Studies on Fe Complexes Produced by Yeast. V. Role of the Ligand in Fe Absorption from an Fe(II)–Oligosaccharide Complex

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The role of the ligand (oligosaccharide) of an Fe(II) complex (B1-c) produced in wine by *Saccharomyces cerevisiae* in gastrointestinal Fe absorption was examined. B1-c was found to consist of Fe(II) and an oligosaccharide having the composition of Ara : Xyl : Man : Glc : GalUA (1 : 1 : 1 : 5 : 1), and the sequence of these constituent monosaccharides was presumed by means of partial hydrolysis of B1-c with glycosidases and a diluted acid. The physicochemical comparison of the partially hydrolyzed, Fe(II)-containing products (Fe-containing fragments) obtained by glycosidase treatment indicated that the Man and GalUA residues of the ligand should be essential for stabilization of the complex form at the physiological pH in the digestive tract. The intestinal Fe absorption *in vivo* showed marked differences among the Fe-containing fragments having different ligands (sugar chains). Furthermore, the inhibitory effects of the Fe-containing fragments on B1-c uptake by brush border membrane vesicles of the small intestine varied with the ligand, probably being related to the composition of the sugar chain. These results suggest that the sugar chain of the ligand of B1-c may be involved, through its coordination with Fe(II), in (i) high stability and Fe solubility of B1-c at the physiological pH in the digestive tract and (ii) recognition of B1-c molecules in its transport system on the intestinal brush border membrane and, thereby, may contribute to excellent intestinal Fe absorption from B1-c.

Keywords ferrous complex; oligosaccharide; physicochemical property; gastrointestinal absorption; ligand role absorption–structure relationship

Introduction

Gastrointestinal Fe absorption from some Fe complexes with sugars or organic acids administered is known to be better than that from inorganic Fe salts,[1-6] and the solubility of the Fe complexes at the physiological pH in the small intestine has been explained as one of the factors inducing such high Fe absorption.[7,8] However, the Fe transport system from Fe complexes on the intestinal mucosa remains unclear, and few reports have dealt with the role of their ligands in the transport process.

We have demonstrated in our previous studies that (i) an Fe(II)–oligosaccharide complex having a molecular weight of about 1500 (B1-c) produced in wine by *Saccharomyces cerevisiae* showed high gastrointestinal Fe absorption and incorporation of Fe into hemoglobin[9]; and (ii) the main reasons for the high Fe absorption from B1-c are the high Fe solubility of B1-c in the digestive tract based on its structure of coordination of Fe(II) with an oligosaccharide[10] and the high rate of Fe transfer from B1-c by a special transport system.[11]

In the present study, we determined the composition of the ligand (oligosaccharide) of B1-c and examined the role of the ligand in the above-mentioned characteristics specifying the Fe absorption from B1-c (physicochemical properties and a special transport system).

Materials and Methods

Preparation of Fe Complex According to the method described in our previous report,[9] $FeCl_3$-supplemented grape juice was fermented by *S. cerevisiae* OC-2, and seven solubilized fractions were separated from the wine by column chromatography with Amberlite XAD-2 (Rohm & Haas Co.) and Sephadex G-15 and G-50 (Pharmacia Fine Chemicals, Inc.) successively. B1-c, which is one of the seven fractions separated and the one showing the highest gastrointestinal Fe absorption, was used for the study. For the experiment for gastrointestinal Fe absorption, ^{59}Fe-labeled B1-c was prepared from $^{59}FeCl_3$ (New England Nuclear Corp.)-supplemented grape juice in a similar way.

Characterization of the Ligand of B1-c An aqueous solution of B1-c (about 30 μg as Fe) prepared as above and containing 1 μmol of mannitol as the internal standard was dried under reduced pressure. After methanolysis and trimethylsilylation in accordance with the method of Bhatti *et al.*,[12] the constituent monosaccharide were assayed by gas chromatography under the following conditions: apparatus, Hitachi 663-30 gas chromatograph; column, 5 mm i.d. × 2 m, 3% OV-1 on Chromosorb W_{HP}; oven temperature, 140—180 °C (temperature increasing rate, 0.5 °C/min).

Separately, according to the method of Akai *et al.*,[13] the aqueous B1-c solution was reduced by $NaBH_4$ (final concentration, 0.05%) at 30 °C for 24 h and then oxidized with the same volume of 0.1 M $NaIO_4$ at 4 °C for 48 h, and formaldehyde formed was determined with chromotropic acid. The polymerization degree was calculated by dividing the total content of the constituent monosaccharides (μmol) by the amount of formaldehyde formed (μmol).

The ligand of B1-c was partially hydrolyzed as follows: 10 mM Hepes buffer (pH 6.8; for treatment with α-glucosidase) or 10 mM Mes buffer (pH 5.0) containing B1-c or its partially hydrolyzed product was allowed to react (a) at 30 °C for 1 h with α-glucosidase (from yeast; Sigma Chemical Co.), α-amylase (from *Bacillus* species, Sigma), or amyloglucosidase (from *Aspergillus niger*, Sigma) (10 unit/ml buffer) or with α-mannosidase (from Jack beans, Sigma) (5 unit/μmol substrate); or (b) at 60 °C for 1 h with H_2SO_4 solution added at the final concentration of 1 N. The reaction products were fractionated at 10 °C on a Sephadex G-25 (Pharmacia) or Bio-gel P-4 (Biochemical Industry Co.) column, and the elution patterns of sugars and Fe were determined by the anthrone method[14] and by atomic absorption spectrometry, respectively.

The constituent monosaccharides of each fraction from the partially hydrolyzed products were assayed by gas chromatography as described above. In addition, the fast atom bombardment (FAB)-mass spectrum of each fragment was determined with a mass spectrometer (JEOL DX-300). The reduced terminal group of the ligand of B1-c was estimated by comparison of the sugar composition of B1-c after $NaBH_4$ treatment with that of intact B1-c. The proportion of Fe(II) to total Fe contained in B1-c and its Fe-containing fragments was determined by the bathophenanthroline method.[15]

Electrophoresis of Fe-Containing Fragments Paper electrophoresis was performed by the method described in our previous report,[10] as follows. Each Fe-containing fragment, which was obtained by partial hydrolysis of B1-c with enzymes, was spotted on the filter paper (Toyo Filter Paper Co., No. 50), and electrophoresis was performed using an Atto SJ-1060 apparatus at 50 V/cm for 30 min with 0.2 M acetic acid–Na acetate buffer (pH 3.8). Fe was detected by spraying with 0.35% *o*-phenanthroline–ethanol solution.

pH Dependency of Fe Solubility in Aqueous Solutions of Fe-Containing Fragments To an aqueous solution of each Fe-containing fragment

obtained by partial hydrolysis of Bl-c, phthalate (pH 3—7), Tris–HCl (pH 7—9), or borax buffer (pH 9—11) was added at the final concentration of 25 mM (phthalate and borax buffers) or 50 mM (Tris–HCl). The buffer solution was adjusted to desired pH's with dilute NaOH or HCl solution added dropwise, using an Iwaki pH meter, and the ionic strength was adjusted to 0.08 with NaCl. The Fe-containing fragment solution thus prepared (40 μg Fe/ml) was centrifuged at 10000 rpm for 30 min, and the concentration of soluble Fe in the supernatant was determined by atomic absorption spectrometry. The results were expressed as percentage of Fe solubility (soluble Fe amount/added Fe amount).

Gastrointestinal Fe Absorption from Fe-Containing Fragments The Fe absorption from the Fe-containing fragments prepared from ^{59}Fe-Bl-c was examined *in vivo* and *in vitro* (uptake by brush border membrane (BBM) vesicles of the small intestine), as follows. The details of the methods are described in our previous reports.[10,11,16]

In the *in vivo* experiment, an aqueous solution of each Fe-containing fragment (250 μg Fe/kg) was orally administered to 6-week-old male Sprague-Dawley rats (Clea Japan, Inc.) which were fasted overnight. The amounts of ^{59}Fe absorbed through the gastrointestinal tract and incorporated into hemoglobin were calculated as percent of the dose.

In the *in vitro* experiment for Fe uptake by BBM vesicles, the BBM vesicles were prepared from the rat duodenum by the Mg precipitation method.[17] The purity of the vesicles prepared was confirmed by their specific sucrase activity (about 20 times higher than that in the first homogenate) and ouabain-sensitive ($Na^+ + K^+$) adenosine triphosphatase (ATPase) activity (1/18 of that in the first homogenate). Electron microscopy revealed that they exclusively contained closed, spherical and right side out vesicles. The BBM vesicles (final concentration, 500 μg protein/ml) were incubated at 37 °C for 2 min in the medium (0.1 M NaCl, 0.1 M mannitol, 20 mM Hepes buffer (pH 7.0)) containing ^{59}Fe-Bl-c or ^{59}Fe–Fe-containing fragment. The reaction of this mixture, 200 μl, was terminated with 5 ml of the ice-cold incubation medium, and the ^{59}Fe uptake by the vesicles was determined by the rapid filtration method.

Results

Characterization of the Ligand of Bl-c 1) Sugar Composition The molar ratios of the constituent monosaccharides to Fe of Bl-c are shown in Table I. More than 90% of Fe contained in Bl-c was Fe(II). The ligand consisted of Ara : Xyl : Man : Glc : GalUA (1 : 1 : 1 : 5 : 1 in molar ratio when the Fe content was taken as 1).

2) Polymerization Degree of the Sugar Chain The polymerization degree of the ligand of Bl-c was about 9, when obtained by division of the total content of the constituent monosaccharides (μmol) assayed above, by the

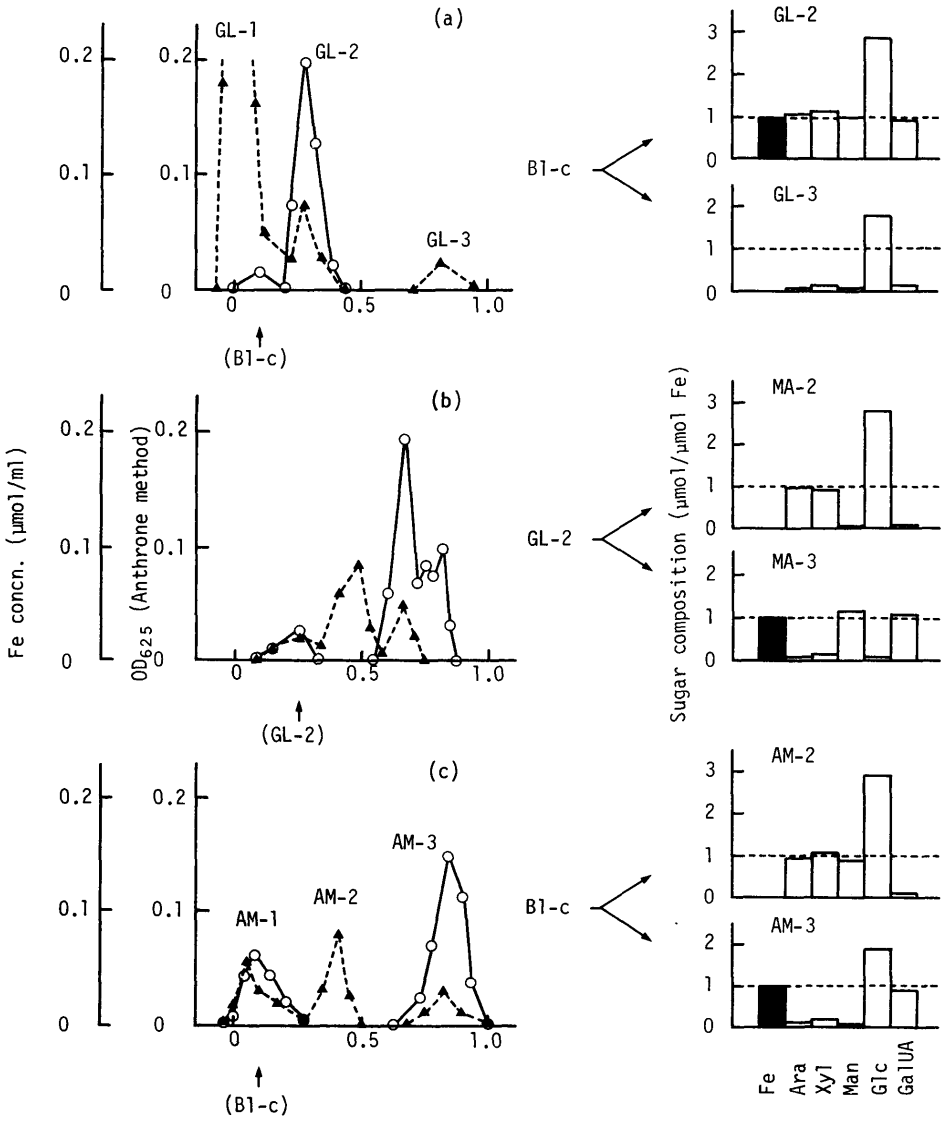

Fig. 1. Analyses of Oligosaccharides after Exoglycosidase Treatment of Bl-c and Its Fragments

(a) Bl-c treated with α-glucosidase; (b) GL-2 treated with α-mannosidase; (c) Bl-c treated with amyloglucosidase. ○, Fe concentration; ▲, OD_{625}/ml determined after reaction by the anthrone method with 1 ml of each fraction (the value represents a relative saccharide concentration).
Chromatographic conditions: column, Sephadex G-25 (1.2 cm i.d. × 110 cm); solvent, H_2O; flow rate, 12 ml/h.

formaldehyde amount (μmol) determined after treatment with $NaBH_4$ and $NaIO_4$.

3) Partial Hydrolysis of Bl-c Exoglycosidases Figure 1 shows the sugar compositions of the Fe fragments obtained by fractionation of Bl-c on a Sephadex G-25 column after single or combined treatment with the exoglycosidases (α-glucosidase, α-mannosidase, and amyloglucosidase).

When Bl-c was treated with α-glucosidase, three peaks were obtained (named GL-1, GL-2, and GL-3 in the order of elution). GL-1 appeared around $K_d=0.1$ as the peak consisting of slightly remaining, unchanged Bl-c and enzyme-derived glycoproteins. GL-2 ($K_d \fallingdotseq 0.3$) consisted of Fe : Ara : Xyl : Man : Glc (1 : 1 : 1 : 1 : 3), corresponding to the composition where two Glc residues were lost from Bl-c. GL-3 was the liberated Glc. The total contents of the constituent monosaccharides and Fe in GL-2 and GL-3 were nearly identical to those of Bl-c (Fig. 1a). In an early phase of this reaction with α-glucosidase, shoulders appeared in the left of GL-2, showing gradual liberation of the Glc residues.

The α-mannosidase treatment did not affect Bl-c directly, but cleaved GL-2 into five fragments (MA-1 to MA-5). MA-1 corresponded to unchanged GL-2, and MA-2 ($K_d \fallingdotseq 0.5$) was a sugar fragment consisting of Ara : Xyl : Glc (1 : 1 : 3) without Fe. MA-3, which accounted for most of the low-molecular-weight fragments, appeared in the position nearly corresponding to that of a disaccharide, and its composition was Fe : Man : GalUA (1 : 1 : 1). MA-4 and MA-5 contained little sugar and are considered to be free Fe yielded as the byproducts. The total contents of the constituent monosaccharides and Fe in MA-2 and MA-3 corresponded to those of GL-2 (Fig. 1b). The α-glucosidase treatment of MA-2 resulted in no further cleavage.

When Bl-c was treated with amyloglucosidase, three fragments were obtained (AM-1 to AM-3), as shown in Fig. 1c. AM-1 corresponded to intact Bl-c. AM-2 ($K_d \fallingdotseq 0.4$) consisted of Ara : Xyl : Man : Glc (1 : 1 : 1 : 3). AM-3 eluted in the monosaccharide position consisted of Fe : Glc : GalUA (1 : 2 : 1).

In the Fe-containing fragments, GL-2, MA-3, and AM-3, more than 85% of the Fe was present as Fe(II).

4) Partial Hydrolysis of Bl-c and MA-2 by an Endoglycosidase (α-Amylase) In the elution pattern of α-amylase-treated Bl-c on a Bio-Gel P-4 column (Fig. 2a), most of the Fe and the sugars were eluted around $K_d=0.7$—0.8 as a mixture of fragments. Therefore the elution pattern of the constituent monosaccharides was determined per fraction. The contents of Glc, Xyl, Man, and GalUA were high around $K_d=0.6$. By contrast, Ara was eluted in a lower-molecular-weight position.

When MA-2 was treated (Fig. 2b), Ara was eluted around the position of a trisaccharide, as in the case of the treatment of Bl-c. On the other hand, Xyl appeared in a lower-molecular-weight position than Ara did, by contrast with the result with Bl-c. Accordingly, it is suggested that (i) Bl-c and MA-2 contain the Glc–Glc linkage inside their ligands; (ii) Ara is located around the reduced terminals of Bl-c and MA-2; and (iii) Xyl is present around the non-reduced terminal of MA-2 (*i.e.*, inside the ligand in the case of Bl-c).

5) Partial Hydrolysis of Bl-c and MA-2 by an Acid When Bl-c was treated with 1 N H_2SO_4 at 60 °C for 1 h, Ara was characteristically eluted around the position of a monosaccharide ($K_d \fallingdotseq 1.0$) in the elution pattern on a Sephadex G-25 column. Glc was distributed in a relatively wide range with $K_d \fallingdotseq 0.6$ as the center, and the other constituent monosaccharides mostly remained in high-mole-

TABLE I. Sugar Composition of Bl-c

Molar ratios					
Fe	Ara	Xyl	Man	Glc	GalUA
1.00	0.93	0.92	0.94	5.13	0.92

The Fe content was obtained by atomic absorption spectrometry. The contents of the constituent monosaccharides were determined by gas chromatography.

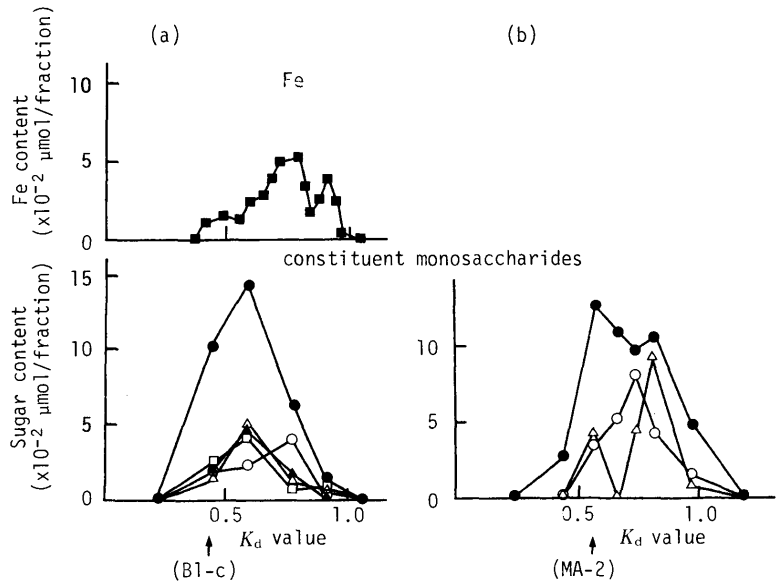

Fig. 2. Analyses of Oligosaccharides after α-Amylase Treatment of Bl-c and MA-2

(a) Bl-c (1.05 μmoles) treated with α-amylase; (b) MA-2 (1.03 μmoles) treated with α-amylase. ■, Fe; ○, Ara; △, Xyl; □, Man; ●, Glc; ▲, GalUA. Chromatographic conditions: column, Bio-Gel P-4 (2.5 cm i.d. × 105 cm); solvent, H_2O; flow rate, 45 ml/h; fraction volume, 4.2 ml.

cular-weight positions. In the case of the treatment of MA-2, Xyl as well as Ara appeared in the position of a monosaccharide, and Glc was eluted in a large amount around the position of a trisaccharide. These results indicate that the Xyl residue present inside the sugar chain of Bl-c hardly undergoes hydrolysis, while in MA-2, Xyl located in the non-reduced terminal is easily cleaved as Ara is.

6) Identification of the Terminal of Bl-c Reduced with $NaBH_4$ The $NaBH_4$ treatment of Bl-c nearly completely eliminated the Ara content, but caused no changes in the contents of the other constituent monosaccharides. This result suggests that Ara is the reduced terminal of Bl-c.

7) Electrophoresis of Bl-c and Fe-Containing Fragments Obtained by Glycosidase Treatment Figure 3 shows the electrophoretic patterns of Bl-c and its Fe-containing fragments (GL-2, MA-3, and AM-3). GL-2 and MA-3 were shifted, like Bl-c, to the anode, and these fragments having both GalUA and Man residues retained the properties of the Fe complex. On the other hand, AM-3 which do not have the Man residue showed the same behavior as an Fe(II) salt ($FeSO_4$), and this fragment is presumed to be present in the form of an Fe(II) salt combined with GalUA.

8) Estimation of Molecular Weights of Partially Hydrolyzed Products of Bl-c by Mass Spectrometry When the FAB spectrum of Bl-c was measured, a relatively high intensity peak appeared at m/z 1488. In the spectra of GL-2, AM-2, MA-2, and MA-3, characteristic peaks appeared at m/z 1152, 913, 791, and 404, respectively. The masses of these peaks are around the molecular weights of the fragments estimated from their sugar compositions, and the peaks are regarded as pseudomolecular ion peaks or similar fragments.

9) Tentative Structures of Bl-c and Its Fe-Containing Fragments Figure 4 shows the sugar sequences of Bl-c and its Fe-containing fragments presumed from the above-described results of the characterization of the ligand of Bl-c. The electrophoretic patterns and elution behaviors after glycosidase treatment of Bl-c and its Fe-containing fragments suggest the necessity of the GalUA and Man residues for maintenance of the complex form, and it is presumed that the Fe atom is coordinated in close vicinity to the GalUA and Man residues.

pH Dependence of Fe Solubility in Aqueous Solutions of Fe-Containing Fragments Figure 5 shows the Fe solubilities in pH 3—11 aqueous solutions of the Fe-containing fragments obtained by the enzyme treatment of Bl-c (GL-2, MA-3, and AM-3). In the GL-2 solutions, like in the Bl-c ones, more than 90% of Fe was soluble up to pH 11.0. In the MA-3 solutions, more than 80% of Fe was present in a soluble form up to about pH 9.0, but the Fe solubility

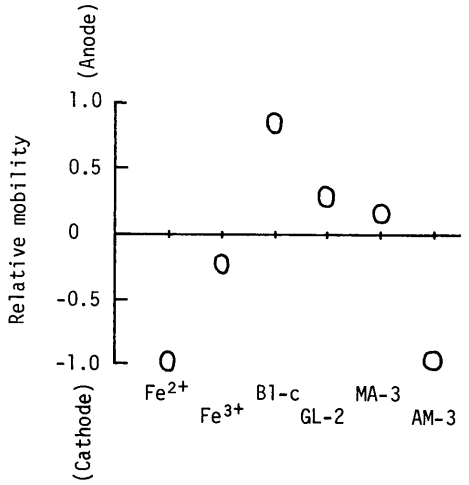

Fig. 3. Electrophoretic Patterns of Bl-c and Its Fe Fragments

Stationary phase, Toyo filter No. 50; buffer, AcOH/AcONa (pH 3.8); detection, *o*-phenanthroline.

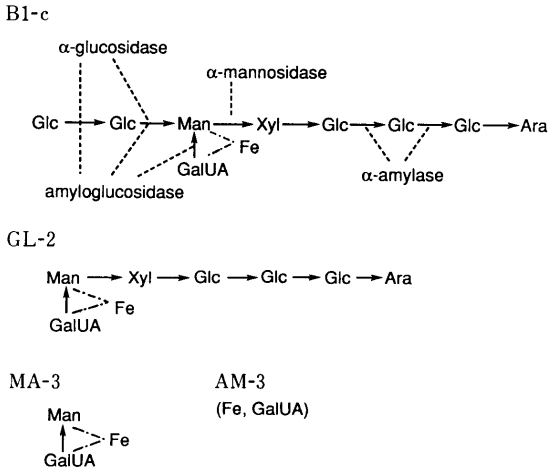

Fig. 4. Tentative Structures of Bl-c and Its Fe-Containing Fragments

TABLE II. ^{59}Fe Absorption and Incorporation into Hemoglobin (Hb) in Rats after Oral Administration of ^{59}Fe-Labeled Bl-c and Its Fe Fragments (250 μg Fe/kg)

Sample	% of dose	
	Absorption	Incorporation into Hb
Bl-c	59.5±4.9[b]	34.8±2.2[b]
GL-2	49.7±1.7	31.0±3.8[a]
MA-3	40.9±6.6	23.9±6.6
AM-3	29.0±5.8	10.2±0.8
$FeSO_4$	37.6±4.0	20.0±1.9

Each value represents the mean±S.E. ($n=3$—7). *a*) Significantly higher than in the $FeSO_4$ group ($p<0.05$). *b*) Significantly higher than in the $FeSO_4$ group ($p<0.01$).

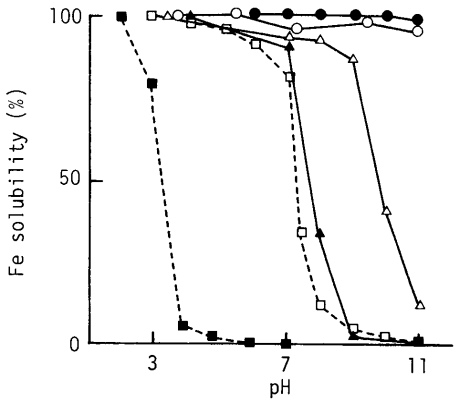

Fig. 5. Effect of pH on Fe Solubility of Bl-c and Its Fe-Containing Fragments (40 μg Fe/ml)

After pH adjustment, each solution was centrifuged at 10000 rpm for 30 min, and the Fe concentration in the supernatant was determined. ●, Bl-c; ○, GL-2; △, MA-3; ▲, AM-3; □, $FeSO_4$; ■, $FeCl_3$.

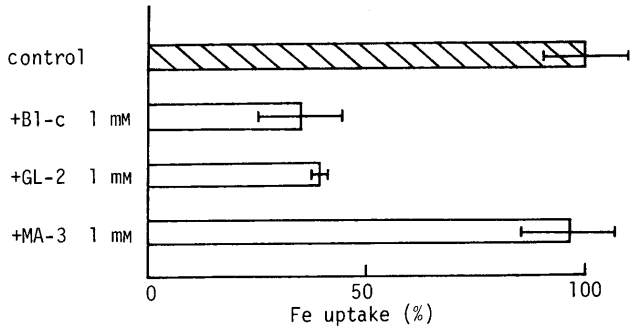

Fig. 6. Effect of Fe-Containing Fragments on Fe Uptake from Bl-c by Rat Duodenal BBM Vesicles

Initial Bl-c concentration, 11.2 μg Fe/ml (200 μM). Each column with a bar represents the mean $\pm$ S.E. ($n=3$).

sharply decreased at and over pH 10.0. On the other hand, Fe solubility in the AM-3 solutions sharply decreased over pH 7.0, as in the case of inorganic Fe(II) salts.

***In Vivo* Gastrointestinal Fe Absorption from Fe-Containing Fragments** Table II shows the percent of the ^{59}Fe dose absorbed and incorporated into hemoglobin in rats after oral administration of the ^{59}Fe–Fe-containing fragments (GL-2, MA-3, and AM-3) compared with those after administration of ^{59}Fe–Bl-c and $^{59}FeSO_4$. Both parameters were significantly higher in the Bl-c group than in the $FeSO_4$ group ($p<0.01$), as reported previously.[9] The values in the GL-2 group were slightly lower than those in the Bl-c group but were significantly higher than those in the $FeSO_4$ group. By contrast, the values in the MA-3 group were as low as those in the $FeSO_4$ group, and the values in the AM-3 group were still lower.

Effect of Fe-Containing Fragments on Fe Uptake from Bl-c by Rat Duodenal BBM Vesicles Figure 6 shows the effect of Bl-c, GL-2, and MA-3 (1 mM) on the ^{59}Fe uptake from ^{59}Fe–Bl-c (initial concentration, 11.2 μg Fe/ml (200 μM)) by rat duodenal BBM vesicles after 2 min of incubation. The addition of unlabeled Bl-c caused saturation, and the uptake rate decreased to 35% when the rate without added Fe was taken as 100%. The addition of GL-2 also showed similar inhibition (39%), while MA-3 had little inhibitory effect.

Discussion

The factors affecting gastrointestinal Fe absorption from Fe compounds are mainly considered to be (i) the existing states of the Fe compounds in the digestive tract based on their physicochemical properties and (ii) the transport systems and rates of the Fe compounds in the digestive tract. In fact, it has been reported that Bl-c produced in wine by yeast and some other Fe complexes have high Fe solubility at the physiological pH in the digestive tract[7,8,10] and that Fe is smoothly absorbed in the form of complexes with ligands from such compounds in the digestive tract.[11,16,18–21]

In the present study, structural analysis revealed that Bl-c is a complex of an Fe(II) and an oligosaccharide consisting of Ara : Xyl : Man : Glc : GalUA (1 : 1 : 1 : 5 : 1) and has a molecular weight of about 1500. This is in good agreement with the result of our previous qualitative determination that Bl-c might be an Fe(II) complex with an oligosaccharide consisting of a uronic acid and neutral sugars.[10] The Fe atom is presumed to be coordinated in close vicinity to the GalUA and Man residues, from the following present findings—(i) Fe showed the same behavior as GalUA when Bl-c was treated with glycosidases; and (ii) the results of the electrophoresis suggest the necessity of the GalUA and Man residues for maintenance of the complex form (Fig. 3). However, the hydroxyl group to which the Fe atom is actually bound remains unclear. Its identification, as well as the determination of the mode of the glycosidic linkage, needs future clarification.

When the pH dependence of the Fe solubilities of Bl-c and Fe-containing fragments obtained by its partial hydrolysis was examined, no difference was observed between Bl-c and GL-2 (a fragment in which two Glc residues are cleaved from Bl-c). Also, MA-3 with the ligand consisting of GalUA and Man showed a high Fe solubility up to pH 9. By contrast, AM-3 sharply decreased the Fe solubility at pH 7. From this result, it is inferred that a part of the ligand other than the GalUA and Man residues may also be involved in stabilizing the complex form.

The Fe absorption from the Fe-containing fragments examined *in vivo* corresponded well to the results on the Fe solubility; *i.e.*, the Fe absorption from GL-2 was almost comparable to that from Bl-c, whereas the absorption from MA-3 was lower than that from Bl-c for its high Fe solubility around the physiological pH and the absorption from AM-3 was as low as that from an inorganic Fe salt. As we suggested previously,[11,16] Bl-c may be transported across the intestinal membrane in the form of a complex with oligosaccharide by a special transport system. It is considered, therefore, that the difference in behavior in the special transport system may lead to such a wide difference in Fe absorption between Bl-c and the fragments, MA-3 and AM-3. As expected, little effect from the addition of MA-3 was observed on the ^{59}Fe uptake from ^{59}Fe–Bl-c by the BBM vesicles in contrast to the results with unlabeled Bl-c and GL-2. These findings suggest the possibility that the oligosaccharide chain may be involved in the recognition of Bl-c in its transport system.

As described above, the present results indicate that the sugar composition of the ligand of Bl-c plays an important role in the high solubility of Bl-c at the physiological pH and in its special transport process on the intestinal mucosa which are the factors specifying the Fe absorption from Bl-c. For further clarification of the roles of the sugars, it will be necessary to determine the coordination bond of the sugars and the Fe atom.

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