Consideration of the Optimum pH for the Analysis of Serum *p*-Phenylenediamine Oxidase Activity in Thoroughbred Horses

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ABSTRACT. The optimum pH for the measurement of serum *p*-phenylenediamine oxidase (Ox) activity was given (pH 6.6), and the relationship between serum Ceruloplasmin(Cp) concentration and its Ox activity was established in healthy adult horses. In adult horses, serum antigenic Cp concentrations were measured by the single radial immunodiffusion (SRID) method with the affinity-purified antibody to equine plasma Cp and compared with its Ox activity. Efficient co-relation between Cp concentration and Ox activity in the sera ($r=0.93$) and its Ox/Cp ratio were given. These results might contribute to the calculation of antigenic Cp concentration from its Ox activity, the analysis of holo-Cp content in serum, and the research for copper metabolism in Thoroughbred horses. — **KEY WORDS:** ceruloplasmin, equine, oxidase.

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Deficiency and excess of trace minerals, such as copper, zinc, selenium, and cobalt, are emphasized the importance for maturity of musculoskeletal system in growing animals, and are thought to cause the insufficiency of economic performance of livestock, especially cattle, porcine and avian. In equine medicine, deficiency of trace minerals is thought to be one of major causes of developmental orthopedic diseases [13]. Particularly, copper, one of these minerals, was pointed up as a cause of osteochondrosis and other developmental disorders in horses [4–6, 13].

Ceruloplasmin, which was named by Holmberg and Laurell in 1948 [10], is a glycoprotein containing blue-colored copper in mammalian blood plasma. Ceruloplasmin is one of major plasma proteins, but its properties easily change. For this difficult nature, there have been many reports on the methods for purification and characterization [7, 9, 14, 16, 17] in some kinds of animals [7, 12, 15, 20]. However, only few reports were found to isolate and characterize equine Cp [16, 18, 20].

Ceruloplasmin is a marker for copper metabolism [10], and used for the evaluation of copper deficiency [11]. Usually, serum Cp concentration is indirectly evaluated by measurement of *p*-phenylenediamine Ox activity. However, the discrepancies between the units of *p*-phenylenediamine Ox per unit of Cp were found, and thought to represent a difference of the pH of the condition buffer used, which were pH 5.2 to 6.7 [6]. In addition, the optimum pH for the measurement of *p*-phenylenediamine Ox was varied in some kinds of animals, including human (pH 5.90), rat (pH 6.20), fowl (pH 6.30), rabbit (pH 6.40), bovine (pH 6.43), guinea pig (pH 6.47), and donkey (pH 6.60) [2], and that in Thoroughbred horses is unknown.

In the present study, the optimum pH for the analysis of equine plasma Cp oxidase activity was considered using *p*-phenylenediamine Ox in serum from adult Thoroughbred horses, and the ratio of its Ox activity to antigenic Cp concentration measured directly by the immunological method, was estimated.

Serum Ox activity to *p*-phenylenediamine was determined by the method reported by Bingley and Dick [3], and the

Table 1. Serum oxidase activity (A_{530}) at each pH (pH 5.0 to 7.5) of 0.1 M acetate buffer

pH	A_{530}
5.0	0.284 ± 0.057
5.5	0.408 ± 0.094
6.0	0.507 ± 0.117
6.2	0.561 ± 0.116
6.4	0.737 ± 0.165
6.6	0.757 ± 0.186
6.8	0.690 ± 0.120
7.0	0.606 ± 0.109
7.5	0.544 ± 0.073

The values of A_{530} indicated mean $\pm$ S.D.

absorption (530 nm; A_{530}) of the mixture was given as its Ox activity. The optimum pH of 0.1 M acetate buffer for this assay was investigated at serial range from pH 5.0 to 7.5 using acetate buffer (Table. 1 & Fig. 1).

Equine plasma Cp was isolated by ammonium-precipitation, DE-52 anionexchange chromatography and EAH-Sepharose 4B chromatography [18]. Antibody to equine Cp was purified by equine Cp binding sepharose 4B affinity chromatography from the antiserum to equine plasma Cp raised in rabbits. The SRID method [18], using purified rabbit antibody to equine Cp mentioned above, was used to measure serum Cp concentration. Protein concentration was determined by measuring at the A_{280} of equine Cp [16].

In serum samples of clinically healthy adult horses (thoroughbred, $n=25$; 8.5 ± 4.6 years old), Cp concentration and its Ox activity were given. There was a high correlation between the serum Cp concentration and its Ox activity in these horses (correlation coefficient=0.93) and the following formula was calculated (Fig. 2).

$$A_{530} = 0.019 \times \text{serum Cp (mg/dl)} - 0.22$$

The Ox activity is known to be affected by the pH of the

Fig. 1. pH-*p*-phenylenediamine oxidase activity plots for adult Thoroughbred horses. Approximately pH 6.6 of the buffer was the optimum condition for the reaction of serum oxidase activity to *p*-phenylenediamine.

condition buffer [3]. The pH-*p*-phenylenediamine Ox activity plots for Thoroughbred horses were given (Fig. 1). The peak value of the A_{530} was reached at the pH 6.6, so this pH was considered as the optimum pH for the measurement of the Ox activity.

Serum Cp concentration was measured directly by the immunological assay described [18] with some modification. In the SRID method, purified antibody to equine Cp purified by equine Cp binding sepharose 4B affinity chromatography was employed, and a more reliable assay system was established. The rang of the SRID was extended (1.6 to 100 mg/dl) compared with previous report [18]. A higher degree of correlation ($r=0.928$) was still found between Cp concentration and oxidase activity of sera from clinically healthy adult horses in this study (Fig. 2).

Serum Cp concentration in human plasma can be indirectly calculated by the data on serum Ox activity. However, the Ox activities of certain amounts of Cp have variation in species and age [2, 18, 21]. Therefore, the relationship between Cp concentration and its Ox activity in adult equine sera was explored. The value of Ox activity/Cp in adult thoroughbred horses was similar to that in human beings [17].

The ratio of Ox/Cp in adult Thoroughbred horses, which was given in this study, would contribute to the calculation of equine Cp concentration from its Ox activity. This ratio might be used in adult horses only, because this value in newborn foals was indicated to be less than that of adult horses [2, 18]. Further study is required to be confirm the ratio of Ox/Cp in younger horses.

The ratio of the Ox activity to antigenic Cp concentration may indicate the relationship between holo- and apo-Cps. The Ox activity in serum samples represents the concentration of copper binding Cp (holo-Cp) [1], and Cp concentration measured by immunological method in this study indicate both holo-Cp and copper-free Cp (apo-Cp). Therefore, this ratio would be of value to analyze holo-Cp content. Serum Ox activity in newborn foals was reported

Serum Ceruloplasmin concentration

Fig. 2. Correlation between serum ceruloplasmin and oxidase activity in adult Thoroughbred horse sera. r: correlation coefficient.

to be one-fifth that in adult horses [2]. This may indicate that the ratio of holo- and apo-Cp in newborn foals might be different from that in adult horses, or that the ability to bind to copper would be immature in newborn foals. This difference of the Ox/Cp ratio between newborn foals and adults might have some relationship to orthopedic abnormalities in foals induced by copper-deficiency [4, 8].

In conclusion, the optimum pH for the measurement of *p*-phenylenediamine Ox activity in equine serum was given, and the relationship between antigenic Cp concentration and its Ox activity was established in sera from adult thoroughbred horses. The optimum pH of the condition buffer for the reaction of *p*-phenylenediamine with serum Ox originating from serum ceruloplasmin was 6.6. In adult horses, serum Cp concentrations were measured by single radial immunodiffusion method with the affinity-purified antibody to equine plasma Cp and compared with its Ox activity. Efficient co-relation was given between Cp concentration and Ox activity in the sera ($r=0.928$). These results might contribute to the calculation of antigenic Cp concentration from its Ox activity, the analysis of holo-Cp content in serum, and the research for copper metabolism in Thoroughbred horses.

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