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A New Method for Assay of Ferroxidase Activity and Its Application to Human and Rabbit Sera

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A simple, sensitive and precise method for serum ferroxidase assay was developed by determining Fe(III) produced enzymatically from Fe(II) added as a substrate in an acetate buffer (pH 6.0). The Fe(III) formed was determined by flow injection analysis (FIA) using thiocyanate as a detection reagent.

The reproducibility of this method throughout all steps was satisfactory (coefficient of variation, C.V. = 3.4%), and the normal value of human serum ferroxidase activity was 0.30 ± 0.02 μmol Fe(III)/min/ml (mean ± standard deviation).

This method was applied to study the behavior of human and rabbit serum ferroxidases on Sephadex G-150 column chromatography.

Keywords——ferroxidase assay; flow injection analysis; human and rabbit sera; ceruloplasmin; thiocyanate; oxidation of Fe(II) to Fe(III)

Ferroxidase (EC 1.16.3.1, ceruloplasmin), which catalyzes the oxidation of Fe(II) to Fe(III), is present in human blood and plays a significant role in the metabolism of iron, promoting the transfer of Fe(II) from storage cells to plasma transferrin.[1–4] The enzyme activity arises mainly from ceruloplasmin (a copper-containing protein) and Osaki *et al.* have termed the name "ferroxidase" instead of ceruloplasmin to indicate its enzymatic function.[2]

Generally, patients with Wilson's disease have low serum ceruloplasmin levels,[5,6] as copper-deficient pigs do.[7,8] However, abnormality is not observed in the metabolism of iron in Wilson's disease patients and the sera have more ferroxidase activity than might be expected.[9,10] Several workers proposed the presence of serum components having ferroxidase activity other than ferroxidase (ceruloplasmin).[10–12] One of such components is citrate, proposed by Lee *et al.* as a source of Fe(II) oxidizing activity in low-ceruloplasmin serum,[11,13] and another is ferroxidase II isolated from the Cohn IV-1 fraction of human serum by Topham and Frieden.[14] They suggested that this enzyme may be responsible for iron metabolism in Wilson's disease patients. Later, Sexton *et al.* demonstrated that ferroxidase II is not an enzyme but is a lipohydroperoxide produced during the isolation.[15] Thus, the mechanism of iron metabolism is still not completely elucidated.

We have been interested in the clinical significance of ferroxidase and ferroxidase-like substances in human serum. The assay of serum ferroxidase activity is currently carried out by the method of Johnson *et al.*[9] This method is based on the principle that the time course of absorbance change at 460 nm reflects the formation of transferrin from apotransferrin and Fe(III). However, the method is tedious and time-consuming, and turbidity of the serum interferes with the determination. We have now established a simple and precise assay method for serum ferroxidase by high performance liquid chromatography (HPLC).[16] However, this method still has the disadvantage of being time-consuming.

In this paper, we describe a simple, sensitive and precise method for the determination of

Fe(III) in the presence of Fe(II) by flow injection analysis (FIA). The FIA method is based on the formation of Fe(III)–thiocyanate complex (λ_{max} 480 nm) in the continuos flow system, and it was applied to the assay of ferroxidase activity in serum. The method was also applied to the study of serum components other than ceruloplasmin having ferroxidase-like activity in human and rabbit sera.

Materials and Methods

Materials——Ferric ion standard solution: Ferric ammonium sulfate ($Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$) was dissolved in 0.5 M $HClO_4$. Ferrous ion solution (substrate): Ferrous ammonium sulfate ($FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$) was dissolved in deoxygenated distilled water just before use. Fresh serum sample: Whole blood from donors was collected and allowed to clot. The serum was separated by centrifugation and stored at 4 °C.

Instruments——FIA system comprised a PSU-2.5 pump, a VMU-6 sample injector (Seishin Co., Ltd., Tokyo), an UVILOG-7 variable wavelength detector (Oyobunko Co., Ltd., Tokyo) and an SS-250F recorder (Sekonic Co., Ltd., Tokyo). PTFE joints and tubings were obtained commercially. Atomic absorptin analysis (AAS) was done with a Shimadzu AA-650 spectrometer fitted with a GFA-2 graphite furnace atomizer (Shimadzu Seisakusho Co., Ltd., Kyoto). Absorbance and fluorescence were measured with a Hitachi 181 UV-VIS spectrophotometer and a Hitachi MPF-4 fluorescence spectrophotometer (Hitachi Co., Ltd., Tokyo), respectively.

Ferroxidase Assay——A mixture of 0.2 ml of 0.3 M acetate buffer (pH 6.0) and 0.1 ml of serum diluted 20- to 50-fold with 0.15 M NaCl was preincubated for 5 min at 30±0.1 °C in a micro test tube (1.5 ml). Then 0.01 ml of 10 mM ferrous ammonium sulfate solution was added and mixed well. The whole was incubated for 5 min at 30±0.1 °C, and the reaction was stopped by adding 0.2 ml of 1.25 M perchloric acid. After centrifugation (10000 rpm, 3 min), the formed Fe(III) in the supernatant was analyzed by FIA at the sampling rate of 120 samples/h.

Ceruloplasmin Assay——Serum ceruloplasmin was measured as *p*-phenylenediamine (PPD) oxidase activity.[17]

Lipohydroperoxide Assay——Lipohydroperoxide was measured by the fluorometric method of Yagi[18] using thiobarbituric acid (TBA).

Serum Copper Assay——Serum copper was analyzed by the flameless atomic absorption technique of Imanari *et al.*[19]

Results

FIA System

Figure 1 shows a schematic diagram of the FIA procedure for the determination of Fe(III). Pump-1 (P-1) was used to deliver sample carrier (0.5 M perchloric acid aqueous solution) at a flow rate of 1.5 ml/min. The sampling valve (S) had two loops of PTFE with volumes of 60 and 140 μl. A stream of aqueous 0.5 M sodium thiocyanate solution was delivered by pump-2 (P-2), and the flow rate was adjusted to 1.5 ml/min. The corresponding sample zone and the reagent stream were mixed at point J. The resultant solution was delivered to a reaction coil (R.C., PTFE tube, 140 cm × 0.5 i.d.), and was then monitored at 480 nm (D). The Fe(III) concentration was determined by the peak height method. Under these conditions, 120 samples/h could be analyzed.

The working curves for Fe(III) at the sample sizes of 60 and 140 μl were linear over the range of 1 to 100 μM ($Y=0.67X$, $r=0.99$, 60 μl loop; $Y=0.45X$, $r=0.99$, 140 μl loop). The repeatability of this method was satisfactory (coefficient of variation, C.V. = 0.8% for 10 μM Fe(III), $n=10$). The same working curve was also obtained in the presence of 140 μM Fe(II).

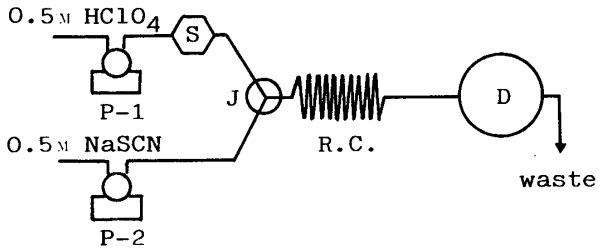

Fig. 1. Flow Diagram of FIA

P-1 and P-2, pumps; S, sampling valve; J, mixing joint; R.C., reaction coil; D, detector.

TABLE I. Recovery of Fe(III) from Serum on Perchloric Acid Deproteinization

Amount added (μM)	Recovery,[a] %
	Mean ± S.D.
90	101.8 ± 4.0
180	101.2 ± 3.0

a) Mean of 6 analyses.

TABLE II. Ferroxidase Activity Found in 10 Duplicate Analyses of Normal Human Sera

Subject	Ferroxidase activity[a] μmol Fe(III)/min/ml	
1	0.27	0.25
2	0.31	0.30
3	0.29	0.29
4	0.27	0.28
5	0.30	0.30
6	0.32	0.33
7	0.32	0.31
8	0.28	0.29
9	0.29	0.29
10	0.30	0.31
Mean ± S.D.	0.30 ± 0.02	

a) Serum volume used was 2 μl.

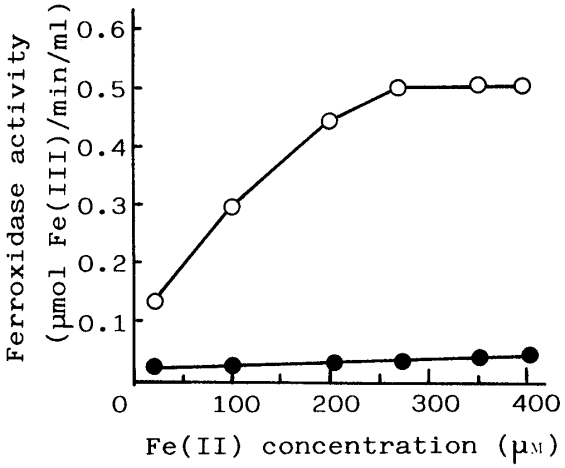

Fig. 2. Effect of Substrate (Fe(II)) Concentration on the Assay of Ferroxidase Activity after Incubation for 5 min with 2 μl of Human Serum (○—○) and without Serum (●—●)

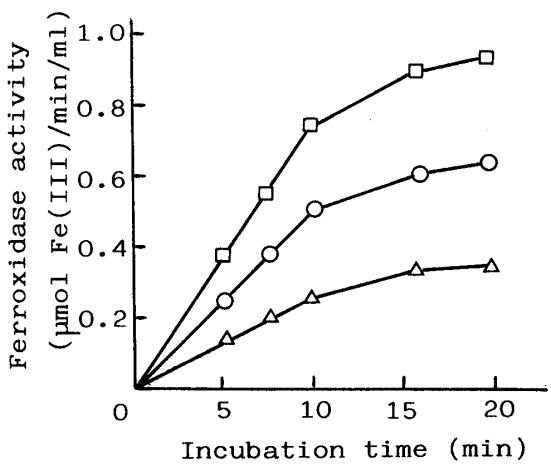

Fig. 3. Effect of Incubation Time on the Assay of Ferroxidase Activity with Saturating Substrate Concentration (333 mM Fe(II)) Using 1 (△—△), 2 (○—○) and 3 (□—□) μl of Human Serum

Serum Ferroxidase Assay by FIA

In order to apply the FIA to the assay of serum ferroxidase activity, the optimum conditions of substrate concentration, reaction time, serum volume and deproteinization were

investigated. The buffer and incubation temperature were set according to the method of Osaki *et al.*[9]

The effect of deproteinizing agent was tested by using perchloric acid. To 10 μl of human serum, 90 μl of 0.3 mM or 0.6 mM Fe(III) (90 μM or 180 μM in final concentration) and 200 μl of acetate buffer (0.3 M, pH 6.0) were added. The mixture was allowed to stand for 10 min at 30 ± 0.1 °C, then 200 μl of 1.25 M perchloric acid was added. The mixture was centrifuged and 60 μl of the supernatant was subjected to FIA. The results showed that Fe(III) was completely recovered (Table I). The effects of substrate concentration, incubation time and serum volume were tested using 2 μl of human serum. A saturating substrate concentration was obtained above 300 μM Fe(II), as shown in Fig. 2. Therefore, the substrate concentration was fixed at 333 μM Fe(II) in the final mixture, because the response for the substrate blank due to spontaneous oxidation of Fe(II) increased with increasing substrate concentration. The ferroxidase activity increased linearly in proportion to the concentration of serum up to at least 3 μl, and to an incubation time up to 10 min (Fig. 3).

The values of ferroxidase activity in normal human sera obtained by this method are presented in Table II.

Ferroxidase-like Active Substances (Non-ceruloplasmin Ferroxidase) in Human and Rabbit Sera

In order to search for a ferroxidase other than ceruloplasmin-ferroxidase, the fractionation of human and rabbit sera was attempted by gel-filtration chromatography. Fresh human and rabbit sera were chromatographed on a Sephadex G-150 column and eluted with 0.01 M Tris-HCl buffer containing 0.15 M NaCl (pH 7.8). The elution profiles of ferroxidase activity and protein concentration in each fraction (2.2 ml) are illustrated in Fig. 4. The PPD oxidase activity in each fraction is also illustrated. Ferroxidase activity in both sera, however,

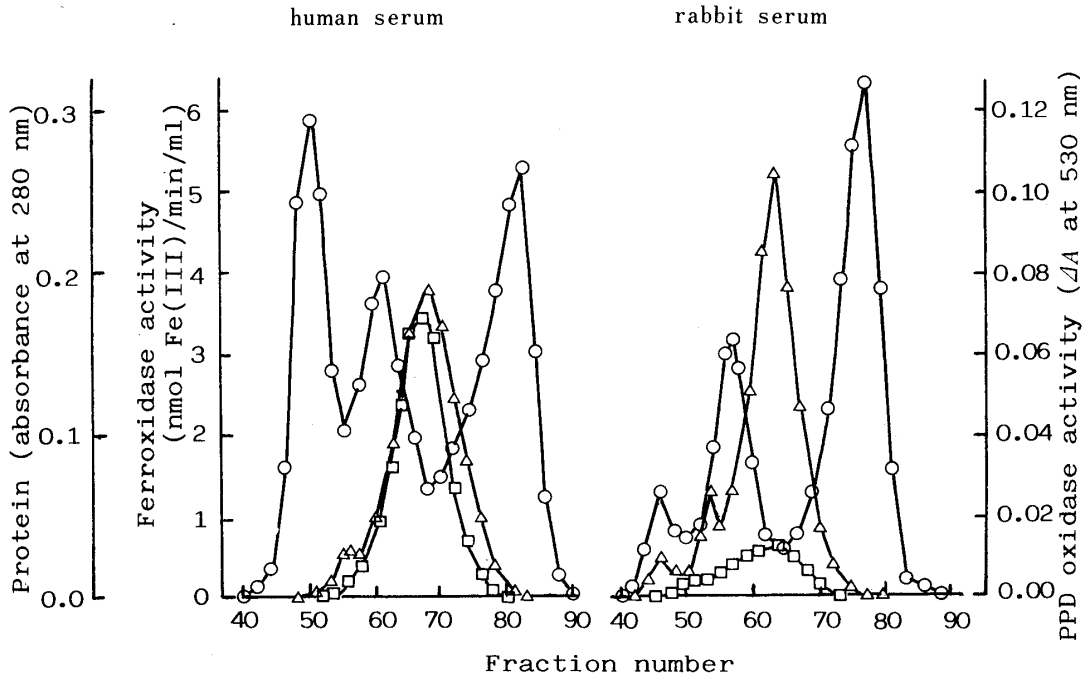

Fig. 4. Elution Profiles of Protein (○—○), Ferroxidase Activity (△—△) and PPD Oxidase Activity (□—□) of Human and Rabbit Sera in Gel-Filtration Chromatography

Chromatography was carried out on an 87 cm × 2.2 cm i.d. glass column packed with Sephadex G-150 using 0.01 M Tris-HCl–0.15 M NaCl buffer (pH 7.8, flow rate at 9 ml/min) as the eluent. The effluent was collected in 2.2 ml fractions.

TABLE III. Correlations among Ferroxidase Activity, PPD Oxidase Activity for Ceruloplasmin and Copper Concentration in Human and Rabbit Sera

	Human ($n=7$)		Rabbit ($n=7$)	
	Equation	r	Equation	r
Fox(Y)[a]–Cu(X)[b]	$Y=0.16X+0.14$	0.956	$Y=0.62X+0.02$	0.997
Cp(Y)[c]–Cu(X)	$Y=0.15X+0.07$	0.976	$Y=0.17X-0.00$	0.994
Fox(Y)–Cp(X)	$Y=0.96X+0.09$	0.919	$Y=3.62X+0.02$	0.989

a) Ferroxidase activity (Fox).
b) Copper concentration (Cu).
c) PPD oxidase activity for ceruloplasmin (Cp).

was observed almost entirely in the ceruloplasmin fractions. No difference was observed in the elution profiles of ferroxidase activity and PPD oxidase activity between human and rabbit sera.

Correlations Among Ferroxidase Activity, PPD Oxidase Activity and Serum Copper Concentration in Human and Rabbit Sera

The correlations among the concentration of serum copper, ceruloplasmin (as PPD oxidase activity) and the ferroxidase activity in normal human and rabbit sera were investigated (Table III).

The ferroxidase activity measured by this method was highly correlated with the concentration of copper and the PPD oxidase activity in the sera of human and rabbit. A good correlation between the PPD oxidase activity and the concentration of copper was also obtained.

Discussion

The present method is basically similar to the method of Osaki *et al.*[9] with regard to measuring Fe(III) produced enzymatically, but our method has several advantages compared with the method of Osaki *et al.*, as follows. (i) Apotransferrin was replaced with thiocyanate as the detection reagent for Fe(III). Thiocyanate is more sensitive and cheaper than apotransferrin. Consequently, it became feasible to use FIA to determine Fe(III) rapidly in a large number of serum samples (120 samples/h), whereas the apotransferrin method is relatively tedious and time-consuming. (ii) Since 0.5 M perchloric acid was used in the FIA system as a carrier solution, it was possible to prevent the spontaneous oxidation of Fe(II). Perchloric acid is especially suitable as a medium for the formation of Fe(III)–thiocyanate complex because of its lower side reaction coefficient value for Fe(III)[20] and also because of its convenience as a deproteinizing agent as well as a stopping reagent for enzyme reactions. (iii) One to 3 μl serum sample and 5 min incubation time were appropriate, since the present method was carried out under conditions of saturating substrate concentration (Fig. 2). The reproducibility throughout all steps was satisfactory (C.V. = 3.4%, $n=6$). Various inorganic anions and tervalent metal cations have been reported to inhibit ceruloplasmin.[21,22] The possibility of interference in the present method was checked by adding 8 metal cations (Cu^{2+}, Zn^{2+}, Cr^{3+}, Ni^{2+}, Cd^{2+}, Pb^{2+}, Ca^{2+} and Mg^{2+}) and 10 inorganic anions (Cl^-, SCN^-, N_3^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, Co_3^{2-}, ClO_4^-, NO_2^- and $S_2O_3^{2-}$) to serum. The cations did not interfere, except for Cu^{2+} and Cr^{3+} which slightly oxidized ferrous iron (about 4%) at final concentration of 3 and 100 μM, respectively. Strong inhibition was observed with azide and thiocyanate, which at 1 and 10 mM concentrations gave 95 and 55% inhibitions, respectively.

These inorganic anions including cyanide are known to be copper-enzyme inhibitors.[23] Nitrite, which oxidizes Fe(II), interfered with the formation of Fe(III)–thiocyanate complex.[24] Other inorganic anions showed less than 5% inhibition at 10 mM final concentration, and these results were the same as those found for ceruloplasmin.[21]

The mean ferroxidase activity of normal human sera was 0.30 μmol Fe(III)/min/ml (Table II). The standard deviation of differences between duplicate analyses was ± 0.008 μmol Fe(III)/min/ml. The C.V. of duplicate analyses performed within a single run was 1.7%. Thus, this analytical system is effective for the determination of ferroxidase activity in serum.

We also investigated the presence of ferroxidase other than ceruloplasmin-ferroxidase in human and rabbit sera by Sephadex G-150 chromatography. In our experiments, most of the ferroxidase activity was observed at the ceruloplasmin fractions obtained from fresh human and rabbit sera (Fig. 4), but at least 5% of the ferroxidase activity was not inhibited by 1 mM azide as also found for purified ceruloplasmin.[15] Thus, we could clearly detect the presence of some other ferroxidase activity.

A minor peak having very low ferroxidase activity was observed in a higher molecular fraction than ceruloplasmin (Fig. 4). This activity may arise from citrate contained in the β-lipoprotein fractions as reported by Lee *et al.*[13]

When the ferroxidase activity of these fractionated sample was stored at 4—6 °C for 72 h after being separated on the Sephadex G-150 column, and then assayed again, a new peak having strong ferroxidase activity appeared at the fractions just after the void volume. These fractions gave a positive TBA test.[18] This result suggests that the new peak contains lipohydroperoxide.[15] However, this phenomenon did not occur in serum which was diluted with saline and stored at 4—6 °C. This result suggests the presence of some kinds of substances inhibiting lipohydroperoxide formation.

The correlation between the concentrations of copper and ceruloplasmin in rat serum and between the PPD oxidase and ferroxidase activities in human serum have already been investigated by Sunderman and Nomoto[17] and Osaki *et al.*,[2] respectively, and they found good correlations. Our results also showed that ferroxidase activity was well correlated with the concentrations of copper ($r=0.956$ and 0.977) and ceruloplasmin ($r=0.919$ and 0.989). Our FIA method gave a better correlation ($r=0.919$) between the PPD and ferroxidase activities in human serum than was obtained by the apotransferrin method.[4] The slopes of the ferroxidase–copper and ferroxidase–ceruloplasmin plots for human serum were different from those of rabbit serum. Thus, there may be a species difference in the structure and properties of ceruloplasmin.

Conclusion

We developed a simple, accurate and sensitive method for the assay of ferroxidase activity in serum. Since the ferroxidase activity was highly correlated with the PPD oxidase activity in the same species, the present method is also effective for the determination of ceruloplasmin. It is superior to the PPD oxidase activity method and the transferrin formation method, in terms of the reproducibility, linearity, simplicity and cost. In addition, this procedure should be applicable to lipohydroperoxide determination in serum with some modifications.

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