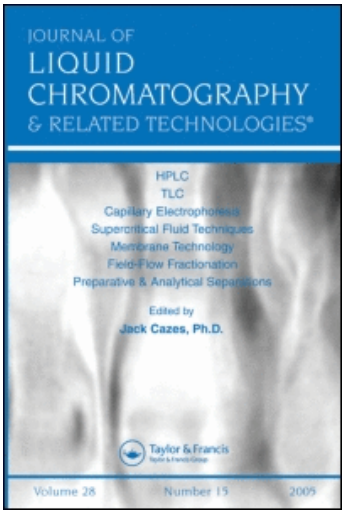

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Kumar, Krishan(1997) 'Iron Assay and Size Exclusion High Performance Liquid Chromatography of Ferritin and Magnetoferritin', Journal of Liquid Chromatography & Related Technologies, 20: 20, 3351 — 3364
To link to this Article: DOI: 10.1080/10826079708005836
URL: http://dx.doi.org/10.1080/10826079708005836

J. LIQ. CHROM. & REL. TECHNOL., 20(20), 3351–3364 (1997)

IRON ASSAY AND SIZE EXCLUSION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF FERRITIN AND MAGNETOFERRITIN

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ABSTRACT

A size-exclusion HPLC method was developed for analysis of the ferritin and magnetoferritin samples. In the ferritin sample three peaks corresponding to aggregate and monomer were observed, which are in contrast to the magnetoferritin sample in which only two peaks corresponding to aggregate and monomer were observed. A sensitive spectrophotometric method was developed for assay of iron in the ferritin and magnetoferritin samples. The method involves solubilization of iron core by HCl or acetic acid, reduction of Fe^{3+} to Fe^{2+} with sodium dithionite or sodium sulfite, and reaction of Fe^{2+} with 2, 2' bipyridyl. Increasing concentration of acetic acid or sodium sulfite increased the iron content, suggesting that higher concentration of these reagents are needed to solubilize the iron core and to reduce Fe^{3+} to Fe^{2+}, respectively. An HPLC analysis of the protein after the experiment demonstrated that the proteins do not survive the analysis conditions.

INTRODUCTION

There are several proteins which contain iron or have strong affinity towards iron, e.g. the iron and oxygen transport proteins, Transferrin and Hemoglobin, respectively, and the iron and oxygen storage proteins, Ferritin and Myoglobin, respectively.[1] Ferritin, a water soluble protein, is synthesized in almost all tissues but is most abundant in liver, spleen, and bone marrow. Ferritin consists of a spherical polypeptide shell (apoferritin with molecular weights of 21 kDa and 19 kDa, respectively. The polypeptide shell surrounds a 450 kDa molecular weight), which has two different types of subunits, heavy (H) and light (L), crystalline hydrated iron oxide/phosphate core (6 nm in size), $(FeOOH)_8 .FeOPO_3H_2$, and can accommodate up to 4500 iron atoms.[2]

Methods for modification of the protein, Ferritin, such as changing the charge, introducing reactive and biologically active groups, radioiodination, and removal of iron from the protein pocket have been published.[3-9] Mann and coworkers[10] changed ferritin to magnetoferritin by replacing the iron core. In their experiments, they[10] removed the iron oxide/phosphate core from the ferritin sample by dialysis of the sample against thioglycolic acid, added Fe(II) slowly, followed by air oxidation to a mixture of Maghemite (γ-Fe_2O_3) and Magnetite (Fe_3O_4). Magnetoferritin has superparamagnetic character, high T_2 relaxivity, which is useful in MRI as a contrast agent.[11,12]

There are numerous reports on the characterization and analysis of ferritin. The structure of horse spleen apoferritin at 2.8 Å resolution and the amino acid sequence have been determined.[13,14] It is possible to isolate different iron containing fractions from a single ferritin preparation by density gradient centrifugation[15] and to determine the average molecular iron content by spectroscopic methods.[16] High Performance Liquid Chromatography (HPLC) was used[17] to analyze subunits in human liver and spleen ferritins and HPLC coupled with ICP-MS[18] and ICP-AES[19] were used to quantitate iron and protein. The average number of iron atoms were determined by an X-radiation absorption technique.[20] However, information on characterization and analysis of magnetoferritin is lacking.

Analysis of ferritin and magnetoferritin for their iron content and their purity is essential for their evaluation and characterization. Gravimetric, solvent extraction, emission spectrography, complexometric, and redox titrations, ICP analysis, and spectrophotometric methods can be used for iron assay of these proteins.[21] Among all of these methods, the spectrophotometric method is by far easiest and sensitive, accurate and precise. In the method, iron in the 2+ or 3+ oxidation state is complexed or chelated to form a highly

colored species which is measured using its visible absorbance spectrum. In the higher oxidation state iron may be complexed with excess sodium thiocyanate. There are two potential problems in using the method to analyze magnetoferritins: (1) The method requires working at low pH due to hydrolysis of Fe^{3+} above pH 2. Under these conditions the protein may decompose into small fragments, and (2) The iron in ferritin or magnetoferritin is a mixture of 2+ and 3+ oxidation states, consequently, one has to use an oxidizing agent such as hydrogen peroxide. An excess of the oxidizing agent may also oxidize the coordinating ligand, thiocyanate, and may give erroneous results.

Iron in the lower oxidation state, Fe^{2+}, is usually determined by its reaction with 2,2' bipyridyl to form $Fe(bpy)_3^{2+}$ followed by it absorbance measurement at 522 nm. Drysdale and Munro[22] used this method to determine iron in ferritin samples. Acetic acid rather than H_2SO_4, $HClO_4$, or HNO_3 was used in the analysis to avoid acid-catalyzed cleavage of the protein as the recovery of the protein after analysis was desirable. No validation of the method or proof of recovery of the protein was given in the report.

The goals of the present work were: (a) to reexamine and develop analytical methods to determine iron content in ferritin and magnetoferritin samples, respectively, (2) to develop a size-exclusion chromatographic (HPLC) method to investigate aggregation of the protein, and (3) to investigate if the protein can cope with these analysis conditions and can be recovered after the iron assay for further use.

MATERIALS AND METHODS

Horse spleen ferritin, (lot # 33H 70302) was purchased from Sigma Chemicals (St. Louis, MO) with 12.0 and 108 mg/mL iron and protein concentrations, respectively. A sample of horse spleen ferritin (100 times diluted Sigma horse spleen ferritin which would correspond to 0.12 and 1.08 mg/mL iron and protein concentrations, respectively) was used in the present work. The magnetoferritin samples were received from Dr. S. Mann of the University of Bath (England). Sodium Chloride, Sodium Hydroxide, Sodium Dihydrogen Phosphate, Sodium Acetate, Acetic Acid, 1.0 N Hydrochloric Acid (all from Fisher), Sodium Azide, Sodium Dithionite, Sodium Sulfite (all from Aldrich), and HEPES, and 2, 2' Bipyridyl (all from Sigma) were used as is. The pH of the buffer was adjusted with Hydrochloric Acid and Sodium Hydroxide (both from Fisher) in the case of HEPES and phosphate buffers, respectively. Distilled deionized water was used for all solution preparations.

Solution pH values were measured with an Orion combination glass electrode and an Orion pH meter model 740. All spectrophotometric measurements were made with an HP 8452A diode-array spectrophotometer interfaced to an HP-310 data station. Solutions for iron content determination were heated in a Fisher heating bath.

The HPLC was a two-pump system (Rainin Instruments) with a Rheodyne injection valve containing a 50 μL loop. The HPLC system was interfaced with a Macintosh SE computer. An Applied Biosystem UV-Vis detector at 280 nm was used. Integration of peak areas was performed using Dynamax software. A silica based size-exclusion column, Bio Sil SEC-400 (300 x 7.8 mm, Bio-Rad), with a molecular weight range of 5,000 to 1,000,000, was used. A Bio Rad protein standard was used for size (molecular weight) vs. retention time calibration curve. The mobile phase condition used was: 0.15 M NaCl, 0.050 M Na_2HPO_4/ NaH_2PO_4 at pH 7.0 (containing 0.01% Sodium Azide). In some experiments HEPES buffer was used in the mobile phase. In all experiments the flow rate was 1.0 mL/min.

For iron assay, experiments were carried out using the literature method[22] (Method I) under variable conditions to validate the method (*vide infra*). The method requires the presence of 75 mM sodium sulfite, 6% acetic acid (1.04 M), and 0.05% 2,2` bipyridyl and heating at 100°C in a water bath for 1 h. The literature method was also modified to determine iron content in the protein samples (method II). In method II: 1.0 mL protein was mixed with 0.1 mL of 1.0 M HCl solution in a scintillation vial. The mixture was incubated at 80°C for 1 h and then cooled to room temperature. 0.5 mL buffered (0.5 M Sodium Acetate, pH 5.3) oxygen free $Na_2S_2O_4$ (10 mM) solution was added to the mixture followed by addition of 0.5 mL 2,2' bipyridyl (15 mM in 0.05 M HCl) solution. The mixture was heated again at 80°C for 1 h to ensure the complete formation of $Fe(bpy)_3^{2+}$. The solution was taken into a volumetric flask and the final volume was made up to 25.0 mL with water and the pH was adjusted to 4.0. The iron content was calculated from a knowledge of the absorbance at 522 nm and the molar extinction coefficient of $Fe(bpy)_3^{2+}$ ($\varepsilon = 8.65 \times 10^3\ M^{-1}\ cm^{-1}$).[23]

RESULTS AND DISCUSSION

Size-Exclusion Chromatography of Ferritin and Magnetoferritin

A Bio-Rad standard was initially injected to calibrate the peak positions as a function of size (molecular weight) of the macromolecules (Figure 1). The

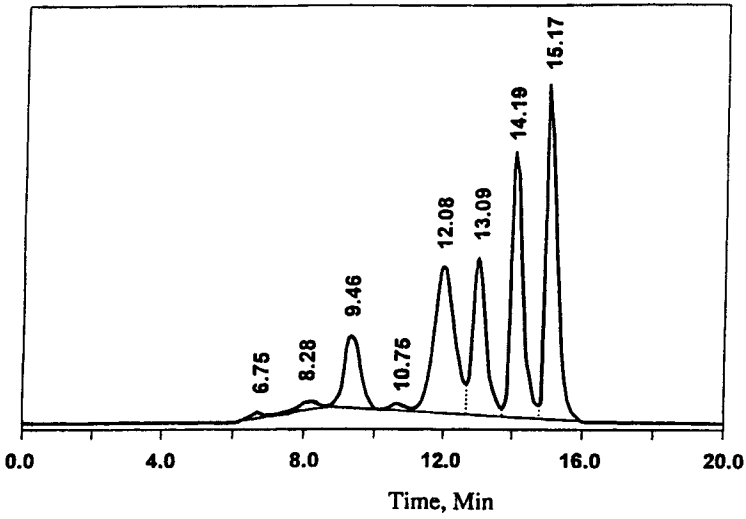

Figure 1. HPLC chromatogram of the Bio Rad standard. The HPLC conditions used are: Column: Bio-Sil SEC-400, Mobile Phase: 0.15 M NaCl, 0.050 M Na_2HPO_4/NaH_2PO_4 at pH 7.0 (containing 0.01% sodium azide), Detector: UV/Vis at 280 nm, and Flow Rate: 1.0 mL/min.

Bio Rad standard is a mixture of five proteins (Mwt in kDa and retention times in minutes are given in parenthesis) and these are: Thyroglobulin (670, 9.5), Immunoglobulin (158, 12.1), Ovalbumin (44, 13.1), Myoglobin (17, 14.2), and Vitamin B_{12} (1.35, 15.1). Smaller peaks at 6.7 and 8.25 minutes were also observed; they are due to aggregates of proteins. When a horse spleen ferritin sample was injected onto a size-exclusion column, three peaks were observed, a first peak at 8.7 min (~35%), a second peak at 9.7 min (~20%), and followed by a third peak at 11.2 min (~45%) (Figure 2). The size of ferritin or magnetoferritin is the same as the size of apoferritin. This is due to the fact that all the iron, $(FeOOH)_8.FeOPO_3H_2$ in the case of ferritin and a mixture of Fe_2O_3 and Fe_3O_4 in the case of magnetoferritin, is inside the pocket of the protein. This suggests that the peaks eluting at 8.8 and 9.8 minutes are probably due to the mixtures of aggregates which could not be separated by this column, and the last peak is a monomer of horse spleen ferritin. Similar chromatographic behavior was observed in the presence of HEPES buffer. Aggregation of ferritin is not reported in the past. The source of aggregation may be the sample. More work is in progress to resolve this. Experiments were carried out with the magnetoferritin samples also, and two peaks were observed (Figure 3). The first peak which appeared at 6.6 minutes corresponds to the aggregate and the monomer of the protein eluted at 11.2 minutes (450

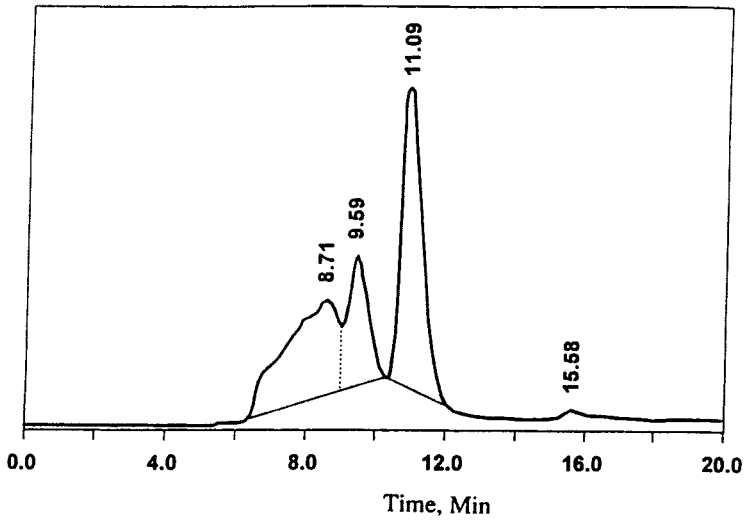

Figure 2. HPLC chromatogram of ferritin, the HPLC conditions are same as in Figure 1.

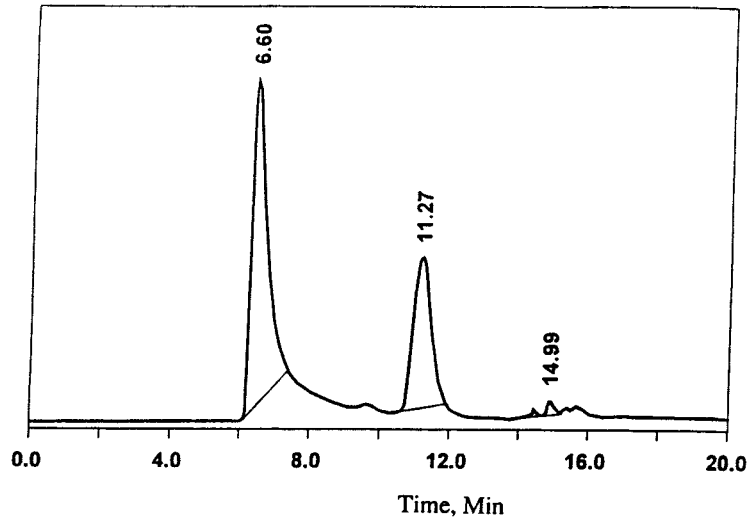

Figure 3. HPLC chromatogram of magnetoferritin, the HPLC conditions are are same as in figure 1.

kDa molecular weight). The ratio of the peak areas was 65:35. Some HPLC experiments with magnetoferritin were carried out at a lower concentration of salt or in the absence of sodium chloride in the mobile phase. The monomer peak was less retained by lowering the concentration of salt. In the absence of salt both peaks coeluted at 6.6 min. This observation is consistent with the previous results that many proteins can undergo hydrophobic interaction with the stationary phase at high ionic strength.[24,25]

Ultrasonication of magnetoferritin was attempted to disrupt the aggregation of the protein. The ratio of the aggregate and monomer remained the same, however, several minor peaks with retention times between 15 and 20 minutes were seen. This suggests that the ultrasonication breaks protein subunits into smaller molecules which have molecular weights <1 kDa as the retention time of Vitamin B_{12} (with molecular weight of 1.35 kDa) is 15.1 minutes.

Iron Assay

Initially a sample of ferritin and four samples of magnetoferritin with variable amounts of iron were analyzed by method I.[22] The pH of the mixture was measured as 3.6. The same samples were analyzed by method II and the values are compared in Table 1. From the table two observations can be made: (1) the iron content determined by method II is consistently higher than the values determined by method I and (2) the difference is lower in the case of ferritin (<5%) than magnetoferritin (11-30%).

The difference in the final pH (3.6 vs. 4.0) in the two methods was thought to be responsible for this discrepancy. The ligand protonation constant of 2,2' bipyridyl is reported as 4.47 at 25°C and $\mu = 0.1$ (KCl).[26] The stepwise stability constants (log K_1, log K_2, and log K_3) of its Fe^{2+} complexes are known to be 4.20, 3.70, and 9.55, respectively.[26] These constants were used to calculate the percentage of $Fe(bpy)_3^{2+}$ complex formed under different pH conditions.[27] Under the experimental conditions (for example: 0.019 mM Fe^{2+}, 0.30 mM bpy, pH 3.6) these calculations revealed 99.9% formation of $Fe(bpy)_3^{2+}$. In other cases the ratio of 2,2' bipyridyl to Fe^{2+} was much higher than this experiment and the percentage of $Fe(bpy)_3^{2+}$ formed should be 100%, suggesting that the pH difference can not explain the difference in iron values determined by the two methods. Moreover, additional experiments were carried out in which pH was raised from 3.6 to 4 and no change in the absorbance was observed.

Table 1

Analysis of Ferritin and Magnetoferritin Samples for the Iron Content by Method I[22] and Method II

Sample #	Method I, μg/mL	Method II, μg/mL
Ferritin	113.8 ± 0.9	119.4 ± 0.7
Magnetoferritin Sample #1	20.7 ± 1.0	26.9 ± 0.3
Magnetoferritin Sample # 2	20.9 ± 0.2	27.0 ± 0.6
Magnetoferritin Sample # 3	27.8 ± 1.0	30.8 ± 0.7
Magnetoferritin Sample # 4	25.6 ± 0.9	29.6 ± 0.9

The iron concentration by the two methods differed by only 4.6% in the case of ferritin, while the difference was as high as 30% in magnetoferritin samples (Table 1). This was thought to be due to the different iron cores in the two proteins, i.e. ferritin contains $(FeOOH)_8.FeOPO_3H_2$ whereas a mixture of Fe_2O_3 and Fe_3O_4 was introduced in the modified protein, magnetoferritin. It is possible that 6% acetic acid can not solubilize iron core in ferritin and, less so, in magnetoferritin. The iron content in ferritin and magnetoferritin samples were determined under variable acetic acid concentrations. The percentage of acetic acid was varied between 0.175 M and 2.58 M (1% to 15% volume by volume). The iron content was found to increase with the increased acetic acid concentration (Table 2, Figure 4). The increase in Fe content was 33.3% for ferritin samples and 59.3% in the magnetoferritin samples. This is probably due to the difference in the nature of iron cores in the two proteins. The iron content in the two proteins at higher acetic acid concentration agrees with the iron content determined by method II. For example, the values of iron content in the ferritin sample are: 119.4±0.7 μg/mL and 122.7±0.8 μg/mL by method II and by method I (with high concentration of acetic acid), respectively. Similarly, the iron content in the magnetoferritin sample are 30.8±0.7 μg/mL and 29.51±0.08 μg/mL by these two methods, respectively. In summary, the data given in Table 2, suggest that a higher concentration of acetic acid is required for solubilization of all iron in the protein cores rather than just 6%.

Experiments were also carried out to investigate the effect of reducing agent Na_2SO_3 on the iron concentration (Table 2, Figure 5) at 0.975 M acetic acid and 3.2 mM 2,2' bipyridyl concentration. The amount of iron increased gradually from 95.9±0.4 μg/mL to 113.8±0.9 μg/mL (15.7%) as the concentration of Na_2SO_3 increased from 10 mM to 75 mM. Under similar conditions, the iron concentration of the magnetoferritin sample increased from

Table 2

Iron Content Determination in Ferritin and Magnetoferritin Samples Under Different Experimental Conditions

S.N.	pH Range	$[CH_3CooH]^a$	$[Na_2SO_3]_b$	[Bipyridyl][b]	Ferritin		Magnetoferritin	
					Absorbance	[Fe], μg/mL[c]	Absorbance	[Fe], μg/mL[c]
1	3.12-3.19	2.58	75.0	3.20	0.380 ± 0.003	122.7 ± 0.8	0.1828 ± 0.0005	29.51 ± 0.08
2	3.28-3.40	1.72	75.0	3.20	0.371 ± 0.006	119.6 ± 1.8	0.170 ± 0.002	27.43 ± 0.24
3	3.54-3.63	0.975	75.0	3.20	0.352 ± 0.003	113.8 ± 0.9	0.164 ± 0.003	26.5 ± 0.5
4	3.69-3.79	0.65	75.0	3.20	0.347 ± 0.009	112.0 ± 2.9	0.136 ± 0.003	22.01 ± 0.52
5	3.88-4.05	0.335	75.0	3.20	0.253 ± 0.015	81.8 ± 4.8	0.106 ± 0.005	17.17 ± 0.77
6	4.20-4.26	0.175	75.0	3.20	0.222 ± 0.008	71.8 ± 2.5	0.0744 ± 0.004	12.0 ± 0.6
7	3.54-3.63	0.975	75.0	3.20	0.352 ± 0.003	113.8 ± 0.9	0.164 ± 0.003	26.5 ± 0.5
8	3.35-3.50	0.975	50.0	3.20	0.343 ± 0.005	110.6 ± 1.5	0.167 ± 0.003	27.0 ± 0.5
9	3.21-3.30	0.975	25.0	3.20	0.323 ± 0.001	104.4 ± 0.3	0.167 ± 0.002	26.9 ± 0.3
10	2.74-2.85	0.975	10.0	3.20	0.297 ± 0.001	95.9 ± 0.4	0.150 ± 0.001	24.2 ± 0.2
11	3.50-3.60	0.975	75.0	12.8	0.358 ± 0.006	115.5 ± 1.9	0.163 ± 0.003	26.3 ± 0.4
12	3.38-3.53	0.975	75.0	6.20	0.352 ± 0.001	113.5 ± 0.4	0.161 ± 0.002	26.0 ± 0.4
13	3.40-3.50	0.975	75.0	1.60	0.347 ± 0.007	112.1 ± 2.2	0.162 ± 0.002	26.1 ± 0.4
14	3.42-3.53	0.975	75.0	0.80	0.344 ± 0.005	110.9 ± 1.5	0.157 ± 0.002	25.3 ± 0.3

[a] Concentration in M
[b] Concentration in mM
[c] Calculated iron content in the stock solution, N = 4

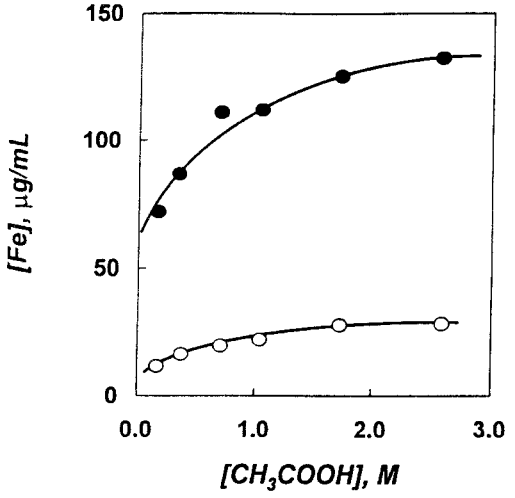

Figure 4. Plot of Fe content in the ferritin (●) and magnetoferritin (o) samples vs. [Acetic Acid].

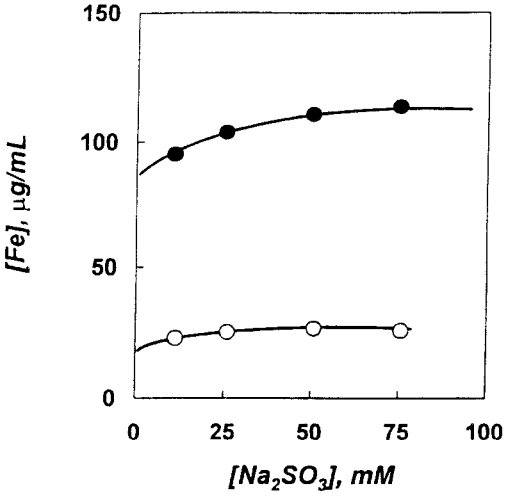

Figure 5. Plot of Fe content in the ferritin (●) and magnetoferritin (o) samples vs. [Na_2SO_3].

24.2±0.2 μg/mL to 27.0±0.5 μg/mL (10%). The plot given in Figure 5, suggests that 75 mM sodium sulfite is the optimum concentration of the reducing agent for these determinations. A higher concentration of sodium sulfite may be needed for samples containing higher concentrations of iron in the protein samples.

Three equivalents of 2,2' bipyridyl are needed for the formation of $Fe(bpy)_3^{2+}$. In the present work, the concentration of 2,2' bipyridyl was varied from 0.8 mM to 12.8 mM, which translates into the bipyridyl to iron ratio of 4 to 64 for ferritin and 16 to 256 for magnetoferritin. The iron concentration was found to be constant in the 2,2' bipyridyl concentration range studied, suggesting that >4 equivalents of bipyridyl concentration is sufficient to determine iron in these samples. Similar results were obtained from the species distribution calculations under these concentration conditions.[27]

The samples of proteins were heated at 70-80°C for iron concentration determination. The determination was also performed at room temperature. Upon addition of Na_2SO_3, 2, 2' bipyridyl, and acetic acid, a pink/red color developed slowly. The completion of color development took 10 h for ferritin and several days for magnetoferritin analysis at room temperature. The rate of the reaction of Fe^{2+} with 2,2' bipyridyl was measured by Wilkins and coworkers[28] in the pH range of 6.0 to 6.8. They calculated a second-order rate constant for the reaction of Fe^{2+} with 2,2' bipyridyl as 1.1×10^5 $M^{-1}s^{-1}$. From these data one can calculate a half-life of the reaction of the order of milliseconds. However, in the present work, acidic conditions were used where the majority of the bipyridyl is in the less reactive protonated form. The ligand protonation constant of 2,2' bipyridyl is 4.47. Krumholz[29] reported an initial rate of reaction of Fe^{2+} (1.37×10^{-5} M) with $H(bpy)^+$ (1.66×10^{-4} M) at $[H^+]$ = 1.60×10^{-3} M (-log $[H^+]$ = 2.8) as 1.85×10^{-6} M min^{-1}. A half-life of the reaction under our experimental conditions can be calculated to be less than 1 h at room temperature. Contrary to this, a longer reaction time at room temperature was observed in the present work, which could be attributed to the slow solubilization of iron core followed by its reaction with 2,2' bipyridyl under acetic acid conditions.

Recovery of the Protein

We used size-exclusion HPLC analysis of the mixture of the protein, 2,2' bipyridyl, sodium sulfite, and variable concentrations of acetic acid. In all of the cases, two broad peaks with retention times of 20 and 33-34 minutes, were observed. The first peak was identified due to $Fe(bpy)_3^{2+}$, as confirmed by an injection of preformed $Fe(bpy)_3^{2+}$. The second peak, which was observed even

at a lowest acetic acid concentration (0.175 M), was assigned to the fragments of the protein. No peak at 11 minutes was observed, suggesting that the proteins can not survive these analysis conditions.

CONCLUSION

A size-exclusion HPLC analysis of ferritin and magnetoferritin demonstrated the formation of aggregates of ferritin and magnetoferritin. Higher concentration of acetic acid is required for determination of iron in the two protein samples. The proteins can not be recovered after iron assay.

ACKNOWLEDGMENTS

The author gratefully acknowledges Dr. S. Mann (the University of Bath, England) and Protein Magnetics (CA) for supplying the magnetoferritin samples and, thanks Dr. A. Nunn for his interest and encouragement to carry out these studies. Suggestions and comments from Drs. R. B. Frankel (California Polytechnic State University), S. Mann (the University of Bath, England), and A. Nunn (Bracco Research USA, Inc.) are highly appreciated by the author.

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Received February 12, 1997
Accepted March 14, 1997
Manuscript 4366