

# Antioxidant properties of caeruloplasmin towards iron- and copper-dependent oxygen radical formation

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The antioxidant activity of caeruloplasmin can be mainly ascribed to its ferroxidase activity which effectively inhibits ferrous ion-stimulated lipid peroxidation and ferrous ion-dependent formation of hydroxyl radicals in the Fenton reaction. In addition, caeruloplasmin can prevent copper ions from stimulating lipid peroxidation.

<i>Caeruloplasmin</i>	<i>Antioxidant</i>	<i>Lipid peroxidation</i>	<i>Hydroxyl radical</i>	<i>Ferroxidase</i>
		<i>Metal ion-dependent free radical</i>		

## 1. INTRODUCTION

Aerobic cells are protected under normal conditions against damage by active oxygens such as superoxide radicals, hydrogen peroxide and hydroxyl radicals by the enzymes superoxide dismutase, catalase and glutathione peroxidase. Extracellular fluids, however, are generally poor in these enzymes [1-3] yet can be subjected to oxygen radicals produced by activated leukocytes, enzymes and autoxidising substances. Nevertheless, it has been shown that extracellular fluids do contain effective antioxidants of their own which are different from those found intracellularly [4,5]. Fractionation of human serum has shown that the copper-containing  $\alpha_2$ -globulin caeruloplasmin and the  $\beta$ -globulin transferrin contribute significantly to these important antioxidant activities [4,6].

The ferroxidase activity of caeruloplasmin [7] and the high affinity binding of the resulting ferric ions to transferrin suggest a co-ordinated role for these two proteins in protection against catalytic iron damage [4,6,8]. Recent studies have shown that the ferroxidase activity of caeruloplasmin cannot account for all its observed protective antioxidant functions since it can protect lipids and

erythrocyte membranes from copper stimulated damage [9,10]. The purpose of this study is to examine in greater detail the protection afforded by caeruloplasmin againsts copper-dependent lipid peroxidation.

## 2. MATERIALS AND METHODS

Caeruloplasmin (type X human), albumin (human, fatty acid-free), catalase (bovine thymol-free),  $\gamma$ -globulin (rabbit Cohn fraction II), papain (type IV), linoleic acid (99% pure), 2-deoxy-D-ribose and Lubrol PX were from Sigma. All other chemicals were of the highest grades available and obtained from BDH. All reagents were prepared in chelex-resin-treated distilled water.

### 2.1. Preparation of lipids

Bovine brain lipids were prepared as in [11]. Multilamellar phospholipid vesicles were prepared by vortex mixing 5 mg phospholipid in 1 ml 0.15 M NaCl (pH 7.4) for 5 min. Resulting liposomes were left at 4°C for 1 h before use. Fatty acid micelles were prepared by adding 1.25 mg linoleic acid to 20 ml 0.15 M NaCl (pH 7.4) together with 1 ml 1% (w/v) Lubrol PX. The mix-

ture was vortex-mixed for 5 min and stored at 4°C for 1 h before use. Autoxidised lipids were prepared as in [12]. Reaction mixtures consisted of 0.2 ml lipid preparation, 0.1 ml protein 2.5 mg/ml in water, and 0.1 ml 1 mM metal salt. Volumes were made to 0.5 ml by the addition of chelex-resin-treated distilled water, and the samples incubated at 37°C for 1 h.

### 2.2. Deoxyribose degradation

This was carried out as described above for lipid samples, but 0.2 ml 5 mM deoxyribose was added instead of lipid. After addition of metal salts 0.1 ml 10 mM hydrogen peroxide was added and the tubes were incubated at 37°C for 30 min.

### 2.3. Measurement of oxygen radical damage

After incubation of the lipid containing samples 0.5 ml 1% (w/v) Lubrol was added, followed by 0.5 ml of 1% (w/v) thiobarbituric acid in 0.05 M NaOH. HCl, 0.5 ml, 25% (v/v), was added to the phospholipid samples, 0.5 ml acid buffer (pH 3.5) to the fatty acid samples, and 0.5 ml 2.8% (v/v) trichloroacetic acid to the deoxyribose samples [13]. All samples were heated at 100°C for 15 min

to develop the colour, which was read at 532 nm. Phospholipid fluorescence was measured as in [11]. Results shown were reproducible at  $\pm 5\%$ .

## 3. RESULTS

Caeruloplasmin inhibits lipid peroxidation and deoxyribose degradation stimulated by iron and copper salts (table 1,2). Copper-dependent deoxyribose degradation is, however, significantly inhibited by most other proteins added, whereas iron-dependent deoxyribose degradation is inhibited only markedly by caeruloplasmin and catalase. Cobalt-dependent damage to deoxyribose is not markedly inhibited by caeruloplasmin (table 1). Caeruloplasmin was more effective against iron-dependent deoxyribose degradation in the absence of hydrogen peroxide (table 1).

Phospholipid liposomal peroxidation stimulated by both iron and copper salts is most effectively inhibited by the addition of caeruloplasmin. Other proteins inhibit copper-stimulated lipid peroxidation although their effect is not as marked as that of caeruloplasmin (table 2). Fatty acid micelles are sensitive to cobalt salt-stimulated peroxidation [14]

Table 1

Effect of caeruloplasmin on iron, cobalt and copper salt-dependent deoxyribose degradation in the presence of hydrogen peroxide

	TBA-reactivity ( $A_{532 \text{ nm}}/0.5 \text{ h}$ )					
	Ferrous salt		Cupric salt		Cobaltous salt	
	$A_{532 \text{ nm}}$	% Inhibition	$A_{532 \text{ nm}}$	% Inhibition	$A_{532 \text{ nm}}$	% Inhibition
Blank ( $\text{H}_2\text{O}_2$ + deoxyribose)	0		0		0	
Control ( $\text{H}_2\text{O}_2$ + metal salt + deoxyribose)	0.38		0.34		0.22	
Control (metal salt + deoxyribose only)	0.14		no degradation		no degradation	
+ caeruloplasmin (no $\text{H}_2\text{O}_2$ )	0.04	74	—	—	—	—
+ caeruloplasmin (+ $\text{H}_2\text{O}_2$ )	0.19	50	0.09	74	0.18	18
+ albumin (+ $\text{H}_2\text{O}_2$ )	0.33	13	0.06	82	0.17	23
+ papain (+ $\text{H}_2\text{O}_2$ )	0.32	16	0.11	68	0.12	45
+ $\gamma$ -globulin (+ $\text{H}_2\text{O}_2$ )	0.33	13	0.13	62	0.19	14
+ catalase ( $\text{H}_2\text{O}_2$ )	0.06	84	0	100	0	100

Final metal salt concentrations were 0.2 mM, hydrogen peroxide 2 mM, and protein, 0.4 mg/ml

Table 2

Effect of caeruloplasmin on iron- and copper salt-stimulated peroxidation of phospholipid liposome

	TBA-reactivity ( $A_{532}/h$ )			
	Ferrous salt		Cupric salt	
	$A_{532}$	Inhib. (%)	$A_{532}$	Inhib. (%)
Blank				
(phospholipid only)	0.02		0.02	
Control (phospholipid				
+ metal salt)	0.20		0.22	
+ caeruloplasmin	0.08	67	0.05	85
+ albumin	0.19	6	0.14	40
+ papain	0.20	0	0.15	35
+ $\gamma$ -globulin	0.20	0	0.21	5
+ catalase	0.22	0	0.13	45

Final metal salt concentrations were 0.2 mM and protein, 0.4 mg/ml. Results were calculated after the subtraction of appropriate blanks

but this, unlike iron stimulated damage, was not markedly inhibited by the addition of caeruloplasmin (table 3).

Incubation of pre-formed products or lipid peroxidation with caeruloplasmin did not substan-

Table 3

Effect of caeruloplasmin on iron and cobalt salt-stimulated peroxidation of fatty acid molecules

	TBA-reactivity ( $A_{532}/h$ )			
	Ferrous salt		Cobaltous salt	
	$A_{532}$	Inhib. (%)	$A_{532}$	Inhib. (%)
Blank (fatty acid only)	0.01		0.01	
Control (fatty acid				
+ metal salt)	0.45		0.65	
+ caeruloplasmin	0.05	91	0.62	5
+ albumin	0.36	20	0.61	6
+ papain	0.46	0	0.56	14
+ $\gamma$ -globulin	0.34	25	0.59	9
+ catalase	0.51	0	0.52	20

Final metal salt concentrations were 0.2 mM and protein, 0.4 mg/ml. Results were calculated after subtraction of appropriate blanks

Table 4

Effect of caeruloplasmin on the products of lipid peroxidation

	TBA-re-activity $A_{532}$	Fluorescence $E_x$ 360 $E_m$ 430 RFI units
Phospholipids autoxi- dised 3 days in air and prepared as liposomes		
Control	0.06	63
+ caeruloplasmin	0.11	78
+ albumin	0.07	75
Fatty acid (18:3) autoxidised 3 days in air and prepared as micelles		
Control	0.07	—
+ caeruloplasmin	0.07	—
+ albumin	0.07	—

Proteins were at a final reaction concentration of 0.4 mg/ml and incubated with the products of lipid peroxidation for 1 h

tially alter their resulting TBA-reactivity and fluorescent properties (table 4).

#### 4. DISCUSSION

The potent antioxidant activity of normal human plasma has been shown to be mainly dependent on the copper-containing protein caeruloplasmin and the iron-binding protein transferrin [4,6]. Caeruloplasmin can protect tissue homogenates, phospholipid membranes, DNA, deoxyribose and erythrocyte membranes from iron-dependent damage [9,15–18]. Caeruloplasmin is a more effective antioxidant against ferrous ions than against a mixture of ferrous ions and hydrogen peroxide [17]. The latter system is a powerful generating source of hydroxyl radicals and these appear to damage some of the protein molecules. In addition, caeruloplasmin can also protect lipids and erythrocyte membranes from copper-stimulated damage [9,10], suggesting an antioxidant function other than that of its ferroxidase activity.

Proposals that caeruloplasmin functions as an extracellular 'superoxide dismutase' [19] have been shown to be incorrect since it reacts stoichiometrically with superoxide radicals and not catalytically [6,20,21]. Similarly, its ability to scavenge hydroxyl radicals [20] cannot explain its protective role against iron-stimulated lipid peroxidation [17]. Alternative proposals are that caeruloplasmin might decompose lipid peroxides or scavenge organic oxygen radicals [16,22,23]. However, these activities were not confirmed in the present studies since caeruloplasmin did not markedly change the TBA-reactivity and fluorescence of peroxidised lipid or inhibit cobalt-stimulated fatty acid peroxidation.

So far, all proteins tested inhibit copper-dependent deoxyribose degradation [24]. This is because copper ions ligate non-specifically to proteins, allowing site-specific hydroxyl radical formation on the protein molecule [24]. In this respect, caeruloplasmin is no different from other protein molecules. The greater inhibitory activity of catalase in the deoxyribose system, however, can be ascribed to its scavenging of hydrogen peroxide, so inhibiting OH<sup>•</sup> radical formation in this reaction [17,24]. Using the same protein concentrations, for a reaction in which lipids are undergoing copper-stimulated peroxidation, caeruloplasmin appears to behave quite differently from other proteins tested. In some way, it effectively prevents copper ions, including those that are protein-bound, from stimulating lipid peroxidation.

This unusual antioxidant property of caeruloplasmin may have important implications in vivo for conditions such as rheumatoid joint disease and Wilson's disease, where changes in copper homeostasis, caeruloplasmin and oxygen radicals are known to occur [6].

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