milk[4]	
EHMP	Iron content	Iron removal	Iron content	Iron removal
(m*M*/L)	(ppm)	(%)	(ppm)	(%)
0	.120	19	.125	11
.01	.099	33	.055	61
.10	.063	57	.050	64
2.00	.063	57	.035	75

[1]1-Ethyl-3-hydroxy-2-methyl-4(1H)-pyridinone.

[2]50 mg of resin and 40 ml of milk with EHMP (pH 6.7) were stirred at 20°C for 24 h.

[3]Initial iron content, .148 ppm.

[4]Initial iron content, .140 ppm.

These results clearly indicate that mediators, as well as pH, have a pronounced effect on iron removal from milk. As has already been mentioned, part of the iron in milk is complexed by Lf. A Lf molecule has two iron-binding sites that can bind iron(III) tightly with carbonate (or bicarbonate), forming a ternary complex. Lactoferrin can resist heating at pH 4.0 and 90°C for 5 min without any significant loss of iron-binding capacity, antigenic activity, or antibacterial activity (2). Recent structural information indicates (3, 4) that there are two conformations for the iron-binding sites in

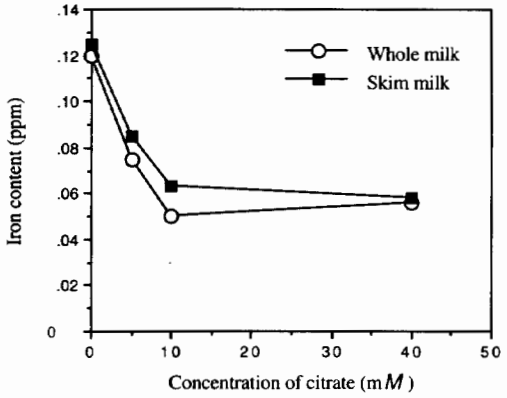

Figure 2. Effect of citrate on the iron removal from milk in the presence of the resin. Experiments were performed with 40 ml of milk (pH 6.7) and 50 mg of resin at 20°C for 24 h.

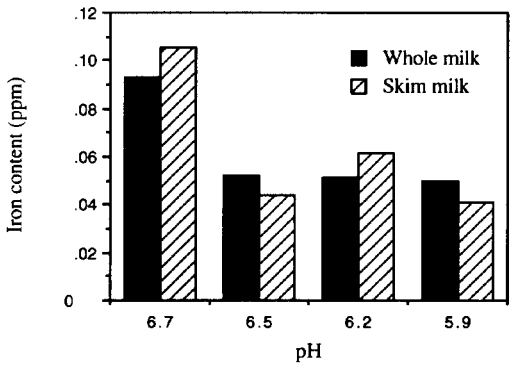

Figure 3. Iron removal from milk by the resin versus pH. Experiments were performed with 40 ml of milk and 50 mg of resin at 20°C for 24 h. The initial concentrations of whole and skim milk were .15 and .14 ppm, respectively.

two different lobes of Lf. The iron complex of Lf favors the "closed" conformation in which the iron is forced well below the protein surface. The iron atoms are quite deeply buried in Lf, around 1 nm below the protein surface (3); therefore, the water-insoluble resin cannot get access to the iron. The "closed" conformation might be influenced by pH, and Lf in solution releases its iron at pH lower than 4.0 (15). The effect of mediators and pH on iron removal may be explained by the release of most of the iron in Lf by H^+ and by the mediators and then removed by the resin.

Table 3 presents data on the growth of spores of *C. tyrobutyricum* in milk, which had been pretreated in various ways. To evaluate the role of iron, the different behavior of water-soluble chelators and of the resin should be taken into account. Addition of Lf to the milk enhanced the Lf concentration, probably resulting in a redistribution of the iron present. A similar effect occurred from addition of EHMP. However, when the resin is used, iron might be released and removed by the water-insoluble resin. Iron removal from milk in the presence of only the resin was low (Table 1), but it increased significantly after addition of EHMP (Table 2) or citrate (Figure 2).

Table 3 illustrates that addition of extra Lf resulted in a marked decrease of the growth of the spores, and the effect was identical for the synthetic chelator EHMP. Feng et al. (12) reported that EHMP could inhibit the growth of *Escherichia coli* and *Listeria inocua* in a brain-heart infusion medium (BHI; Difco, Detroit, MI). The antibacterial activity was due to a limitation of iron available to the microorganisms because, when extra ferric ions were added to the medium, bacterial growth started again. Also, inhibition of bacterial growth associated with Lf was abolished when iron was added to saturate the iron-binding sites of Lf (26). Both Lf and EHMP acted by competing with bacteria for iron, but it is impossible to separate the water-soluble iron chelator (EHMP) from the milk, which may create problems, such as toxicity. A comparable influence on the growth was found when the resin (with or without extra Lf) was used, although only a small part of the iron was removed by the resin (Table 3). In a preliminary experiment, we found that growth was completely inhibited by the resin (20 mg) with sodium bicarbonate (67 mg). Addition of sodium bicarbonate increased the pH of the milk slightly, which may have influenced the growth conditions of the spores and the properties of, e.g., Lf. King and Mabbitt (18) and Rowe (29) reported that CO_2 inhibited the growth of bacteria in milk, possibly by a direct effect of CO_2 on the cells by a mass action effect on decarboxylating enzymes and other enzymes. However, the mechanism of the action of CO_2 on the inhibition of bacterial

TABLE 3. Growth of spores of *Clostridium tyrobutyricum* in milk at pH 6.7.[1]

Additive[2]	Iron content of milk after treatment	Bacterial counts after[3] 3 d	5 d
	(ppm)		
	.15	8000	$>3 \times 10^7$
Lf (40 mg)	ND[4]	40	3×10^4
EHMP (15 mg)	.15	200	2×10^4
Resin (20 mg)	.10	70	1×10^5
Resin (20 mg) + Lf (40 mg)	ND	200	$>3 \times 10^5$

[1]Mixtures of the additive and 40 ml of whole milk were stirred at 10°C for 24 h. The mixtures, or the supernatants when using the resin, were removed and used for the determination of the spores growth.

[2]Lf = Lactoferrin; EHMP = 1-ethyl-3-hydroxy-2-methyl-4(1H)-pyridinone.

[3]Initial counts: 70 to 80 cfu/ml.

[4]Not determined.

growth has not yet been satisfactorily explained. More study is necessary, but, in our opinion, it is also possible that the addition of $NaHCO_3$ might enhance the activity of apolactoferrin, because HCO_3^- is considered to be a co-anion in the complex of apolactoferrin with iron(III) (3).

The results of our experiments indicate the advantages of the AHMP-DMAA resin for the growth reduction of spores of *C. tyrobutyricum* in milk because of its iron-chelating ability, water insolubility, and the possibility of reuse. However, more experiments are required to investigate the effects of other additives and to study the relationship between iron removal and bacterial growth as well as the applicability of the resin.

CONCLUSIONS

An AHMP-DMAA resin was able to remove iron from wine, beer, and milk. The resin easily removed iron from wine and beer (71 to 83%), but the iron removal from milk was more difficult using the same conditions (11 to 19%). Iron removal from milk by the resin was increased in the presence of water-soluble mediators such as EHMP or citrate. In the treated milk, growth of spores of *C. tyrobutyricum* was reduced, and growth was completely inhibited by addition of the resin and sodium bicarbonate.

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