A RADIOIMMUNOASSAY FOR MANGANESE CONTAINING SUPEROXIDE DISMUTASE

A. BARET, P. SCHIAVI, P. MICHEL, A. M. MICHELSON+ and K. PUGET+
CERB, BP 610, Toulon Naval and *+Institut de Biologie Physico-Chimique, 13, rue P. et M. Curie, 75005 Paris, France*

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1. Introduction

The comprehension of the mechanisms implicated in a wide variety of problems connected with the metabolism of molecular oxygen requires the elaboration of sensitive, specific and easy assays for superoxide dismutases. The enzymatic techniques [1] already available which are based on superoxide generating systems and superoxide detecting systems, have a number of disadvantages and they cannot be applied easily to the study of crude tissue homogenates, since various small molecules readily consume $O_2^{\cdot-}$ and hence give rise to false estimations. Moreover, the distinction between copper and manganese containing enzymes, based, for example, on the difference of sensitivity to cyanide, is not absolute [2,3].

Radioimmunoassay techniques can contribute to the solution of these problems. They already allow the assay of bovine [4,5], rat and human [4] Cu-SODs without any interference from Mn-SOD. This report describes a radioimmunoassay for Mn-SOD, which, in conjunction with the techniques in [4] for Cu-SOD, allows the precise estimation of both enzymes in crude extracts. Simultaneous estimation of enzymic activities under conditions where this is possible, can be used to follow changes in specific activity (or apparent specific activity, since natural inhibitors of SOD may well exist) with age or in various diseases, for example.

Abbreviations: h Mn-SOD, human manganous superoxide dismutase; Cu-SOD, copper containing superoxide dismutase NSHPP, *N*-succinimidyl-3-(4-hydroxy-5-[125 I]iodophenyl) propionate; BSA, bovine serum albumin; pO_2, partial pressure of oxygen

2. Materials and methods

2.1. *Superoxide dismutases*

These were prepared as in [6]. Apoenzymes were prepared by dialysis of the enzymes at pH 3.8 in presence of EDTA at 4°C. Loss of the metal was followed by decrease in enzymic activity.

2.2. *Labelling of human Mn-SOD*

The ^{125}I-labelling was done by coupling the Mn-SOD (10 μg) with 200 μCi of [^{125}I]NSHPP (Radiochemical Centre, Amersham) as in [7]. The tracer was purified on Ultrogel AcA 44 (Industrie Biol. Française) with 0.1 M phosphate (pH 7.6) buffer containing 0.25% gelatin as eluent buffer.

^{57}Co labelling of the apoenzyme: 400 μCi carrier-free $^{57}Co^{2+}$ (Radiochemical Centre, Amersham) were added to 40 μg Mn-SOD apoenzyme in 0.1 M sodium acetate (pH 3.6). Cobalt was incorporated into the apoenzyme by a slow neutralization (18 h) to pH 8 with ammonia under reduced pressure. The excess of $^{57}Co^{2+}$ was separated from the labelled enzyme by Ultrogel AcA 44 chromatography with 0.1 M phosphate (pH 7.5) containing 0.5% BSA as eluent buffer.

2.3. *Anti-human Mn-SOD antibodies*

Rabbits were immunized by injection of 1 mg human Mn-SOD for injection 1, then with 0.5 mg for subsequent monthly injections. The preparations of Mn-SOD were emulsified with complete Freund adjuvant (Difco) and administered subcutaneously. Appreciable titers of antibodies were obtained at injection 3, and the greatest affinity was obtained with one of the antisera (RS3) which was therefore used in the radioimmunoassay.

2.4. *Radioimmunoassay*

All dilutions were done with the following buffer: 0.1 M phosphate (pH 7.6) containing 0.5% BSA.

The final volume of the incubation mixture is 0.5 ml and contains: purified or crude preparations of Mn-SOD (0.1 ml); labelled enzyme (0.3 ml, 5000 cpm); 2500-fold diluted RS3 antiserum (0.1 ml).

The incubation was for 18–24 h at 4°C. Bound enzyme was separated from the free enzyme by addition of 100 μl 2% normal rabbit serum in diluent buffer, 15 μl goat antirabbit γ globulin antiserum (Institut Pasteur Production) and 500 μl 12% (w/v) polyethyleneglycol 6000 in 0.1 M phosphate buffer (pH 7.6). The tubes were shaken and then centrifuged at 1500 × *g* for 10 min. The supernatant was discarded and the radioactivity of the precipitates counted in a Packard 5360 γ spectrometer.

2.5. *Subcellular fractionation of rat liver and platelets*

Rat liver was fractionated as in [8] using homogenization in a Dounce homogeniser with 0.33 M saccharose, 0.025 mM EDTA, 15 mM Tris-HCl (pH 7.4) as buffer. Three centrifugations, 10 min at 750 × *g*, 10 min at 8200 × *g* and 2 h at 100 000 × *g* were performed. The pellets from 8200 × *g* and 100 000 × *g* centrifugations were resuspended in 0.1 M phosphate (pH 7.5) containing 0.5% BSA, and stored at −20°C. Isolation and subcellular fractionation of rat platelets was done according to [9]. The subcellular fractions and the whole homogenates were treated with 0.1 vol. 1% (v/v) Triton X-100 and stored at −20°C.

3. Results

3.1. *Labelled human Mn-SOD*

Fig.1 shows the chromatographic patterns which are obtained by gel filtration of the ^{125}I and $^{57}Co^{2+}$ labelling mixtures. In both cases 2 peaks are obtained. Peak I corresponds to the fractions which show the greatest immunoreactivity (95%), as determined by incubation of the tracer (10 000 cpm/400 μl diluent buffer) in an excess of antibody (100 μl antiserum RS3 100-fold diluted in the buffer). The specific radioactivity of the ^{57}Co purified tracer is 6 μCi/μg, corresponding to an incorporation of 2 atoms cobalt for 1 molecule of Mn-SOD apoenzyme. The specific activity of the iodinated tracer is 18 μCi/μg, corresponding to the binding of 1 mol [^{125}I]NSHPP/mol Mn-SOD.

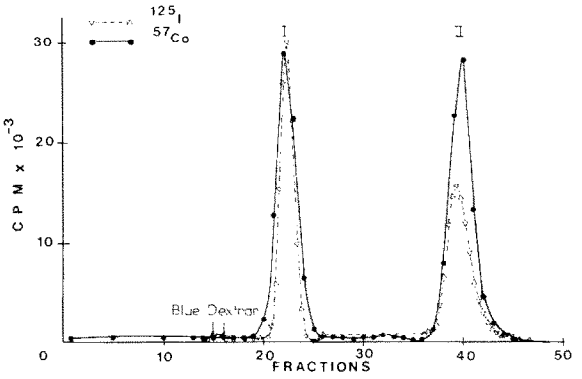

Fig.1. Ultrogel AcA 44 chromatography of the ^{57}Co and ^{125}I-labelling mixtures.

Stability assays have shown that the ^{57}Co tracer when stored at −20°C can be used for >5 months. However, in the absence of cobalt or manganese, the apoenzyme itself rapidly undergoes an irreversible aggregation which renders impossible any incorporation of ^{57}Co. The iodinated tracer is degraded more quickly and cannot be stored at −20°C for >1 month. It must be purified by Ultrogel chromatography before each series of assays.

3.2. *Standard curves*

The standard curves obtained with the two tracers for a 12 500-fold final dilution of RS3 antiserum, are identical (fig.2). The sensitivity, defined as the quantity of enzyme which displaces 5% of the radioactivity

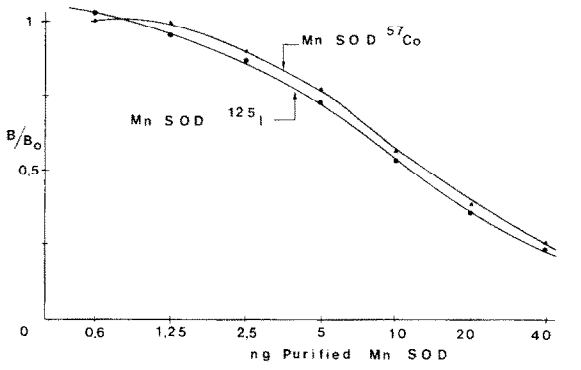

Fig.2. Standard curves of purified human Mn-SOD with two tracers (^{57}Co human Mn-SOD, ^{125}I Mn-SOD). Final dilution of RS3 antiserum: 1/12 500. In ordinates: ratio of radioactivities bound to antibody in presence (B) and in absence (B_0) of purified human Mn-SOD.

from the antibody sites, is ~300 pg, corresponding to 3.5 fmol protein.

3.3. *Specificity*

The dilution curves which are obtained with the homogenates of human liver and platelets appear to parallel those obtained with human purified Mn-SOD (fig.3). A similar result is obtained with human platelet-rich plasma treated with 0.5 vol. 10% (v/v) Triton X-100, and also with a serum containing a high level of Mn-SOD.

On the other hand, the dilution curves of rat and bovine liver homogenates, and rat lysed platelets do not parallel those obtained with human purified Mn-SOD (fig.4).

None of the following SOD containing preparations interfer in the system ^{125}I-labelled human Mn-SOD–RS3 anti-Mn-SOD antiserum: Mustellus liver extract (Mn-SOD); scyllium liver extract (Mn-SOD); purified bacterial Fe-SOD (*Photobacterium leiognathi*); purified human Cu-SOD; *Pleurotus olearius* Mn-SOD; bovine catalase (often contaminated with Cu-SOD).

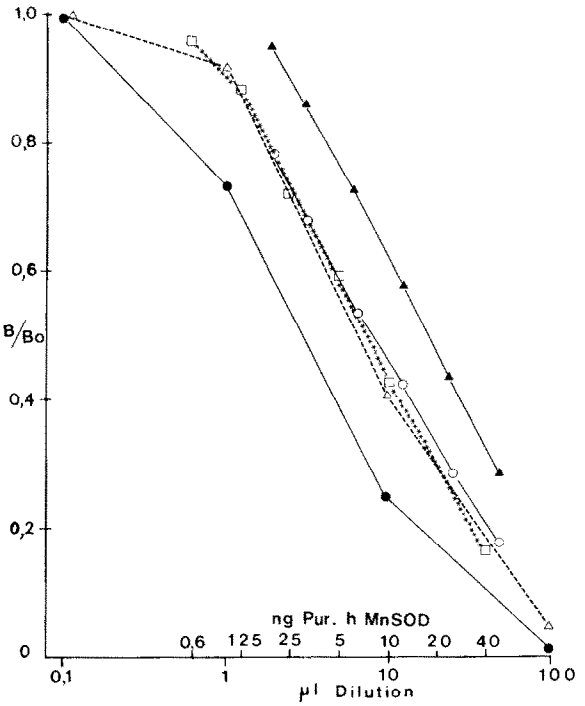

Fig.3. Dilution curves using human ^{125}I Mn-SOD and anti-human Mn-SOD antibodies (RS3, 12 500-fold diluted) with human material. Purified Mn-SOD (□***□); liver homogenate (△– – –△); Triton X-100 treated platelets (●——●); Triton X-100 treated normal platelet-rich plasma (○——○); and high level Mn-SOD serum (▲——▲).

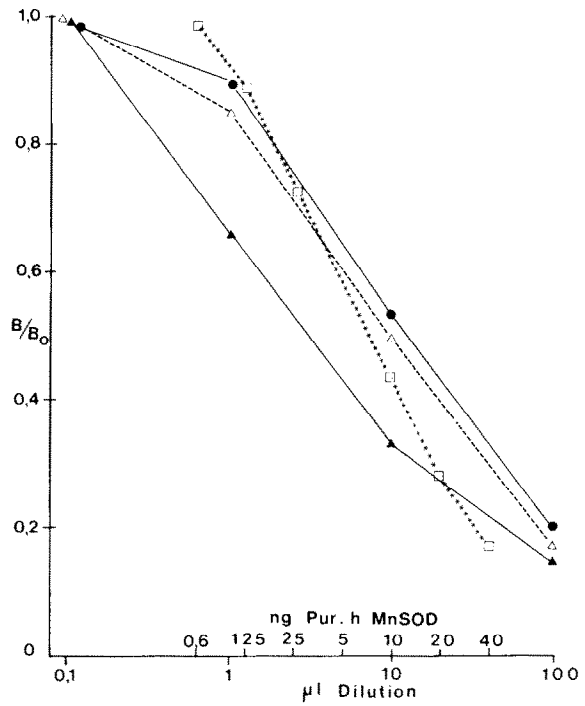

Fig.4. Crossreactions of purified human Mn-SOD (□***□); rat liver homogenate (▲——▲); Triton X-100 treated rat platelets (●——●); and bovine liver homogenate (△– – –△); in the system using human ^{125}I Mn-SOD and antihuman Mn-SOD antibodies (RS3, 12 500-fold diluted).

All of these preparations were tested for quantities of up to 10 µg SOD. It may be noticed that commercial bovine catalase which is contaminated by Cu-SOD [4,10] does not exhibit any crossreaction.

3.4. *Subcellular fractionation of rat liver and platelets*

The results of the assays made on the different subcellular fractions are shown in fig.5. The liver and platelet immunoreactive material are essentially present in the 8200 × *g* pellet, which corresponds to the mitochondrial fraction. Less than 1% of the liver Mn-SOD and 10% of platelet Mn-SOD are present in the cytosolic fraction.

4. Discussion

The lower limits of sensitivity of the radioimmunoassay of human Mn-SOD depend upon the respective qualities of the tracer and the antibody.

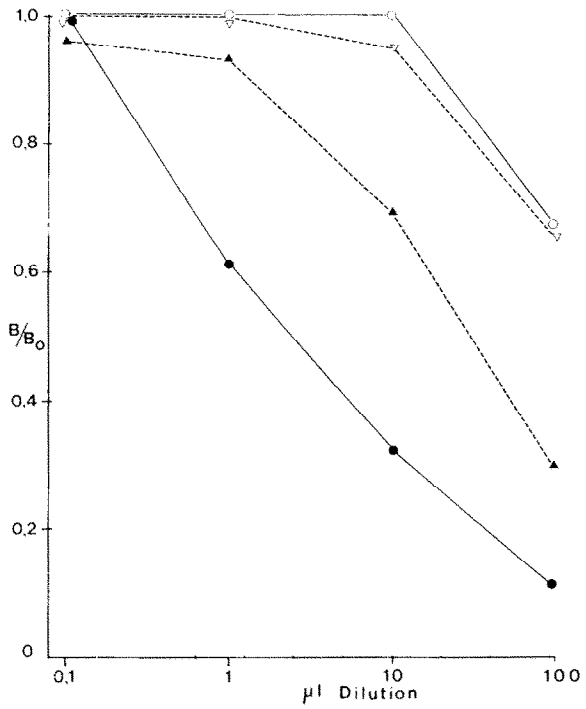

Fig.5. Dilution curves of rat liver and platelet subcellular fractions: 8200 × *g* pellet (●——●) and 100 000 × *g* supernatant (○——○) of rat liver homogenate. 8200 × *g* pellet (▲——▲) and 100 000 × *g* supernatant (△——△) of rat platelet homogenate.

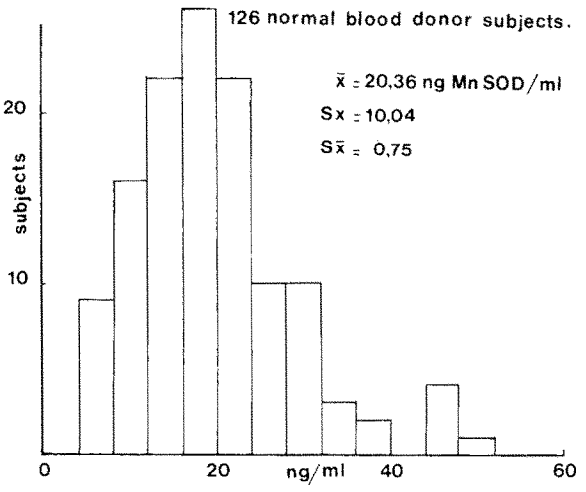

Fig.6. Distribution of values of serum Mn-SOD in normal male subjects.

Radioactive labelling of Mn-SOD had not been described, perhaps due to the instability of the enzyme [11]. The chloramine T technique gives aggregation of Mn-SOD with a loss of enzymatic and immunological activities but iodination with [^{125}I]NSHPP gives a suitable tracer. Labelling of the apoenzyme with ^{57}Co gives a much more stable tracer with the same characteristics as the iodinated molecule but with no enzymatic activity.

The specificity of the radioimmunoassay towards other SODs and other proteins is absolute. This result corresponds to that obtained with the radioimmunoassay of Cu-SOD [4]. This specificity is well established by:

(i) The parallelism of dilution curves of human serum or homogenates of human liver and platelets to purified Mn-SOD;
(ii) The lack of crossreactions with other preparations;
(iii) The localization of immunoreactive material in rat liver mitochondrial fractions [2].

Preliminary studies of crossreactions show an interspecies crossreaction between human, bovine and rat Mn-SOD in the homologous system human labelled Mn-SOD–antihuman Mn-SOD antibody. Since the standard curve obtained with purified human Mn-SOD does not parallel the dilution curves of the rat and bovine liver fractions, the crossreaction is not complete, but yet allows the assay of rat and bovine Mn-SOD with a homologous human system (human tracer and antihuman Mn-SOD). However, the interspecies specificity is less strict than that observed for the Cu-SOD radioimmunoassay, since in this case cross reactions only appear in the heterologous system [4].

As an example of application, we have used the radioimmunoassay of Mn-SOD and Cu-SOD in crude lung homogenates of rats submitted to hyperoxia (520 Torr and 570 Torr during 5 days) [12]. The results show that inductions of the two enzymes are not parallel. Indeed, under both pO_2 conditions there is no variation of Cu-SOD but an increase of Mn-SOD is observed.

As a second example, levels of Mn-SOD were determined in sera from 126 male blood donor subjects aged 18–60 years. The results are shown in fig.4. The average value is 20.36 ng/ml serum with a most frequent value of 19.3 ng/ml. A subdivision of the population (table 1) indicates that there is no significant variation of serum Mn-SOD with age (18–60 years).

Conjunction of the Cu-SOD and Mn-SOD radio-

Table 1

Age	n	Serum Mn-SOD $\bar{x}$[a] (ng/ml)	Sx[a]	$S\bar{x}$[b]
18–30	69	20.4	9.8	1.18
30–40	29	18	8.5	1.6
40–50	20	21.05	10.5	2.35
50–60	8	19.9	9	3.18

[a] Sx, standard deviation; [b] $S\bar{x}$, standard error of the mean

immunoassays provides an easy technique for the estimation of both enzyme proteins with high sensitivity in various biological systems.

The radioimmunoassay of Mn-SOD permits the detection and the assay of this enzyme in human serum, particularly in various pathological conditions. Preliminary results show that the most striking variations of Mn-SOD levels occur in liver diseases such as hepatitis and cirrhosis, with very high increases of serum Mn-SOD.

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