Standardization of Serum Ceruloplasmin Concentrations in International Enzyme Units with o-Dianisidine Dihydrochloride as Substrate

H. Peter Lehmann, Karl H. Schosinsky, and Myrton F. Beeler

We describe a method for calculating the absorptivity (in terms of substrate consumed) of the colored solution obtained when o-dianisidine dihydrochloride is oxidized by ceruloplasmin. By oxidizing o-dianisidine dihydrochloride with known amounts of hydrogen peroxide we could determine that the molar reacting ratio of o-dianisidine to hydrogen peroxide is 2:1. So calculated, absorptivity is 9.6 ml μmol−1 cm−1, the figure used to estimate ceruloplasmin oxidase activity in terms of International Units.

Additional Keyphrases: enzyme activity • normal values

The most widely used method for determining serum ceruloplasmin concentrations have been based on immunological techniques (1) and on the enzymatic diame oxidase activity of the protein with N,N-dimethyl-p-phenylenediamine (2) or p-phenylenediamine (3, 4) as substrates. The units in which ceruloplasmin activity has been reported have varied from milligram per deciliter of serum, when serum oxidation is compared to that obtained from standard ceruloplasmin solutions (5), to arbitrary units that are based either on the increase in absorbance of the solutions per unit time under the reaction conditions (3) or on a comparison of the absorbance of the sample with that of artificial reference standards (3).

The International Union of Biochemistry has recommended that the unit of enzymatic activity be the International Unit, defined as “The amount of enzyme which catalyses the transformation of one micromole of substrate per minute under defined conditions including optimal pH and substrate concentrations” (6). The current recommended temperature is 30 °C.

Ceruloplasmin enzymatic activity has been reported in International Units, with p-phenylenediamine as substrate, based on the assumption that the oxidation product is “Bandrowski’s base,” a trimer of p-phenylenediamine (7). Other studies indicate that the colored product is a free radical semiquinone, “Wuester’s red,” a dimer of the substrate (8).

We recently reported a method for measuring ceruloplasmin oxidase activity by use of o-dianisidine dihydrochloride (hereafter referred to as “o-dianisidine”) as substrate (9). The method is demonstrably more sensitive and less susceptible to nonenzymatic oxidation of substrate than is the case for procedures in which p-phenylenediamine or its N-dimethyl derivative are used. The enzymatic oxidation of o-dianisidine results in formation of a yellow-brown reaction product which, on acidification, is converted to a stable purple-red compound that absorbs maximally at 540 nm (10). Although it is thought to be a dimer, the identity of the final product is not exactly known.

In this communication we report results of experiments in which the absorbance of the final colored solution is related to the amount of o-dianisidine consumed, a procedure that allows measurements for serum ceruloplasmin activity to be expressed in International Units. In some other procedures (11-13) oxidation of o-dianisidine is used as the final step for quantitating such serum constituents as glucose, haptoglobin, and uric acid. Because, in these cases, results are compared with results of simultaneously assayed standards, knowledge of the composition of the final colored product is not necessary. Quantitation of the reported method for ceruloplasmin based on standard ceruloplasmin solutions was not considered because of the expense and variable purity of commercially available ceruloplasmin, and because of the recommendation for reporting serum enzymes in International Units, i.e., in terms of activity.
Procedure

The procedure for measuring serum ceruloplasmin oxidase activity with o-dianisidine as substrate has been described (9). Briefly, it is as follows: Two tubes, each containing 0.7 ml of acetate buffer (pH 5, ionic strength 0.1) and 0.05 ml of serum are incubated at 30 °C, and 0.2 ml of o-dianisidine reagent (250 mg/dl) is added to each tube. The reaction is stopped after 5 and 15 min by adding 2 ml of sulfuric acid (9 mol/liter). The absorbance of the final red solution is measured at 540 nm in a cuvet having a 1-cm light path. The blank is de-ionized water.

o-Dianisidine was oxidized by adding 0.05 ml of hydrogen peroxide [standardized by iodometric titration (14)] and 0.01 mg of peroxidase (Sigma Chemical Co., Type II, RZ 1.0 to 1.5) to 0.75 ml of acetate buffer (pH 5) and 0.2 ml of o-dianisidine reagent. The solutions were then incubated at 30 °C until the reactions were complete (2 min). Two milliliters of 9 mol/liter sulfuric acid was then added, yielding colored solutions whose absorbances were measured at 540 nm in a 1-cm cuvet vs. de-ionized water blanks.

Results and Discussion

In other reported procedures in which oxidation of o-dianisidine has been used as the quantitative measurable step (11–13) hydrogen peroxide is formed in the initial reaction. This is then coupled, in the presence of peroxidase, to the oxidation of o-dianisidine, giving a colored product. We have found that the absorption spectrum of the final colored solution obtained when o-dianisidine is oxidized by ceruloplasmin in pooled pregnancy serum under the described reaction conditions is identical to that obtained when hydrogen peroxide, in the presence of peroxidase, is the oxidant (Figure 1). We therefore concluded that the oxidation product of o-dianisidine is the same whether ceruloplasmin or hydrogen peroxide-peroxidase is the oxidizing agent, a necessary condition for basing measurement of enzyme activity on calculation of substrate consumed by the method used.

We determined the molar relationship between hydrogen peroxide and o-dianisidine in the reaction by measuring the absorbance of the acidified oxidation product in a number of solutions. Figure 2 illustrates the effect on absorbance of the final product of varying the amount of hydrogen peroxide acting on a fixed quantity, 0.315 µmol, of o-dianisidine in the reaction mixture. This amount of o-dianisidine was selected to give absorbance measurements in the range of 0.000 to 1.000. The curve in Figure 2 shows the increasing absorbance with hydrogen peroxide concentration to a maximum corresponding to a molar ratio (in the reaction mixture) for o-dianisidine to hydrogen peroxide of 0.315:0.16, or 2:1. If more hydrogen peroxide is present, the absorbance at 540 nm decreases, the color of the solutions becoming blue-violet. The absorption spectra of some of the colored solutions obtained in these experiments (Figure 3) show a shift in the absorbance maximum toward the red as hydrogen peroxide concentrations are increased.

The results plotted in Figure 4 show that when a fixed amount of hydrogen peroxide was incubated with different amounts of o-dianisidine there was an increase in absorbance to a limiting value, at which
point no further change in absorbance was observed to the highest o-dianisidine concentration measured. The curves in Figure 4, which correspond to 0.117 and 0.147 μmol of hydrogen peroxide in the reaction mixture, show that the lowest amounts of o-dianisidine that yield the maximum amount of product are 0.221 and 0.284 μmol, respectively. In both cases the molar ratio of o-dianisidine to hydrogen peroxide, beyond which no more product formed, was 2:1. The color of the final solution corresponding to the ascending portion of the curves in Figure 4 was similar to colors corresponding to the descending portion of the plot in Figure 2. The absorption spectrum of a solution on the plateau region of Figure 4 was identical with curve 2 of Figure 3.

Comparison of Figures 2 and 4 indicates that in the presence of excess hydrogen peroxide, the oxidation product is structurally modified. If excess o-dianisidine is present, however, no change in the final product is observed. In both cases the limiting point is reached when the molar ratio o-dianisidine to hydrogen peroxide is 2:1, suggesting 2:1 stoichiometry for the reaction of this sort. The final purple-red color could therefore be that of an acidified form of the dimer of o-dianisidine, in agreement with conclusions of a previous study on oxidation of o-dianisidine by hydrogen peroxide (15). However, we were unable to reproduce the earlier work on the absorption spectrum of the acidified oxidation product obtained from 0.25 μmol of o-dianisidine and 0.5 μmol of hydrogen peroxide. Also, when we calculate an extinction coefficient for the final product from the data in Table 1, assuming it to be a dimer of o-dianisidine, we obtain a value of $1.92 \times 10^4 \text{ liter mol}^{-1} \text{ cm}^{-1}$, as compared to $4.57 \times 10^4 \text{ liter mol}^{-1} \text{ cm}^{-1}$ calculated by Moller and Ottolenghi (15) for the same product.

Because of these discrepancies we calculated absorptivity in terms of o-dianisidine oxidized rather than in terms of dimer formed. The data presented in Table 1 are for experiments carried out to relate the absorbance of the final colored solution to the amount of substrate consumed. In all cases, the amounts of hydrogen peroxide shown were incubated for 10 min at 30 °C, to ensure complete reaction, in solutions containing 0.01 mg of peroxidase and 1.58 μmol of o-dianisidine in acetate buffer at pH 5. The amounts of o-dianisidine consumed were determined on the basis of the molar reaction ratio of 2:1 previously described. The absorptivities were then calculated from the measured absorbance of a 3-ml solution (the final volume) by the expression:

$$\text{absorptivity (ml μmol}^{-1} \text{ cm}^{-1}) = \frac{\text{absorbance} \times 3}{(o-dianisidine) \times 1} \quad (1)$$

where $3 = \text{volume-correction factor; (o-dianisidine)} = \text{amount of o-dianisidine oxidized (μmol/3 ml)}$; and $1 = \text{optical path length (cm)}$.

A mean value for the absorptivity of 9.60 ml μmol\(^{-1}\) cm\(^{-1}\) was calculated and used in conversions of the measured absorbance into serum enzyme activity in terms of International Units as shown:

$$U/\text{ml} = \frac{o-dianisidine \text{ oxidized (μmol ml}^{-1} \text{ min}^{-1})}{\text{absorbance} \times 60/\text{absorptivity} \times 10 \times 1}$$

where absorbance = experimental measurement, absorptivity = 9.60 ml μmol\(^{-1}\) cm\(^{-1}\), 60 = volume correction factor (0.05 ml of serum is incubated in a 1 ml reaction mixture, which is diluted to 3 ml with 9 ml/liter sulfuric acid for color development and absorption measurements), $1 = \text{optical path length (cm)}$, and 10 = incubation time (min).

Substituting the values in the above expression and converting the final volume to 1 liter gives the expression

enzyme activity = \frac{\text{absorbance} \times 6.25 \times 10^2 \text{ U/liter}}{\text{absorbance} \times 6.25 \times 10^2 \text{ U/liter}},

which was used to calculate ceruloplasmin activity as measured by the method reported here.
The method described in the Procedure section to measure serum ceruloplasmin concentrations makes use of a two-point test because of a lag phase of 1 to 2 min found for many sera. The enzymatic activity of all sera is therefore calculated from the formula

\[
\text{enzyme activity} = (A_{15} - A_5) \times 6.25 \times 10^2 \text{ U/liter},
\]

where \(A_5\) and \(A_{15}\) are the measured absorbances at 5 and 15 min, respectively.

With this standardization technique, we have established a normal range for serum ceruloplasmin activity of 62–140 U/liter from measurements on 78 clinically healthy young men.

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References

1. Markowitz, H., Gubler, C. J., Mahoney, J. P., et al., Studies on copper metabolism. XIV. Copper, ceruloplasmin and oxidase activity in sera of normal human subjects, pregnant women, and patients with infection, hepatolenticular degeneration and the nephrotic syndrome. J. Clin. Invest. 34, 1496 (1955). (This is representative of many reports in which this technique is used for serum ceruloplasmin assay.)


10. Color determined by comparison with ISCC-NBS Centroid Color Charts.


