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FORMS OF CERULOPLASMIN IN DEVELOPING PIGLETS*

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SUMMARY

Ceruloplasmin was purified and resolved into two forms, ceruloplasmin I and ceruloplasmin II, according to the order of elution by chromatography on DEAE-cellulose, hydroxylapatite and Sephadex G-150 from pigs ranging in age from 2 days to 10 weeks. The sera of the 2-day-old pigs contained only one form of ceruloplasmin, ceruloplasmin II. As the pigs grew older and the overall level of ceruloplasmin increased, the amount of ceruloplasmin II remained relatively constant, while the level of ceruloplasmin I increased dramatically. The two forms of ceruloplasmin differed in their specific enzymatic activity towards *p*-phenylenediamine and in their copper content, with ceruloplasmin I being the greater in both cases.

INTRODUCTION

In a previous communication from this laboratory[1], it was reported that the piglet is born with a low level of serum ceruloplasmin activity which increases to about one-third of the adult level during the first week and levels off at this point unless dietary copper is furnished. In the latter case the ceruloplasmin activity rises to adult level in about 2 weeks.

Since the piglets were born with rather high levels of liver copper, the changes observed in ceruloplasmin activity in the presence and absence of dietary copper prompted further investigation of the ceruloplasmin of the growing piglet. In the course of these studies, it was noticed that the ceruloplasmin from newborn and 3-day-old pigs eluted differently from hydroxyapatite and had a lower specific enzymatic activity than ceruloplasmin from older pigs.

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MATERIALS AND METHODS

Separation and purification of two forms of ceruloplasmin

Ceruloplasmin from pigs ranging in age from 2 days to 10 weeks was purified by modification of procedures described by DEUTSCH[2] and BROMAN AND KJELLIN[3] as follows: Pig serum was dialyzed against 0.05 M acetate buffer (pH 5.5) for at least 4 h. The dialyzed serum was then centrifuged to remove the precipitate and placed on a DEAE-cellulose column which had been equilibrated with the acetate buffer. The column was washed with the acetate buffer until the effluent was nearly protein-free. The ceruloplasmin-rich fraction was eluted with 0.5 M NaCl. This fraction appeared bright blue from older pigs and yellowish from younger pigs (0–3 days). The ceruloplasmin-rich fraction was then dialyzed against two changes of 0.075 M phosphate buffer (pH 6.8) and placed on a hydroxyapatite column equilibrated with the same buffer. Elution was carried out with a phosphate concentration gradient of 0.075–0.4 M (pH 6.8). In this step, the ceruloplasmin may be resolved into two components called ceruloplasmin I and ceruloplasmin II according to the order of elution. The fractions representing the different ceruloplasmin peaks were pooled, respectively and concentrated to about 5 ml. The ceruloplasmin was then passed through a Sephadex G-150 column in the final step of purification.

Protein was determined by the method of LOWRY *et al.*[4]. Copper was analyzed by the method described by MATRONE *et al.*[5]. Enzymatic activity towards *p*-phenylenediamine was determined by the procedure of HOUCHIN[6], as modified by RICE[7]. Spectra were run in a model SP800B Unicam Spectrophotometer.

Kinetic studies

The reactivities of the two forms of ceruloplasmin were compared over a range of 0.1–2 mM *p*-phenylenediamine in 1.0 M acetate buffer (pH 5.5) at 37°. The change in absorbance was followed at 540 mμ on a Unicam recording spectrophotometer. Since the formation of colored product(s) was nonlinear during the first few minutes with respect to time, the reaction velocity was determined after about 2–3 min during the period when ΔA was linear with time. This nonlinearity of *p*-phenylenediamine oxidation has been observed by others[8].

RESULTS

A summary of the purification of ceruloplasmin from serum of a 10-week-old pig is shown in Table I. There was an overall recovery of 20% of the activity as measured in the 100 ml of pig serum, the source of the ceruloplasmin. The greatest loss (approx. 42%) occurred in the first purification step (DEAE-cellulose). Of the 17.9 mg of ceruloplasmin recovered after the final purification step from 100 ml of serum, 87.3% of the protein and 91.3% of the activity resided in the ceruloplasmin I fraction, whereas 12.7% of the protein and 8.7% of the activity resided in the ceruloplasmin II fraction. After the hydroxyapatite and after the Sephadex purification steps, the ratios of total activity of the ceruloplasmin I/ceruloplasmin II fractions were approximately the same (10 to 1). On the per mg basis, the final ceruloplasmin II preparation showed 92 times more activity than the serum and 1.5 times the activity of the ceruloplasmin II fraction.

TABLE I

PURIFICATION OF CERULOPLASMIN FROM 10-WEEK-OLD PIG SERUM

Serum taken from single animal.

Purification stage	*Vol. (ml)*	*Protein (mg/ml)*	*Total protein (mg)*	*p-Phenylene-diamine oxidase (ΔA/0.1 ml)*	*Specific activity (ΔA/15 min per mg protein)*
1. Serum	100	80.00	8000.00	0.149	0.0186
2. DEAE-cellulose	27	23.875	644.63	0.315	0.132
3. Hydroxyapatite					
Fraction I	65	0.870	56.55	0.065	0.747
Fraction II	26	0.793	20.62	0.015	0.189
4. Sephadex G-150					
Fraction I	40	0.391	15.625	0.0685	1.723
Fraction II	30	0.076	2.275	0.009	1.184

Utilizing this purification procedure, the sera of 2-day, 9-day, and 10-week-old pigs were fractionated. The results, ceruloplasmin activity *vs.* fraction number, are shown in Figure 1. Primarily ceruloplasmin II was recovered from the serum of the 2-day-old pig, whereas approximately 68% of the ceruloplasmin activity was in the ceruloplasmin I fraction and 32% in the ceruloplasmin II fraction of the ceruloplasmin

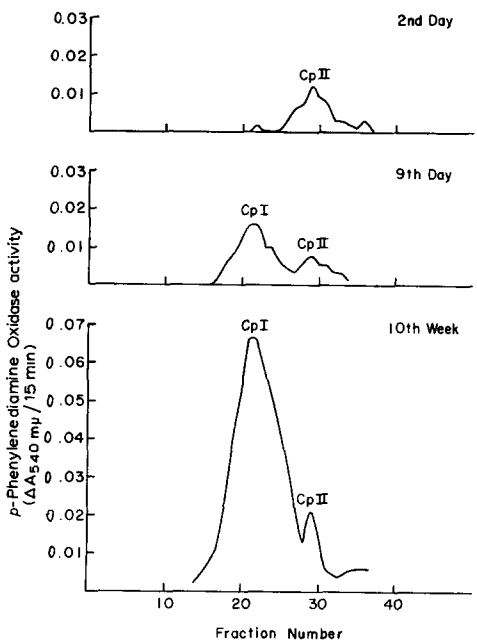

Fig. 1. Effect of age on elution of ceruloplasmin (Cp) from hydroxyapatite. Ceruloplasmin-rich fraction from DEAE-cellulose step, representing 10 ml of serum, was eluted from a hydroxyapatite column (1.3 cm × 9.0 cm) with a linear concentration gradient of 0.075–0.4 M phosphate of pH 6.8 (100 ml + 100 ml). 3-ml fractions were collected.

TABLE II

SUMMARY OF SPECIFIC ACTIVITIES AND RECOVERIES OF CERULOPLASMIN FROM DIFFERENT-AGED PIGS

Number of animals are given in parentheses. n.s., not significant.

Age	*Recovery (mg/100 ml serum)*		*Specific activity (ΔA/15 min per mg protein)*	
	I	*II*	*I*	*II*
2 days (3)	n.s.	3.49		0.644
2 days (9)	n.s.	3.037		0.485
4 days (3)	2.875	2.75	1.174	0.727
10 weeks (1)	15.625	2.275	1.723	1.184
10 weeks (1)	20.45	2.28	1.498	1.253

recovered from the serum of the 9-day-old pig, and finally for the serum of the 10-week-old pig, approx. 94% of the activity resided in the ceruloplasmin I fraction and 6% in the ceruloplasmin II fraction. In other words, there was a positive correlation between the appearance and quantity of ceruloplasmin I and the age of the pig. The increase in ceruloplasmin I is also correlated with the increase in total ceruloplasmin activity of pig serum with age. At birth, piglet serum has low ceruloplasmin activity, approx. 1/20 that of the adult. When pigs are about 3–4 days old, the level of ceruloplasmin activity increases very rapidly until it reaches adult levels in 2–3 weeks[1]. This period is accompanied by a large increase in ceruloplasmin I, while there is relatively little change in ceruloplasmin II. Ceruloplasmin I is the predominant form found in older animals.

Table II shows recoveries and specific activities of the two types of ceruloplasmin from different-aged pigs. Differences were found between the two forms of ceruloplasmin, particularly with respect to the specific enzymatic activities. The specific activities of ceruloplasmin II were lower than those of ceruloplasmin I. The specific

Fig. 2. Polyacrylamide gel electrophoresis of ceruloplasmin I and ceruloplasmin II for 45 min in Tris–barbiturate buffer (pH 8.0) by method of WILLIAMS AND REISFELD[15]. Direction of migration was toward the anode. Protein was stained with Coomassie blue, as described by CHRAMBACH *et al.*[16]. 0.033 mg placed on gel for (CpI) and 0.029 mg for ceruloplasmin II (CpII).

activity of ceruloplasmin II of the 10-week-old pig appeared to be higher than the ceruloplasmin II of the younger pigs. However, this could be due to some contamination by ceruloplasmin I, since it was difficult to adequately resolve the two types when there was a high level of ceruloplasmin in the serum.

Preparations of ceruloplasmin I from 10-week-old pigs and ceruloplasmin II from 2-day-old pigs were further purified by rechromatography on Sephadex G-150 and concentrated prior to analysis by ultrafiltration on a Diaflo membrane*. The electrophoretic mobilities of the two forms of ceruloplasmin appeared to be similar at pH 8.0 in Tris–barbituate buffer, as indicated in Fig. 2.

TABLE III

SOME PROPERTIES OF THE TWO TYPES OF CERULOPLASMIN

Type of source	*I* *10-week-old pig*	*II* *2-day-old pig*
K_{av} Sephadex G-150	0.173	0.173
Protein concn. (mg/ml)	0.560	1.010
$A_{610\ m\mu}$	0.034 ± 0.002*	0.052 ± 0.002*
$A_{280\ m\mu}$	0.715 ± 0.002*	1.165 ± 0.005*
$A_{610\ m\mu}/A_{280\ m\mu}$	0.0476 (0.0446–0.0505)	0.0446 (0.0427–0.0466)
Specific enzymatic activity (ΔA/15 min per mg protein)	1.929	0.861
Copper content (%)	0.277 ± 0.034**	0.139 ± 0.025***

* S.D. of three analyses of same preparation.
** S.D. of two preparations including one above.
*** S.D. of three preparations including one above.

Table III summarizes some of the properties of the two types of ceruloplasmin. The major difference between ceruloplasmin I and ceruloplasmin II, aside from the enzymatic activity, is the copper content. Assuming molecular weights of 150 000 and $A_{610\ m\mu}/A_{280\ m\mu}$ of 0.048 for pure preparations, this would correspond to 7–8 coppers for ceruloplasmin I and about 4 coppers per mole for ceruloplasmin II. Apparently, the 610-mμ chromophore is affected very little by the difference in the copper content, whereas the enzymatic activity seems to be related directly to the copper.

Kinetic studies

The initial curvature encountered in the progress curve of *p*-phenylenediamine oxidation is shown in Fig. 3. Results of the kinetic studies using *p*-phenylenediamine as the substrate are shown in Fig. 4. At 0.12 μM enzyme concentrations, the v_{max} of 0.0216 A/min for ceruloplasmin I is almost twice that for ceruloplasmin II (0.0135 A/min), as would be expected from the results of the specific enzymatic activities. The apparent K_m of ceruloplasmin I also appeared to be slightly higher than that of ceruloplasmin II, 0.31 mM *vs.* 0.23 mM at this enzyme concentration. Both of these values are considerably lower than those reported for human[9] and chick[10] ceruloplasmin under slightly different conditions.

* Amicon Corporation, Lexington, Mass., U.S.A.

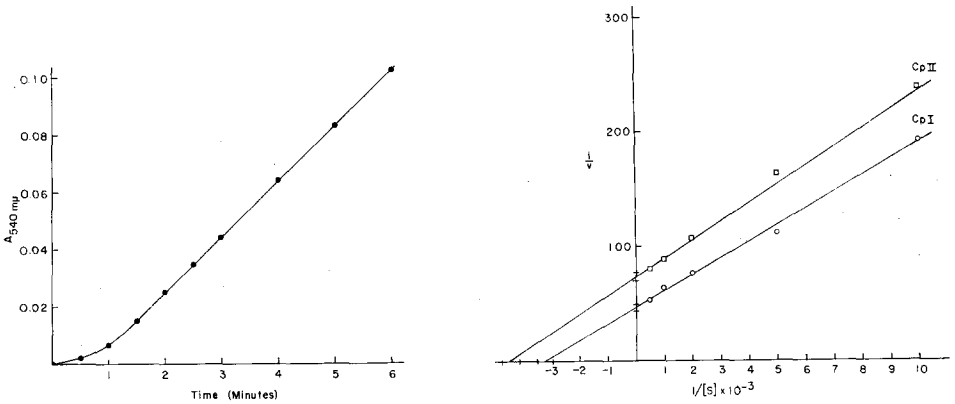

Fig. 3. Progress of oxidation of *p*-phenylenediamine at 37°. Reaction mixture contained 1 ml of 1 mM *p*-phenylenediamine in 1.0 M acetate buffer (pH 5.5) equilibrated at 37° for 10 min and 0.5 ml of 2day ceruloplasmin II in 0.075 M phosphate (pH 6.8). Concentration of the ceruloplasmin in final reaction mixture was approximately 0.32 μM.

Fig. 4. Lineweaver–Burk plots for determination of K_m's of ceruloplasmin (CpI) and ceruloplasmin II (CpII) with *p*-phenylenediamine as substrate. Enzyme concentrations were 0.12 μM. The Experiments were run at 37° in 1.0 M acetate buffer (pH 5.5).

DISCUSSION

The evidence presented showing a gradual change from only ceruloplasmin II in the neonatal piglet to predominantly ceruloplasmin I in the older pig is strong presumptive evidence that the changes observed are physiological. Changes in the relative amounts of two types of ceruloplasmin have been reported in humans[11]. However, only subtle differences have been found in the two forms of human ceruloplasmin. MORELL AND SCHEINBERG[12] found that there were no differences in either the copper contents or the enzymatic activities between these two forms. It has been shown that human ceruloplasmin is composed of 2 α and 2 β peptide chains[13] with the β chain showing some heterogeneity. The possibility has been suggested (K. SIMONS, personal communication) that the two human ceruloplasmins differ in the β peptide structure.

On the other hand, the two types of porcine ceruloplasmin, which show similar electrophoretic homogeneity and $A_{610\ m\mu}/A_{280\ m\mu}$ ratios, exhibit about a 2-fold difference in copper content and in enzymatic activity towards *p*-phenylenediamine. The apparent K_m's and v_{max}'s appeared to be related to the copper concentrations of the preparations. Recently, LØVSTAD[14] has demonstrated two different activities of ceruloplasmin in human serum towards *N,N*-diphenyl-*p*-phenylenediamine, with cord serum exhibiting one activity and adult or pregnant adult serum showing a combination of two activities. To resolve these differences, he suggests that there are two different active sites on two different molecules. From our results, we suggest the possibility that ceruloplasmin II with the reduced copper content has only one active site, while ceruloplasmin I, the adult form, could have two active sites.

A ceruloplasmin with a reduced copper content has been isolated from chickens which had been infected with *Salmonella gallinarum*[10]. Both this and baby pig ceruloplasmin II are present under conditions of very low serum copper[1,10]. Also, in both of

these cases, the ceruloplasmin can be classed as induced or newly synthesized. These observations would suggest that newly synthesized ceruloplasmin in these two species is released into the serum with an incomplete copper complement.

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REFERENCES

1 G. G. GOMEZ-GARCIA AND G. MATRONE, *J. Nutr.*, 92 (1967) 237.
2 H. F. DEUTSCH, *Arch. Biochem. Biophys.*, 89 (1960) 235.
3 L. BROMAN AND K. KJELLIN, *Biochim. Biophys. Acta*, 82 (1964) 101.
4 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
5 G. MATRONE, W. J. PETERSON, H. M. BAXLEY AND C. D. GRINNELS, *J. Dairy Sci.*, 30 (1947) 121.
6 O. B. HOUCHIN, *Clin. Chem.*, 4 (1958) 519.
7 E. W. RICE, *Anal. Biochem.*, 3 (1962) 452.
8 J. PEISACH AND W. G. LEVINE, *Biochim. Biophys. Acta*, 77 (1963) 615.
9 C. G. HOLMBERG AND C. B. LAURELL, *Acta Chem. Scand.*, 5 (1951) 476.
10 B. STARCHER AND C. H. HILL, *Biochim. Biophys. Acta*, 127 (1966) 400.
11 R. RICHTERICH, *Wilsons Disease: Some Current Concepts*, Blackwell, Oxford, 1961, p. 81.
12 A. G. MORELL AND I. H. SCHEINBERG, *Science*, 131 (1960) 930.
13 K. SIMONS AND A. G. BEARN, *Biochim. Biophys. Acta*, 175 (1969) 260.
14 R. A. LØVSTAD, *European J. Biochem.*, 8 (1969) 303.
15 D. E. WILLIAMS AND R. A. REISFELD, *Ann. N.Y. Acad. Sci.*, 121 (1964) 373.
16 A. CHRAMBACH, R. A. REISFELD, M. WYCOFF AND J. ZACCARI, *Anal. Biochem.*, 20 (1967) 150.