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A RADIOIMMUNOASSAY FOR COPPER CONTAINING SUPEROXIDE DISMUTASE

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SUMMARY. - Highly radioactive rat and bovine copper containing superoxide dismutases (Cu-SOD) up to 1700 Ci/mmole of protein were prepared by iodination with ^{125}I and chloramine T. Labelled human Cu-SOD was obtained by coupling the enzyme with N-succinimidyl 3-(4-hydroxy, 5 - (^{125}I) -iodophenyl) propionate. These tracer molecules can be used with anti-bovine or anti-human Cu-SOD antisera to estimate various Cu-SODs as protein (rat, bovine, human) with a sensitivity of 20-80 picogrammes, using standard radio-immunochemical techniques.

Control in vivo of the steady state concentration of the radical ion superoxide $O_2^{\cdot-}$ strongly depends upon the quantity of superoxide dismutase (SOD) present (1). There are several types of enzyme, which are ubiquitous among all aerobic species, but differ in their subcellular location and in the nature of the metal at the active site (2).

It is thus of interest to develop a sensitive, specific, and easy assay of the enzymes, particularly if discrimination between mangano and cuprozinc SODs can be achieved. Radioimmunological techniques answer these requirements and can contribute to the solution of a wide variety of problems connected with the metabolism of molecular oxygen.

MATERIALS AND METHODS

Superoxide dismutases. Rat, human and bovine cuprozinc superoxide dismutases were prepared according to the technique of McCord and Fridovich (3). After purification, the specific activity of the bovine enzyme was 3300 NBT-riboflavin units/mg (4). In addition, a commercial bovine blood SOD (Sigma) has been used (SA : 2700 McCord and Fridovich units/mg). Before use, the purity of these preparations was tested by polyacrylamide gel electrofocalisation (Multiphor LKB).

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Antibovine SOD and antihuman SOD antibodies. Antibovine and antihuman SOD antisera were obtained by immunisation of rabbits, with, for the first injection, 1 mg of protein followed by three further injections of 0.5 mg at monthly intervals. The Cu-SOD preparation was emulsified with complete Freund adjuvant and administered subcutaneously.

^{125}Iodine labelling of cuprozinc SOD. The labelling of bovine and rat enzymes was effected with 2 µg of protein in 2 µl of buffer, 200 µCi of Na ^{125}I (IMS 30, Amersham) in 2 µl, and small quantities of chloramine T in 0.5 M phosphate buffer pH 7.5 (5). The percentage of ^{125}I incorporation into the protein was measured by precipitation of the iodinated enzyme by 10 % trichloracetic acid in presence of 1 mg/ml of bovine serum albumin (BSA) as carrier. For each labelling, the quantity of chloramine T (generally about 1.5 µg) giving a yield of iodination between 40 % and 50 % of the iodine used is determined. When this percentage is reached, the reaction is stopped by addition of sodium metabisulfite, the quantity of which is twice that of chloramine T, and the labelling medium is diluted with 1 ml of diluent buffer (0.1 M phosphate pH 7.6, containing 2 % BSA). The product is purified on Sephadex G 100 with the same buffer as eluent (Fig. 1).

Labelling of the human enzyme was effected by coupling human Cu-SOD with N-succinimidyl 3-(4-hydroxy, 5-(^{125}I) iodophenyl) propionate using the technique described by Bolton and Hunter (6). The tracer is purified on Ultrogel Aca 44 (Industrie Biologique Française) with 0.1 M phosphate buffer pH 7.6 containing 0.5 % gelatin as eluent buffer. Two peaks of radioactivity are observed. The first corresponds to the immunoreactive fractions used as tracer, whereas the second corresponds to the coupling product of the excess of reagent with glycocol, introduced before the chromatographic step to terminate the reaction (6). This technique was used since treatment of human Cu-SOD with chloramine T gives rise to aggregates.

Radio-immunoassay. The incubation mixture for radio-immunoassay contains purified or unpurified preparations of SOD appropriately diluted (0.1 ml), labelled enzyme (0.3 ml, 5000 cpm), and diluted antiserum (0.1 ml) in a final volume of 0.5 ml. All dilutions are made with 2 % BSA in 0.1 M sodium phosphate pH 7.6. The duration of the incubation at ambient temperature is 18 to 24 hours. The separation of the bound fraction from free enzyme is effected by addition of 1 ml of a solution of 30 % polyethylene glycol 6000 in 0.1 M phosphate pH 7.6 after addition of 100 µl of normal bovine serum in each tube. After centrifugation, the supernatant is discarded and the radioactivity of the precipitate is counted in a Packard 5360 scintillation spectrometer.

RESULTS

High specific activity iodinated SOD. Figure 1 shows the chromatographic pattern which is obtained on gel filtration of the iodination mixture of rat Cu-SOD on a column of Sephadex G 100. Three peaks are observed. The elution volume at peak n° II is identical to that of unlabelled purified SOD. The immunoreactivity of the different fractions was determined by incubation of 300 µl of tracer (about 10 000 cpm) in the presence of an antibody excess

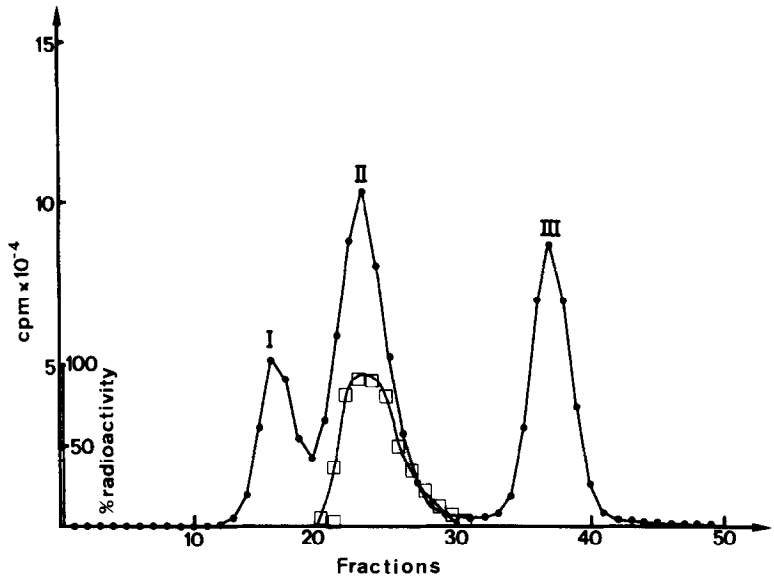

Fig. 1 - Chromatography of ^{125}I - iodinated rat Cu-SOD on Sephadex G 100 -●—●-, cpm ;-□—□-; % radioactivity bound to antibody.

(100 µl of a 10^{-3} dilution). The immunoreactivity presents a maximal value corresponding to the maximum radioactivity of peak n° II and a zero value for peaks I and III. Fractions corresponding to the maximum of peak n° II are diluted to constitute the tracer. The specific radioactivity of the bovine iodinated SOD has been determined by radio-immunoassay. Its value is 51 µCi/µg, corresponding to 0.8 atom of iodine per molecule of SOD and a specific activity of 1700 Ci per mmole of protein.

Assays of stability have shown that the tracer is stable for at least 4 days at 4° C, with no decrease in immunoreactivity. Moreover, chromatography on Sephadex G 100 after 21 days at -20° C shows only slight degradation of the iodinated protein.

Standard curves. Two homologous and one heterologous system were used. Figure 2 shows standard curves obtained with bovine iodinated Cu-SOD using the anti-bovine Cu-SOD antiserum (R. S. 5), at a final dilution of $\frac{1}{800\,000}$. Under these conditions, it is possible to measure 80 pg of bovine SOD. The sensitivity is defined as the quantity of enzyme which displaces 5 % of the radioactivity from the antibody sites. Hence with homologous sera, about 2.4 femtomoles of bovine Cu-SOD can be estimated with precision as protein. The

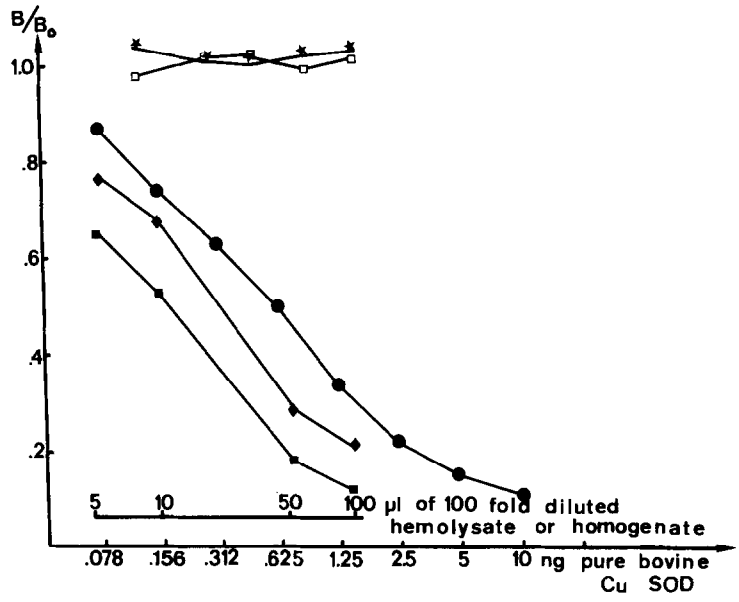

Fig. 2 - Dosage curves using bovine ^{125}I Cu-SOD and anti-bovine Cu-SOD antibodies (RS 5) with purified bovine Cu-SOD (-●—●-), bovine liver homogenate (-■—■-) and bovine hemolysate (-◆—◆-) ; lack of cross reactions with human (-★—★-) and rat (-□—□-) hemolysates. B/Bo is the ratio of radioactivity precipitated in presence of the different cold SODs compared with that precipitated in their absence.

technique is thus about twice as sensitive as the enzymic assay using xanthine oxidase and luminol (7) which is valuable in the region of 3-6 femtomoles, but which gives an enzymic activity of SOD. Conjunction of the two techniques can give valuable information since both protein and activity are measured, using extremely small samples. This double approach can thus be used for human biopsy samples.

Standard curves obtained with the heterologous system rat ^{125}I Cu-SOD plus antibovine Cu-SOD antisera are shown in Fig. 3. The final dilution of the antiserum (R. S. 5) is 1/50 000. Under these conditions, it is possible to detect 80 pg of rat enzyme, 20 pg (0. 6 femtomoles) of bovine enzyme and 800 pg of human enzyme.

Figure 4 presents standard curves obtained with the homologous system human ^{125}I Cu-SOD plus antihuman Cu-SOD antisera. The final dilution of the antiserum (R. S. 6) is 1/25 000. It is possible to detect 80 pg of human enzyme (2. 4 femtomoles).

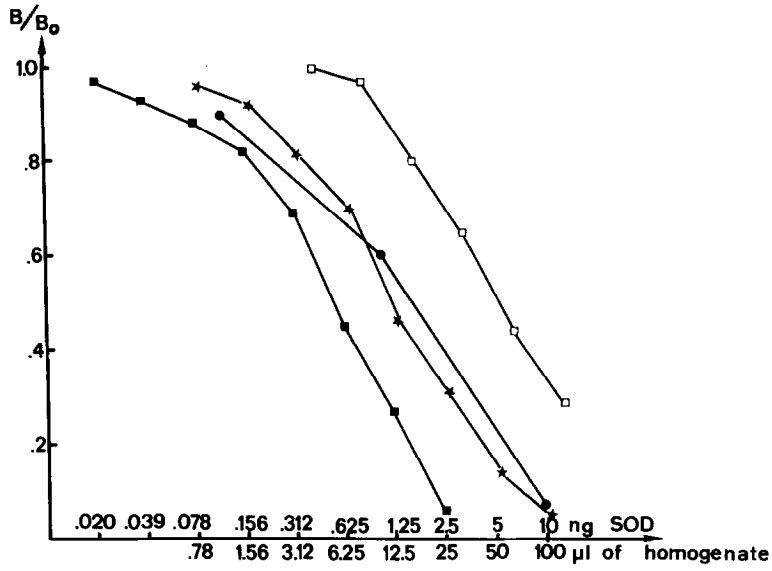

Fig. 3 - Cross reactions of purified rat (-★—★-), human (-□——□-) and bovine (-■—■-) Cu-SODs and rat liver homogenate (-●—●-) in the system using ^{125}I rat Cu-SOD as tracer and anti-bovine Cu-SOD antibodies (RS 5). B/Bo as in fig. 2.

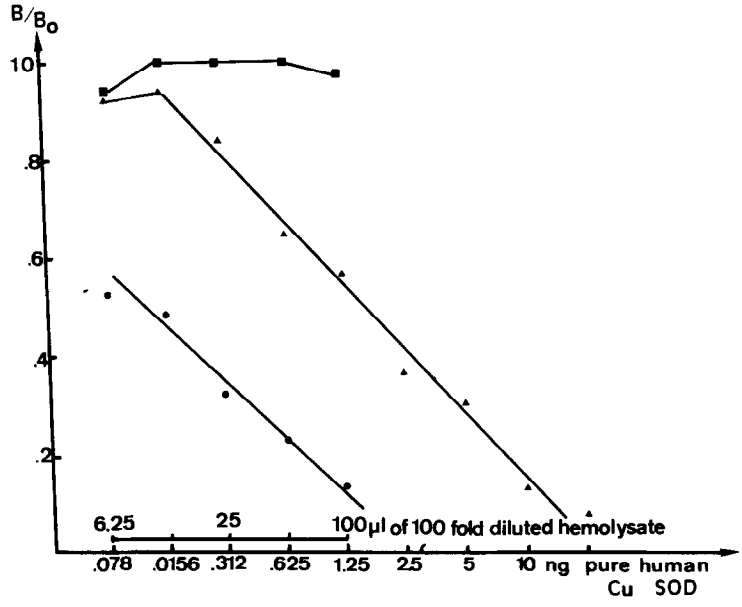

Fig. 4 - Dose response curves in the homologous human Cu-SOD system using ^{125}I-human Cu-SOD and anti human Cu-SOD antibody diluted 25 000 fold. Purified human Cu-SOD (-●—●-), human hemolysate (-●—●-). Lack of cross reaction is shown with rat hemolysate (-■—■-).

Interspecies specificity. With the two homologous systems used, the interspecies specificity is very marked (Table I). Neither rat nor human erythrocyte SOD inhibit interaction between the bovine iodinated SOD and the antibovine SOD antibodies (Fig.2). The same result is obtained with rat liver homogenate. A similar result is obtained with the homologous human system (Fig. 4). No cross reactions are observed between human ^{125}I-Cu-SOD and bovine or rat enzymes. On the other hand, cross reactions between bovine, human and rat Cu-SODs are evident when the heterologous system is studied. Moreover, the dilution curves which are obtained with the homogenates of different rat organs appear to parallel those obtained with the purified rat SOD (Fig. 3). No cross reaction occured between purified human Mn-SOD and Cu-SODs in any system (Table I), nor with bacterial (P. leiognathi) Cu-SOD.

Cross reactions with other bovine proteins. Only catalase preparations presented a cross reaction (2 %) with bovine SOD. Purification of the catalase on Sephadex G 100 showed that this apparent cross reaction is due to contamination by a material whose immunological characteristics are identical to those of SOD. Contamination of catalase by SOD has already been described (8).

Determination of the enzymatic specific activity of human erythrocyte SOD. Individual enzymatic specific activities of human erythrocyte SOD were determined by conjunction of the radioimmunological and enzymatic techniques using the blood of 40 donors (Table II). The results are expressed in enzymatic units determined by the riboflavin-NBT method (4) per μg of protein SOD determined by radio-immunoassay. The value obtained, 3.08 units/μg, may be compared with the specific activity of the purified human enzyme (2925 NBT units/mg) as determined by protein assay.

DISCUSSION

The preparation of stable and high specific radioactivity iodinated erythrocuprein is an essential condition for the elaboration of the radio-immunoassay of this enzyme. Previously, a low specific radioactivity (40 Ci/mmole) iodinated bovine SOD has been used for metabolic studies (9). The greatest difficulty in labelling the enzyme which has been encountered is aggregation of the SOD with complete lack of immunoreactivity (action of chloramine T).

TABLE I

Cross reactions of SODs studied in homologous and heterologous systems

Enzyme	Species	Homologous system		Heterologous system
		Human	Bovine	(rat tracer plus antibovine SOD antiserum)
Cu-SOD	Human	100 %	0 %	25 %
	Rat	0 %	0 %	100 %
	Bovine	0 %	100 %	190 %
	Swordfish	0 %	0 %	0.5 %
	Bacterial	0 %	0 %	0.01 %
Mn-SOD	Human	0 %	0 %	0 %

TABLE II

Specific activity of normal human erythrocyte Cu-SOD

	Enzyme activity		Immunoassay of protein		Specific activity
	units/ml	units/gHb	µg/ml blood	µg/gHb	units/µg
number of subjects	39	39	40	40	39
mean	183	1223	59.75	407.71	3.08
standard deviation	2.88	34.9	1.02	6.37	0.069

This aggregation, which is complete for the human Cu-SOD, could correspond to a dimerisation of the protein, due to formation of disulfide bridges between two cysteine residues. This is suggested by the value of the retention volume of the aggregated enzyme which corresponds to a molecular weight two fold that of the intact iodinated enzyme. Hence, with the rat and bovine enzymes, the quantity of oxidant must be strictly controlled in order to obtain a high proportion of iodinated enzyme which retains all its immunological properties. For the human enzyme, use of N-succinimidyl 3-(4-hydroxy, 5-(^{125}I) iodophenyl) propionate provides a tracer suitable for the radio-immunoassay.

Studies of the cross reactions have shown that the homologous systems are highly specific for an antigenic amino acid sequence of the human or bovine SODs. (This is also true of the rat homologous system). The common amino acid sequences of SODs from different species are only revealed by the heterologous system with RS 5 anti-bovine SOD antiserum and iodinated rat Cu-SOD. The percentage differences of cross reactions indicate conformational or sequence differences of the analogous parts of the enzymes.

The radio-immunoassay of erythrocuprein allows detection and assay in tissues without interference from Mn-SOD which does not show any cross reaction. Moreover it should be the routine technique of choice since no prior purification of the material to be tested is needed. Comparison with other techniques will distinguish between modifications of enzyme activity or enzyme concentration (10). As an example of application, the specific activity of the human erythrocyte Cu-SOD, as determined by radio-immunoassay, corresponds closely to that of purified enzyme as determined by protein estimation and shows no significant individual variation among normal subjects (standard deviation less than 2.5 % of the mean value). In conjunction with a similar radio-immunoassay for the mammalian Mn-SODs which we are presently developing, estimation of both enzyme proteins at very low levels should present little difficulty.

ACKNOWLEDGEMENTS

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REFERENCES

1. Michelson, A.M., Puget, K., Durosay, P. and Bonneau, J.C. (1977) in "Superoxide and Superoxide Dismutases". ed. A.M. Michelson, J.M. McCord and I. Fridovich, p. 467-499, Academic Press, London.
2. Fridovich, I. (1975) Ann. Rev. Biochem. 44, 147-149.
3. McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.
4. Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276-287.
5. Roth, J. (1975) in "Methods in Enzymology" Vol. XXVI B. ed. B.W. O'Malley and J.G. Hardman, p. 223-233, Academic Press, New York.

6. Bolton, A. E. and Hunter, W. M. (1972) J. Endoct. 55, XXX-XXXI.
7. Puget, K. and Michelson, A. M. (1974) Biochimie 56, 1255-1267.
8. Halliwell, B. (1973) Biochem. J. 135, 379-381.
9. Huber, W. and Saifer, M. G. P. in "Superoxide and Superoxide Dismutases" ed. A. M. Michelson, J. M. McCord and I. Fridovich, p. 11-17, Academic Press, London.
10. Crapo, J. D. and McCord, J. M. (1976) Am. J. Physiol. 231, 1196-1203.