Iron(III)-Chelating Resins. IX. Antibacterial Activity of a Water-Insoluble Iron(III)-Chelating Resin

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Antibacterial activity of a water-insoluble iron(III)-chelating resin with covalently bonded 3-hydroxy-2-methyl-4(1*H*)-pyridinone (HMP) groups was evaluated in a brain heart infusion (BHI) medium. The activity of the resin against *Escherichia coli* was lower than that of soluble HMP iron(III) chelators, whereas against *Listeria inocua*, an activity approximately equal to those of the soluble chelators was found. It was observed that the growth of *E. coli* and *L. inocua* was reduced by increasing the amounts of the resin from 2 to 40 mg of resin/mL of medium. Inhibition of bacterial growth in the presence of the resin (10 mg/mL) was abolished by addition of ferric ion to the medium, indicating that the growth of *E. coli* and *L. inocua* was dependent on the available iron in the medium. Reducing the iron concentration in the medium from 14.2 to 0.16 μM (by action of the resin) resulted in a decrease in the growth response from 100% to 19% for *E. coli* and from 100% to 10% for *L. inocua*. In addition, the influence of citrate was studied, but only small effects of citrate supplementation on the growth of bacteria and on the antibacterial activity of the resin were observed.

Introduction

Iron is an essential element for all living things because a large number of cellular enzymes and other proteins require iron in order to function properly.[1] It is generally considered that the growth of bacteria may be inhibited by depriving iron from the growth media.[2-4] The antibacterial activity of iron chelators, such as the proteins lactoferrin and transferrin, and desferrioxamine B (a natural siderophore) has been studied extensively.[5-11] Synthetic iron(III) chelators were also found to inhibit the growth of bacteria[12] and malaria parasites,[14,15] and we recently reported on the antibacterial activity of soluble iron(III) chelators containing the 3-hydroxy-2-methyl-4(1*H*)-pyridinone (HMP) moiety.[16] However, it is impossible to separate water-soluble iron chelators from the treated systems, which may create problems such as toxicity and thus limits their application to a large extent. In addition, with soluble iron chelators, no relationship could be determined between iron concentration and bacterial growth because iron was only chelated and not removed from the system.[12,16]

Application of iron-chelating resins might be advantageous for inhibiting bacterial growth because the resins can be easily separated from the treated systems. DeVoe and Holbein reported, in a patent, on the inhibition of microbial growth by insoluble compounds containing natural siderophores.[2] Resins with immobilized desferrioxamine B (DFO) or HMP groups have been synthesized in our laboratory.[17-20] These resins showed iron(III)-chelating properties and could be used for preparation of apolactoferrin,[21] iron detoxification of poisoned human blood plasma,[22] or iron removal from milk and other media.[23] One of these iron(III)-chelating resins with a high affinity and selectivity for iron(III) was obtained by copolymerization of 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinone (AHMP) and *N*,*N*-dimethylacrylamide (DMAA) in the presence of a cross-linking agent.[20,24]

Table 1. Antibacterial Activity of the Iron(III)-Chelating Resin and the Water-Soluble HMP Chelators[a]

iron-chelating agent	conc of ligand (mM)	growth response (%)	
		E. coli	*L. inocua*
AHMP-DMAA	17[b]	35	11
AHMP-DMAA	33[c]	19	10
AHMP[d]	20	0	13
PHMP[d]	20	0	14
control	0	100	100

[a] Assay tubes were incubated at 37 °C for 7 h. [b] 20 mg of the resin. [c] 40 mg of the resin. [d] Reference 16.

In this paper, results are presented about the antibacterial activity of the AHMP-DMAA resin and the factors influencing the antibacterial activity. The antibacterial activity of the resin against *Escherichia coli* and *Listeria inocua* was studied, and the results are compared with those obtained with the corresponding soluble HMP chelators. Data are also given about the effect of the iron concentration on the growth of bacteria and the influence of the addition of citrate.

Results

The antibacterial activity of the iron(III)-chelating resin was determined, and it was observed that the growth of both *E. coli* and *L. inocua* was reduced by the insoluble iron(III)-chelating resin (Table 1). (Structures of the iron-(III) chelators are shown in Chart 1.) The resin showed a lower activity against *E. coli* than the corresponding soluble chelators but decreased the growth of *L. inocua* to the same extent as the soluble chelators. It is noted that the resin as well as the soluble chelators exhibited different activities against different bacteria.

In order to get more information about the effect of the amount of the resin on the growth of *E. coli* and *L. inocua*, various amounts of the resin were added, and the results are given in Figures 1 and 2. It can be seen that increasing the amount of the resin resulted in a decrease in bacterial growth response; however, the influences on *E. coli* and *L. inocua* were different. For *E. coli*, the growth response was reduced gradually from 100% to 19% with increasing

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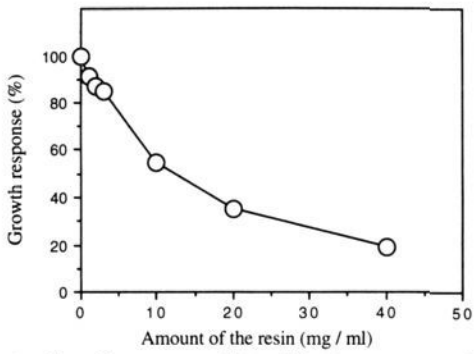


Figure 1. Growth response of *E. coli* in the presence of various amounts of the iron(III)-chelating resin. Experiments were carried out at 37 °C for 7 h.

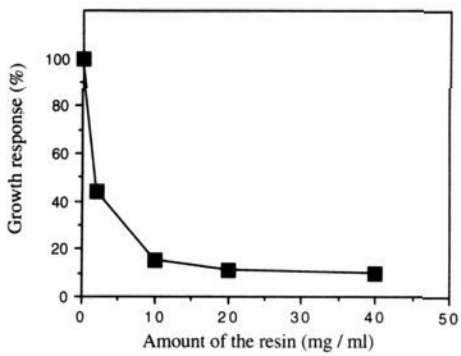
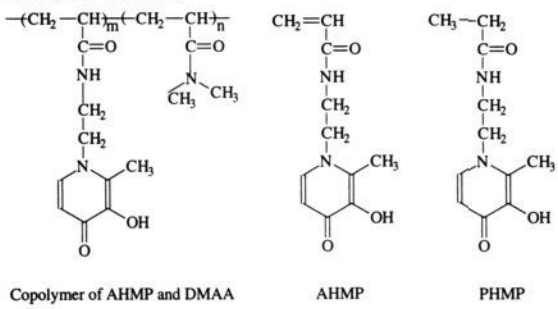


Figure 2. Growth response of *L. inocua* in the presence of various amounts of the iron(III)-chelating resin. Experiments were carried out at 37 °C for 7 h.

Chart 1. Structures of AHMP–DMAA Copolymer, AHMP, and PHMP



amounts of the resin from 0 to 40 mg/mL of medium. Initially, a large decrease in the growth response of *L. inocua* was observed (15% with a resin amount of only 10 mg/mL), and an almost constant growth response was found for amounts of more than 10 mg of resin/mL.

Figures 3 and 4 show the growth response as a function of time for *L. inocua* and *E. coli* under various conditions. By using an amount of the resin of 10 mg/mL, the effect of the resin was maximal after about 6 h. It can also be seen that addition of iron(III) in an amount to saturate the resin abolished the inhibitory activity of the resin for both *L. inocua* and *E. coli*.

Because bacterial growth seemed to depend on the available iron (Figures 3 and 4), the effect of the iron concentration on the bacterial growth was studied in more detail. The effects of the iron(III)-chelating resin on the bacterial growth, as illustrated in Figures 1 and 2, are summarized in Table 2. As can be seen from Table 2, the growth response of both *E. coli* and *L. inocua* decreased with decreasing iron concentrations. However, complete

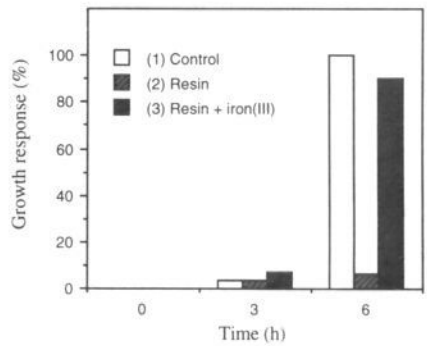


Figure 3. Effect of iron(III) on the growth of *L. inocua*. Experiments were performed at 37 °C with (1) medium only, (2) medium + 10 mg/mL resin, and (3) medium + 10 mg/mL resin + 2.8 μmol/mL iron(III).

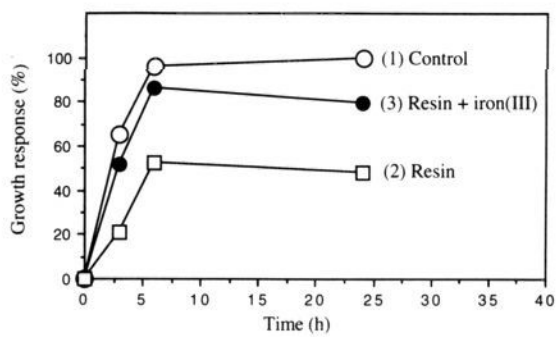


Figure 4. Effect of iron(III) on the growth of *E. coli*. Experiments were performed at 37 °C with (1) medium only, (2) medium + 10 mg/mL resin, and (3) medium + 10 mg/mL resin + 2.8 μmol/mL iron(III).

Table 2. Influence of the Iron Concentration on Bacterial Growth[a]

resin (mg/mL)	conc of iron[b] (μM)	growth response (%)	
		E. coli	*L. inocua*
0.	14.2	100	100
2.5	3.1	87	44
10	0.63	72	*c*
20	0.44	35	11
40	0.16	19	10

[a] Bacteria were incubated in the media at 37 °C for 7 h. [b] Iron concentration in medium after contact with various amounts of resin. [c] Not determined.

inhibition of bacterial growth was not observed even at an iron concentration of 0.16 μM. In addition, it was found that the growth response of *L. inocua* did not decrease any more at an iron concentration below 0.44 μM.

It has been reported that some bacteria utilize iron–citrate complexes to sequester iron from iron-poor environments,[25] so the effect of the addition of citrate on the bacterial growth and the activity of the resin were investigated (Figures 5 and 6). It was found that addition of citrate at a concentration of 10 μmol/mL had only a slight effect on the bacterial growth and the activity of the resin for *E. coli* and almost no influence for *L. inocua*.

Discussion

From the results shown in Table 1, it is evident that by using the iron(III)-chelating resin the bacterial growth of *E. coli* and *L. inocua* could be reduced. Iron removal from the medium was supposed to occur by the immobilized iron(III)-chelating groups on the resin. It was found that the resin was stable in water[20] and that no detectable

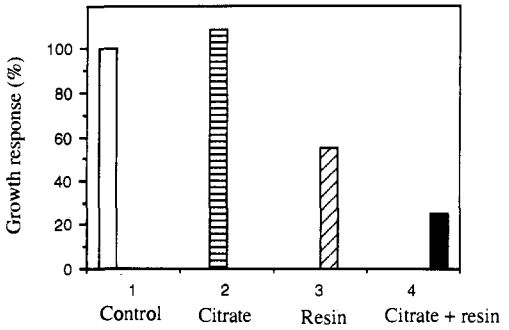


Figure 5. Effect of citrate on the antibacterial activity of the resin for *E. coli*. Growth of *E. coli* took place at 37 °C for 7 h. Citrate (10 μmol/mL) and the resin (10 mg/mL) were added to the corresponding samples.

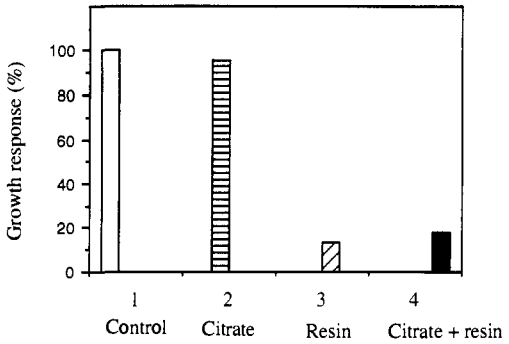


Figure 6. Effect of citrate on the antibacterial activity of the resin for *L. inocua*. Growth of *L. inocua* took place at 37 °C for 7 h. Citrate (10 μmol/mL) and the resin (10 mg/mL) were added to the corresponding samples.

amounts of iron(III)-chelating groups (HMP) were released from the resin under the experimental conditions. The activity of the iron-chelating resin was comparable to that of the soluble HMP iron chelators for *L. inocua* but was lower for *E. coli*. Because the action of soluble iron(III) chelators on bacterial growth is by competing available iron with the bacteria, the difference in the activity between the resin and the soluble iron chelators was probably due to a different mechanism.

The growth of the bacteria was reduced with increasing amounts of the resin, which might be due to the fact that more iron was removed from the medium when more resin was added. However, it seemed impossible to inhibit the growth of *E. coli* and *L. inocua* completely by the resin even by increasing the amount of the resin to 40 mg/mL (Figures 1 and 2). Theoretically, the addition of 40 mg of resin/mL would lead to chelation of 11.1 μmol of iron(III), while the iron content in the BHI medium was about 0.014 μmol/mL (determined by AAS). This means that the capacity of the resin present in the medium was about 800 times the iron concentration. Because it was not possible to obtain lower iron concentrations in the medium than 0.16 μM (Table 2), this might indicate that a small amount of iron in the medium was tightly bound by other substances. It is possible that the remaining iron is used by bacteria for growth because bacteria can produce iron-chelating compounds (siderophores) to sequester iron from iron-poor environments.[5,8-10,26,27]

It was observed that when the resin was saturated with iron its activity to inhibit the bacterial growth was blocked (Figures 3 and 4), indicating that the basis for the antibacterial activity of the resin was its removal of iron required for bacterial growth. Moreover, the bacterial growth response decreased with a decrease of iron concentration in the medium (Table 2). However, a complete inhibition of the bacterial growth was not observed. It was also found that the growth dependence of *E. coli* and *L. inocua* on the iron concentration was different, indicating a different iron requirement for the growth of the different bacteria. An important presently unsolved problem is the determination of the minimal quantities of iron required for the growth of different bacteria and the maximal quantities of iron at or below which the growth of bacteria will be completely inhibited. Weinberg indicated that Gram-negative bacteria need 0.3–1.8 μM iron; most Gram-positive bacteria need 0.4–4.0 μM iron for their growth, and commonly used complex culture media contain 3.0–12.0 μM iron.[28] The minimal quantity of iron required for complete cell growth of *E. coli* in "demineralized" synthetic media was reported to be 0.5 μM.[29-32] In our case, incomplete bacterial growth was observed below 3.1 μM iron for both *E. coli* and *L. inocua*, which may have resulted from the different media used. Because a complete inhibition of the growth of *E. coli* and *L. inocua* was not observed, the maximal iron quantities for completely inhibiting the growth of *E. coli* and *L. inocua* could not be determined.

Addition of citrate to the medium (10 μmol/mL) in the absence of the resin resulted in negligible effects for *E. coli* and *L. inocua*. Contrasting to the reported results with lactoferrin,[9,10] citrate enhanced the activity of the resin for *E. coli* but not for *L. inocua*. One possible explanation for this observation may be that the resin removed more iron in the presence of citrate. This explanation is in agreement with the fact that the growth response of *E. coli* decreased with decreasing iron concentration whereas the growth response of *L. inocua* did not decrease any more at an iron concentration below 0.44 μM.

Conclusions

A cross-linked AHMP–DMAA resin containing covalently bonded HMP groups exhibited antibacterial activity *in vitro* with *E. coli* and *L. inocua*. The antibacterial activity of this water-insoluble iron(III)-chelating resin on *E. coli* was lower than those of soluble HMP iron(III) chelators, whereas for *L. inocua*, the resin showed almost the same activity as the soluble chelators.

The antibacterial activity increased with increasing amounts of the resin, and it was observed that the growth of both *E. coli* and *L. inocua* was dependent on the available iron in the medium. The minimal quantity of iron required for complete cell growth of both *E. coli* and *L. inocua* in the medium was 3.1 μM, and decreasing the iron concentration in the medium resulted in a reduced bacterial growth.

Addition of citrate on the growth of bacteria and on the antibacterial activity of the resin for *E. coli* and *L. inocua* was studied. In the absence of the resin, citrate did not influence the growth of the bacteria. However, citrate was able to enhance slightly the antibacterial activity of the resin for *E. coli*.

Experimental Section

AHMP–DMAA resin was prepared by the reverse-suspension polymerization of 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinone and *N,N*-dimethylacrylamide, using *N,N'*-ethylenebisacrylamide (EBAA) as a cross-linking agent.[20] To a reaction flask with a mechanical stirrer were added AHMP (6.0 mmol), DMAA (51.0 mmol), EBAA (3.0 mmol), $(NH_4)_2S_2O_8$ (0.6

mmol), water (40 mL), hexane (100 mL), CCl_4 (60 mL), and sorbitan monostearate (100 mg). The mixture was stirred and flushed with N_2 for 20 min and then *N,N,N',N'*-tetramethylethylenediamine (0.20 mL) was added, and the polymerization took place for 2 h at 40 °C. The resin was washed with solvents and dried at 80 °C for 48 h. The ligand (HMP) density of the resin was 871 μmol/g of resin, as described previously.[20]

E. coli CA5 (*E. coli*) and *L. inocua* 6B (*L. inocua*) bacteria cultures were provided by NIZO (Netherlands Institute for Dairy Research, Ede). Test strains were subcultured every 3 months to ensure cultural viability and purity. Prior to each assay, bacteria were grown in brain heart infusion (BHI) broth (Difco; iron content, about 14 μM) at 37 °C in a shaking incubator water bath. Iron was minimized in all experiments by using acid-washed glassware as well as chemicals of highest purity. Iron contents in growth media were determined with a Perkin-Elmer Zeeman 5000 atomic absorption spectrophotometer (AAS).

Determination of Bacterial Growth. Bacterial cultures were prepared according to a reported method.[16] Stock cultures (BHI slants) were streaked for growth on BHI plates and incubated at 37 °C for 24 h. A single loopful of bacteria was transferred to 10 mL of BHI medium and incubated at 37 °C for 1 h. A 1.0-mL sample of the culture was transferred to 200 mL of BHI medium and incubated for 2 h at 37 °C in a water bath with shaking.

The iron-chelating resin was tested *in vitro* for its antibacterial activity against *E. coli* and *L. inocua*. Determined amounts of the resin and/or other additives were added to tubes, each containing 9.0 mL of BHI medium. The mixtures were sterilized at 121 °C for 15 min and incubated at 37 °C in a water bath for 18 h. A 1.0-mL portion of the bacterial culture was transferred to the tubes (final volume in all assay tubes, 10.0 mL). The tubes were incubated at 37 °C in a water bath with shaking. Plate counts (CFU) were determined using the standard plate-counting technique, as previously described.[33] The data were expressed as the percentage of growth response (GR), calculated using the formula given by Nonnecke et al.[10]

$$\text{GR (\%)} = \frac{\text{growth under experimental conditions (CFU/mL)}}{\text{maximum growth in basal medium only (CFU/mL)}} \times 100$$

(CFU = colony-forming units.)

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