〈Original Article〉

Study of a novel method for measuring serum diamine oxidase activity

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Summary We inferred from previous reports that the reaction of *o*-tolidine reacting protein (OTRP) is diamine oxidase-like activity using *o*-tolidine and SAT-3 as substrates. The main protein with diamine oxidase activity is ceruloplasmin. The *p*-phenylenediamine (PPD) method measures ceruloplasmin using diamine oxidase activity. The PPD oxidase activity of ceruloplasmin in serum is known to be responsible for 90-95% of that measured. We compared OTRP activity measured by the SAT-3 method with the ceruloplasmin concentration measured by the PPD method. Furthermore, OTRP activity was compared with the ceruloplasmin concentration measured by ELISA. As a result, a significant positive correlation ($r = 0.8544$, $P < 0.0001$) was found between the SAT-3 method and PPD method. ELISA showed a significant positive correlation with the SAT-3 method ($r = 0.6217$, $P < 0.0001$) or the PPD method ($r = 0.5020$, $P < 0.0001$). However, these correlation coefficients were not stronger than the relation between the SAT-3 method and PPD method. In conclusion, these results suggest that OTRP activity mainly measures diamine oxidase activity in serum.

Key words: SAT-3, *o*-tolidine, Ceruloplasmin, *p*-phenylenediamine

1. Introduction

We previously reported that *o*-tolidine reacting protein (OTRP) is an independent marker of free-radical injury[1]. It has been shown to belong to a protein of the globulin fraction by ammonium sulfate precipitation and specifically reacts with o-tolidine in 0.2 M citrate buffer (pH 5.0-5.2)[2-4]. We also improved the measurement method of OTRP by utilizing N,N'-bis (2-hydroxy-3-sulfopropyl)tolidine (SAT-3) instead of *o*-tolidine[5]. However, the reactive mechanism of OTRP and SAT-3 is not clearly defined. Mizoguchi et al. reported that colored matter having an absorption maximum at 674 nm was generated after SAT-3 was oxidized in 1M sodium acetate buffer (pH 5.2)[6-8]. This report was almost consistent with our result that the reaction of OTRP and SAT-3 in 0.2 M citrate buffer (pH 5.2) resulted in an increase in absorbance at about 660 nm[5]. Therefore, it was thought that this reaction is diamine oxidase activity because

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o-tolidine and SAT-3 have the structure of diamine. The main protein that has diamine oxidase activity in blood is ceruloplasmin[9], which accounts for 90-95% of diamine oxidase activity in serum[10]. In view of the above, we inferred that OTRP activity may represent the diamine oxidase activity of ceruloplasmin. The *p*-phenylenediamine (PPD) method, which was established by Sunderman and Nomoto, is known as a measurement method of ceruloplasmin using diamine oxidase activity[11]. In the present study, to clarify the relationship between OTRP and ceruloplasmin, we compared OTRP activity measured by the SAT-3 method with the concentration of ceruloplasmin measured by the PPD method. In addition, the measurement results of the two methods were also compared with the concentration of ceruloplasmin measured by ELISA.

2. Materials and methods

1. Blood sample

Two hundred samples of serum without hemolysis, jaundice and chyle were obtained from the clinical laboratory of Fujita Health University Hospital. These samples were remained after the medical check-up of hospital staff, and serum was pooled from these sera. Written informed consent was obtained from our hospital staff.

2. Reagents and Materials

All reagents used were of analytical reagent grade. SAT-3 was provided by Dojindo Laboratories (Kumamoto, Japan). The Pro-bindTM 96-Well Plate from Becton, Dickinson and Company (Franklin Lakes, NJ) was used as the microplate. Rabbit anti-human ceruloplasmin polyclonal antibody was purchased from Nordic Immunological Laboratories (Eindhoven, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-human ceruloplasmin antibody was purchased from EY Laboratories, Inc. (San Mateo, CA). Human ceruloplasmin was purchased from Cosmo Bio Co., Ltd (Tokyo, Japan). The other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Katayama Chemical Industries Co., Ltd. (Osaka, Japan).

3. Procedure of OTRP activity measurement

SAT-3 (6.62 mM) was dissolved in 0.2 M citrate buffer solution (pH 5.0) at 85℃, and the SAT-3 reagent was adjusted to pH 5.0 again. OTRP activity was measured using the following steps: (a) Three test tubes, labeled SR (sample reaction), RB (reagent blank) and SB (sample blank), were prepared. SR and RB received 2.0 mL SAT-3 reagent, and SB receives 2.0 mL of 0.2 M citrate buffer solution. (b) Then, 0.05 mL serum was added to tubes SR and SB, and 0.05 mL distilled water was added to tube RB. (c) These tubes were placed in a water bath at 37℃ to induce a linear reaction. (d) After 15 minutes, the absorbance of SR and SB was measured for 15 min at 660 nm using a Hitachi 7010 Clinical Spectrophotometer (Tokyo, Japan) against the RB. (e) One unit of OTRP activity was defined as a 0.001 increase in absorbance. OTRP activity was calculated by subtracting the absorbance of the SB from one of the SR.

4. Analysis of absorbance spectrum of oxidized SAT-3

The absorbance spectrum of oxidized SAT-3 was examined using sodium hypochlorite of different concentrations (500-1000 ppm). The absorbance spectrum of SAT-3 in the presence of the pooled serum was also measured.

5. Measurement of PPD oxidase activity

The PPD oxidase activity of ceruloplasmin in serum was measured by the PPD method according to Sunderman and Nomoto[11]. Ceruloplasmin catalyzes the oxidation of PPD to yield a colored product in 0.1 M acetate buffer solution (pH 5.4). The absorbance of the colored oxidation product is measured at 530 nm. The formation rate of the colored oxidation product is proportional to the concentration of serum ceruloplasmin if a correction is made for the non-enzymatic oxidation of PPD as the blank.

6. Measurement of ceruroplasmin concentration using the ELISA method

The microplate was coated with rabbit anti-human ceruloplasmin polyclonal antibody of the capture antibody diluted in 0.1 M carbonate buffer (pH 9.5),

and the coated microplate was incubated overnight at 4℃. The microplate was washed with phosphate-buffered saline (PBS) with 0.05% Tween-20 (pH 7.4). The remaining protein-binding sites in the coated microplate wells were blocked by adding PBS with 5% non-fat dry milk as the blocking buffer. These samples and human ceruloplasmin as the standard were diluted with the blocking buffer as necessary. The samples and standards were run in duplicate on each plate to ensure accuracy. HRP conjugated goat anti-human ceruloplasmin antibody was used as a detection antibody. The substrate solution was mixed with 5.0 mL substrate buffer (0.1 M citrate buffer, pH 5.0), 2 μL hydrogen peroxide (30%) and one OPD tablet. The reaction was carried out to avoid exposing the microplate to light. After incubation at room temperature for 30 minutes, the reaction was stopped by 2N H_2SO_4. The absorbance of each well at 492 nm (dominant wavelength) and 620 nm (reference wavelength) was determined in a Multiscan Bichomatic (Helsinki, Finland). The absorbance was calculated by subtracting the absorbance of the reference wavelength from the dominant wavelength, and then the ceruloplasmin concentration was evaluated from the obtained standard curve.

7. Statistical analyses

Statistical calculations for this study were carried out using software package "Prism 4" purchased from GraphPad Software, Inc. (San Diego, CA).

3. Results

1. Absorbance spectrum of oxidized SAT-3

Figure 1 presents the absorbance spectra of SAT-3 in the presence of increasing concentrations of sodium hypochlorite having an oxidizing effect (500 to 1000 ppm). When sodium hypochlorite reacted with SAT-3, the absorbance spectrum exhibited two peaks at approximately 400 nm and 675 nm. Next, Fig. 2 shows the absorbance spectrum of SAT-3 in the presence of the pooled serum. Both of the absorbance spectra were very similar.

2. Relationship between the SAT-3 method and two measurement methods of ceruloplasmin

Serum OTRP activities in 200 healthy persons were measured by the SAT-3 method. The ceruloplasmin concentrations in their sera were measured by the PPD method and ELISA. A significant positive correlation ($r = 0.8544$, $P < 0.0001$, $n = 200$) was found between the SAT-3 method and PPD method, and the regression equation is Y (PPD method) = 0.2958 X (SAT-3 method) − 1.5947 (Fig. 3). The

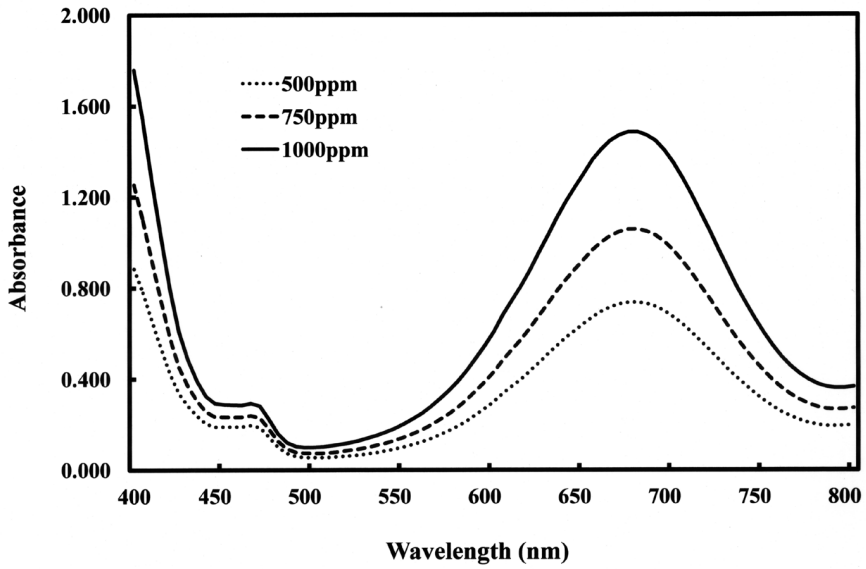

Fig. 1 Absorbance spectrum of SAT-3 in the presence of sodium hypochlorite.

relationship between the SAT-3 method and ELISA showed a significant positive correlation (r = 0.6217, P <0.0001, n = 200), and the regression equation is Y (ELISA method) = 0.2384 X (SAT-3 method) − 5.6511 (Fig. 4). In addition, the relationship between the PPD method and ELISA showed a significant positive correlation (r = 0.5020, P <0.0001, n = 200), and the regression equation is Y (ELISA method) = 0.5560 X (PPD method) − 4.9381 (Fig. 5).

4. Discussion

We previously reported that this protein had clinical significance in several diseases[3]. OTRP exists in the globulin fraction and it reacts with *o*-tolidine and SAT-3 specifically at around pH 5.0[1-4], but its nature is still unclear. There was an increase of absorbance between 600 and 700 nm when *o*-tolidine and SAT-3 with the structure of diamine were oxidized[6-8]. This absorbance spectrum was very similar

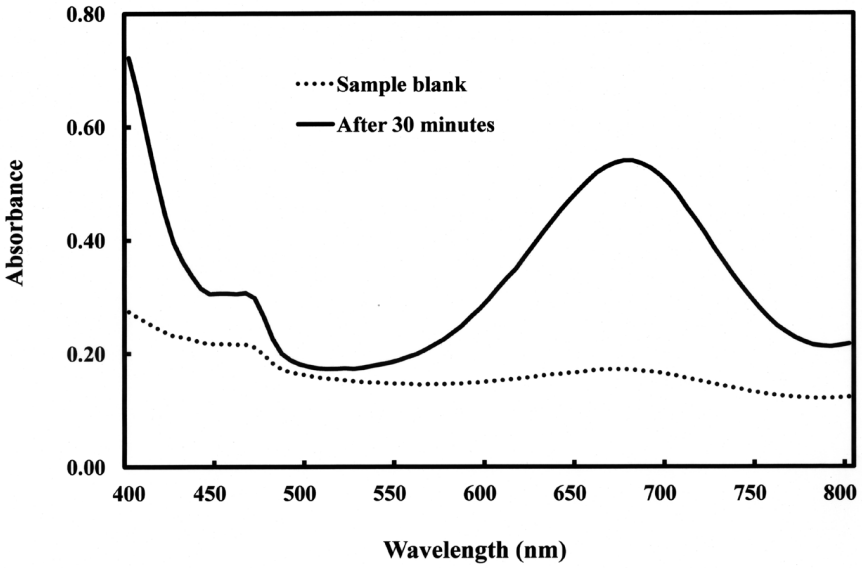

Fig. 2 Absorbance spectrum of SAT-3 in the presence of pooled serum.

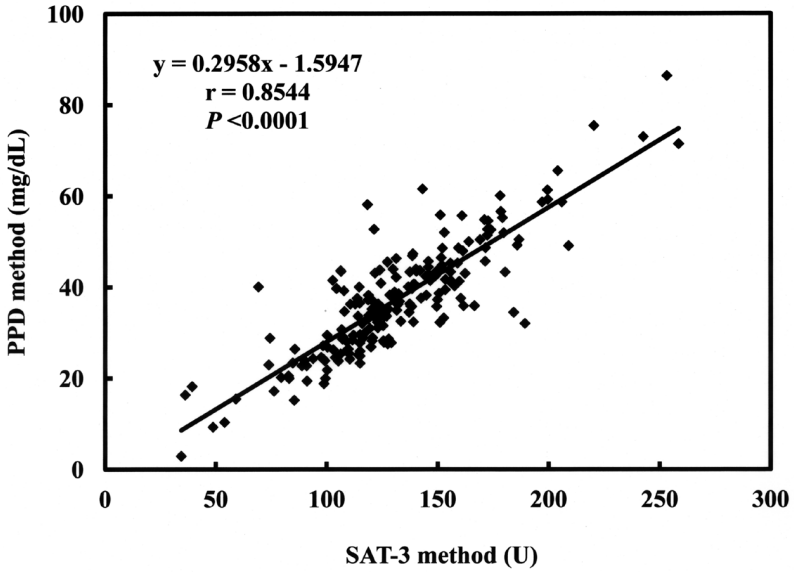

Fig. 3 Correlation between SAT-3 method and PPD method.

to that in Fig. 2. Based on this result, we thought that STA-3 was oxidized by the action of diamine oxidase in serum. Ceruloplasmin is known as the main protein with diamine oxidase activity in blood[11]. Therefore, we inferred that OTRP activity also measures the diamine oxidase activity of ceruloplasmin. Next, we compared OTRP activity with the ceruloplasmin concentration measured by the PPD method. Furthermore, OTRP activity was also compared with the ceruloplasmin concentration measured by ELISA. A significant positive correlation (r = 0.8544) was found between the SAT-3 method and PPD method. This result suggests that OTRP activity mainly measured diamine oxidase activity. Although there was a significant correlation between ELISA and the two oxidative methods (SAT-3 method: r = 0.6217, PPD method: r = 0.5020), these correlation coefficients were not stronger than the relationship between the SAT-3 method and the PPD method. This result was considered as follows. The presence of copper is essential for

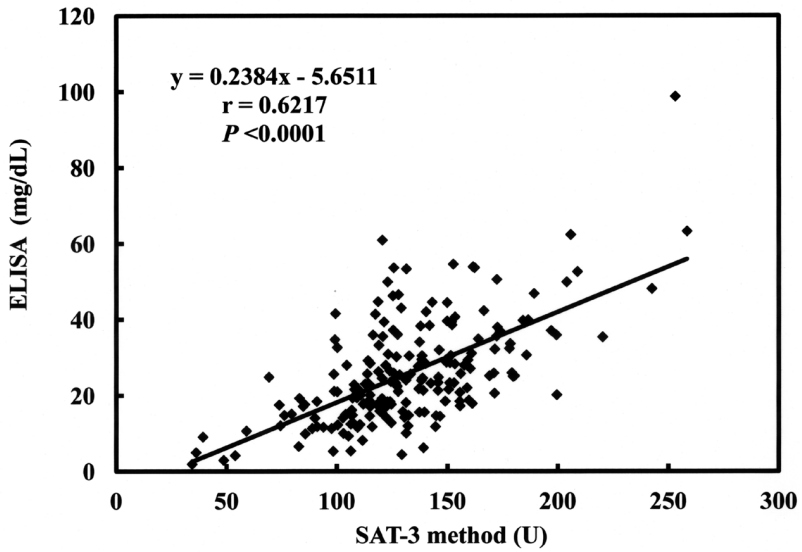

Fig. 4 Correlation between SAT-3 method and ELISA.

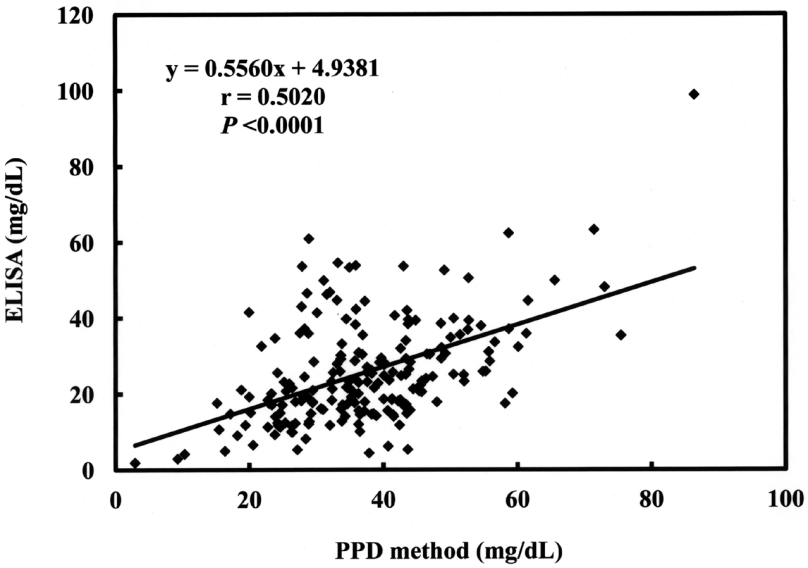

Fig. 5 Correlation between PPD method and ELISA.

the oxidase activity of ceruloplasmin in serum. If copper is released from ceruloplasmin, the oxidase activity of ceruloplasmin is inactivated easily[12]. Ceruloplasmin which has lost copper cannot be measured by an oxidation method such as the SAT-3 method and PPD method. Moreover, ceruloplasmin is known to fragment easily by various radicals[13]. Even if ceruloplasmin is fragmented more than 90%, the oxidase activity of ceruloplasmin has been reported to remain about 30%. Ceruloplasmin which has fragmented highly cannot be measured by ELISA; however, it is possible that the SAT-3 method can measure the oxidase activity of fragmented ceruloplasmin. From these reports, the correlations of the two oxidase methods and ELISA were considered to become worse. The screening test of the ceruloplasmin concentration in serum is useful to diagnose diseases such as Wilson's disease and Menkes syndrome[10]. A decrease in serum ceruloplasmin is usually observed in patients with Wilson's disease[11]. However, some patients with Wilson's disease have serum ceruloplasmin protein that has immunological antigenic properties, but it is functionally inactive[14]. Although the immunochemical analysis of ceruloplasmin can measure its total concentration, an enzymatic activity method can measure only the activity type. The SAT-3 method may be effective in screening Wilson's disease patients with a normal amount of ceruloplasmin[11, 15]. In conclusion, we developed a novel assay of diamine oxidase activity. These results suggest that OTRP activity measured the diamine oxidase activity of ceruloplasmin and its fragments.

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