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DISTRIBUTION OF SUPEROXIDE DISMUTASE AND GLUTATHIONE PEROXIDASE IN THE CARP : ERYTHROCYTE MANGANESE SOD

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SUMMARY. - A non copper containing superoxide dismutase (Cu-SOD), presumably manganese superoxide dismutase (Mn-SOD), has been identified in carp erythrocytes. Erythrocyte catalase is low, glutathione peroxidase (GPX) is extremely high, and superoxide dismutase (SOD) is relatively low. The distribution of Cu-SOD, Mn-SOD and glutathione peroxidase in various tissues is described. Highest activities of both enzymes are found in the liver and lowest in white muscle and the swim bladder.

We have earlier described levels of SOD, catalase and glutathione peroxidase in the blood of various species (1) of warm blooded animals. This report presents studies of the levels and tissue distribution of these enzymes which are concerned with protection against uncontrolled oxidative processes in a typical cold blooded vertebrate, the carp, a fish which is noted for its longevity. We hope that subsequent studies will deal with the accelerated aging at a certain period of its life cycle of another species of fish, the salmon.

In many fish species, fat deposition occurs in non-differentiated tissue and the proportion of lipids in these tissues may reach very high values. For instance, the fat content reported for the musculature of Siscowet trout is 67 % (2) and shark liver may contain as much as 56 % of oil (3). Moreover, as a result of biological or ecological factors, fish lipids are unusually rich in polyunsaturated fatty acids (4) which may explain why numerous species quickly become rancid after death (5). Efficient protection against autoxidative processes must thus be present in cellular structures and storage organs throughout the biological cycle, from development of the egg, through growth and fattening to gonad maturation, since all these operations involve massive transportation of lipids as well as intense oxidative breakdown of fatty acids for energetic purposes. Tocopherols are generally considered as a good natural

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protection against autoxidation in fish tissues (5). Enzymatic processes known to limit free radical formation in living organisms have not been extensively investigated in fish as yet.

MATERIAL AND METHODS

Sexually immature carp weighing 200-500 g were kept in well-aerated free-running tap water (temperature 11-14° C). They were starved till utilisation which occured between 30 and 120 days after the beginning of storage.

ENZYMATIC ACTIVITIES IN RED CELLS

Blood (1 ml) was withdrawn by cardiac puncture with a heparinized syringe and hemoglobin (Hb) estimated according to Drabkin and Austin (6). After centrifugation for 10 min at 3000 g, the serum was removed and the red cells were washed with 0.6 % NaCl and then lysed by addition of one volume of distilled water followed by freezing at -79° C. DNase (50 µg/ml) was added to the lysate which was incubated for 2 hr at room temperature. After determination of hemoglobin, the lysate was either adjusted to 5 % Hb for GPX and catalase determinations or used directly for estimation of SOD.

Glutathione peroxidase was determined as described previously (1) using samples of 5 µl of lysate. Values are expressed as µM NADPH consumed/ min/ g Hb.

Catalase estimations were also performed with 5 µl of lysate using the extremely sensitive luminescent technique described earlier (7). Results are expressed using crystalline beef catalase as reference.

Superoxide dismutase. To 0.5 ml of lysate was added 0.2 ml of chloroform-ethanol (3 : 5) and 0.1 ml of water to precipitate the hemoglobin which was removed by centrifugation. The supernatant was used for SOD determination by the nitroblue tetrazolium method in absence or presence of 5×10^{-3} M KCN to determine both Cu-SOD and Mn-SOD (8).

ENZYMATIC ACTIVITIES IN TISSUES

Carp were frozen in dry ice just before sampling and various tissues (1 g) were homogenized in 2 ml of 10^{-2} M phosphate buffer pH 7.8 containing 10^{-4} M EDTA and 5×10^{-3} M mercaptoethanol, to which 0.2 ml of 10 % Triton 100 were added. One ml of buffer was used to wash the homogenizer and pooled with the homogenate which was centrifuged for 10 min at 3000 g. The supernatant was dialysed against the same buffer overnight. An aliquot of the dialysate was precipitated with 0.3 volumes of chloroform-ethanol (3 : 5 v/v) (P1 extracts) or 0.4 volumes of chloroform-ethanol plus 0.2 volumes of water (P2 extracts). After centrifugation, GPX determinations were carried out on P1 supernatant as described for blood, and SOD determinations on either P1 or P2 supernatants. Proteins were measured by micro-biuret estimations on dialysed extracts of the homogenate supernatants.

ACRYLAMIDE GEL ELECTROPHORESIS

Acrylamide gel electrophoresis was carried out according to Davis (9) with bromothymol blue as marker. Superoxide dismutase activity on gels was revealed by the photochemical method, in absence and presence of 5×10^{-3} M KCN (final concentration).

SEDIMENTATION VELOCITY

Measurements were made with a Spinco L 2-65 B ultracentrifuge using a SW 65 K rotor, according to Martin and Ames (10), with a linear 5 to 20 % sucrose gradient. Centrifugation was carried out for 17 hr at 45 000 rpm, at 3° C with yeast and horse liver alcohol dehydrogenases, bovine Cu-SOD, human Mn-SOD, and horse radish peroxidase as markers.

RESULTS AND DISCUSSION

In fish, the red cell concentration in blood and the hemoglobin concentration in red cells fluctuate to a high degree, due to immediate or delayed effects of stress and to altered physiological status, conditions interfering with the osmotic balance of the fish, and thus with heme concentration (11). However, the GPX, SOD and catalase contents as expressed per erythrocyte or per g of hemoglobin show relatively small variations (Table I). Compared with other vertebrates (1) the catalase level in carp red cells is slightly higher than in birds. However, the level is low with respect to certain mammals, being only 8.4 % of the human level. This level is consistent with the low figure reported by Aebi and Suter for a different species of fish, Ophiocephalus (16.6 % of the human value). These authors also report very low values for birds as well as for two mammals, dog and goat. As pointed out by Aebi and Suter (12), low catalase levels may be offset by an increase in GPX. Although Maral et al. (1) show that this proposition is restricted, it seems to hold true for carp where the red cell GPX has the highest value ever described in vertebrates (3927 % of the human value).

In contrast, the SOD content of carp erythrocytes is lower than any of the values reported by Maral et al. (1) for the various vertebrates studied. Nevertheless the level falls within the variation range (factor of two) described by these authors, and is consistent with the statement that among vertebrates "SOD has a relatively constant value with respect to hemoglobin content". The range of SOD levels is much narrower than that of GPX or

TABLE I

Enzymatic activity in Erythrocytes

Carp	Blood		GPX μM NADPH/min			Catalase μg			SOD units			% SOD non inhibited by 10^{-3} M KCN
	Hb %	red cell x 10^{9}/ml	g Hb	per ml blood	red cell x 10^{-8}	g Hb	per ml blood	red cell x 10^{-8}	g Hb	per ml blood	red cell x 10^{-8}	
1	9.75	3.15	291	28.4	0.90	350	34.0	1.08	913	89.0	2.83	16.6
2	5.96	1.44	223	13.3	0.92	292	17.4	1.21	818	48.8	3.32	12.7
3	10.49	1.67	204	21.4	1.28	286	30.0	1.80	760	79.7	4.77	–
4	8.68	2.41	223	19.4	0.80	366	31.8	1.32	844	73.3	3.04	5.0
5	8.94	2.49	187	16.7	0.67	334	29.9	1.20	635	56.8	2.28	15.1
6	10.12	2.46	223	22.6	0.92	418	42.3	1.72	965	97.7	3.97	10.7
Average	8.99 ± 0.21	2.27 ± 0.103	225 ± 6	20.3 ± 0.86	0.92 ± 0.03	341 ± 8	30.9 ± 1.34	1.39 ± 0.05	823 ± 19	74.2 ± 3.1	3.38 ± 0.15	12.0 ± 0.91
% of human value			3927	2796		8.4	6.0		67.2	47.9		

TABLE II

SOD of carp red cells freed from white cells

	Total SOD (Cu-SOD + Mn-SOD) units/ml/medium	Mn-SOD units/ml/medium	% Mn-SOD
Experiment N° 1	25	2.3	9.2
Experiment N° 2	41.5	4.2	10.1

catalase. From an evolutionary standpoint, this observation suggests that SOD is the primary enzymatic mechanism for red cell protection against uncontrolled oxidation. However, it must be noted that the present survey does not take into consideration the physiological status of the fish i.e. feeding versus starving, nor the nature of the species i.e. active versus sluggish etc...

An interesting feature of carp erythrocyte SOD lies in the fact that part of the activity is not inhibited by 5×10^{-3} M KCN. This is characteristic of Mn-SOD (or in procaryotes, Fe-SOD). The Cu-SOD is completely inhibited at this concentration of KCN. In separate experiments, blood was withdrawn from several fish and pooled. After centrifugation, the layer of white cells was removed by aspiration, and the erythrocytes washed in 0.6 % NaCl, centrifugated and again separated from the remaining white cells. Lysis and chloroform-ethanol treatment gave an extract containing 10 % SOD activity which may be attributed to Mn-SOD (Table II). Before precipitation, an aliquot of the lysate was centrifugated at 100 000 g for 1 hr at 3° C in order to sediment the sub-cellular particles. The Mn-SOD content was found to account for 10.01 % of total activity in the supernatant and 8.78 % in the bottom phase indicating cytoplasmic localisation of both Cu-SOD and Mn-SOD. These extracts were concentrated by evaporation and Minicon filtration. Acrylamide gel electrophoresis of the concentrate showed two bands of R_f 0.22 and 0.36 after revelation of SOD activity. If the staining solution contained 5×10^{-3} M KCN, a single band of R_f 0.22 appeared, which may be considered as Mn-SOD. Sedimentation velocities in sucrose gradients were measured on the concentrate. The KCN inhibited copper enzyme has a molecular weight of 32 000 whereas the postulated Mn-SOD gave a value of 85 600 daltons, similar to that of eucaryote Mn-SODs. These characteristics are in accord with

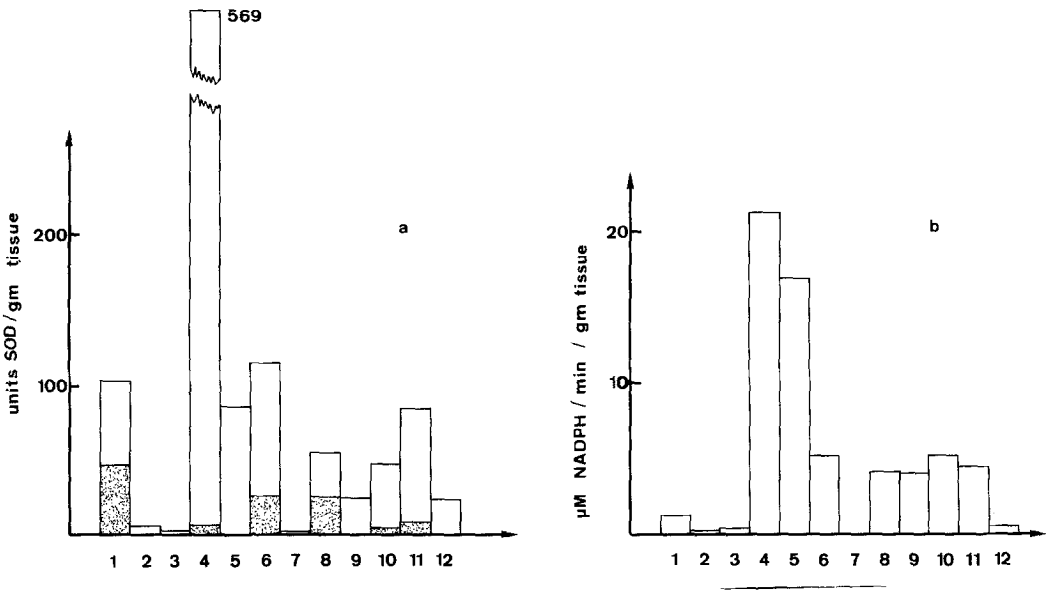

Fig. 1a) Average superoxide dismutase levels in different organs of carp. The proportion of Mn-SOD in the total (Cu-SOD + Mn-SOD) is indicated by the shaded areas.

1b) Average glutathione peroxidase levels in various carp organs. 1. Red muscle ; 2. White muscle (dorsal) ; 3. White muscle (ventral) ; 4. Liver ; 5. Spleen ; 6. Kidney ; 7. Swim bladder ; 8. Heart ; 9. Gils ; 10. Stomach ; 11. Intestines ; 12. Brain.

those of other copper containing or manganese containing superoxide dismutases from various sources.

It thus appears that in contrast with all other species so far described, carp erythrocytes do not contain uniquely a Cu-SOD, but also a Mn-SOD (non-particulate, or very loosely bound). This is almost certainly associated with the fact that carp erythrocytes are nucleated. Indeed addition of DNAase during lysis was necessary (as mentioned in methods) to avoid formation of solid gels due to liberation of DNA from the nuclei. It would be of interest to examine other species known to possess nucleated erythrocytes for the presence of red cell Mn-SOD.

In tissues, the GPX and SOD contents are very variable (Fig. 1). It must be pointed out that organs such as spleen or heart are more or less filled with blood. Moreover, in some cases, SOD estimations are misleading

since non-linear dosage effects or even negative values are observed suggesting the presence of an endogenous source of superoxide ion in the extract. In this respect, our results are rather a measurement of the overall protection against superoxide ion than absolute SOD determinations. For both SOD and GPX, the liver exhibits the highest activity while a hematopoietic tissue such as spleen is comparable with blood. The whole bulk of white muscles which account for half the animal in weight seems essentially devoid of glutathione peroxidase and SOD, as is also the case for the swim bladder which nevertheless contains an oxygen concentrating structure (13). The dark muscle of the carp lateral line contains a significant amount of SOD with the highest proportion of Mn-SOD found in any organ. Acrylamide gel electrophoresis of concentrated extracts (P2) of dark muscle shows two bands of SOD activity at R_f 0.16 and 0.32. In presence of KCN, only the first band corresponding to Mn-SOD appears. The peculiarities of carp dark muscle may be related to its physiological significance. This organ, loaded with lipids, is the site of an intense metabolic activity involving fatty acid breakdown, and its mechanic activity nearly accounts for the whole non-emergency motion of the fish (14). Dark muscle is particularly suceptible to autoxidation when compared to white muscle (15).

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