QUANTITATIVE DETERMINATION OF ACTIVITY OF MULTIPLE FORMS OF SUPEROXIDE DISMUTASE

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A quantitative method of determination of the activity of multiple forms of superoxide dismutase (SOD), based on separation of the forms of the enzyme of gel electrophoresis, elution from the gel, and determination of inhibition of the reduction of nitro-BT in a system of NADH and phenazine metasulfate, is described. Partially purified bovine erythrocyte SOD was shown to separate into three forms; the activity of form 2 is much lower than the degree of its staining for protein.
KEY WORDS: superoxide dismutase; multiple forms; method of determination of activity.

Many workers have shown that multiple forms of the enzyme superoxide dismutase (SOD, EC 1.15.1.1) exist and can be separated by column chromatography and electrophoresis in polyacrylamide gel (PAG). In particular, SOD isozymes have been found in the tissues of man [2], bovine liver [5], plants [8], and microorganisms [11]. In the modern view the function of SOD is natural protection of the organism against oxygen anion-radicals [6]; quantitative determination of the relative activity of the different forms of this enzyme is therefore very interesting.

Bohnenkamp and Weser [3] estimated SOD activity by densitometry of achromatic zones on gel columns after staining in a light–riboflavin–nitro-BT system. Beckman et al. [2] used a visual method of assesment after electrophoretic separation of the forms of SOD and staining for activity. The disadvantages of the densitometric method and, even more, of the visual method are well known and are fully discussed in the specialized literature [1].

A method of determining relative activity of the forms of SOD, based on fractionation of the enzyme by electrophoresis in PAG, followed by elution of the separate fractions and spectrophotometric determination of the activity of the eluates, is described below.

EXPERIMENTAL METHOD

Electrophoresis in PAG was carried out by Davis's method [4] in a "Reanal" apparatus with a voltage of 400 V and current of 4 mA applied to the tube for 7 h.

The method of Nishikimi et al. [10], with minor modifications, was used to stain the gels in order to detect SOD activity, and also for the spectrophotometric determination of the enzyme activity. After electrophoresis the gels were incubated for 20 min in 0.17 M pyrophosphate buffer, pH 8.3, containing 1.2 mM nitro-BT and 28 mM phenazine metasulfate (PMS); the gels were then washed with water and kept in 150 mM NADH, dissolved in the same buffer, until the pale bands of SOD activity were clearly developed (1-1.5 h).

Regions corresponding to the pale bands on the stained gel were cut out of the unstained gels. The pieces of gel thus cut out were homogenized in 1.90 ml 0.017 M pyrophosphate buffer, pH 8.3 (total volume together with gel 2.25 ml). The homogenates were kept for 12 h at 40°C, then centrifuged for 30 min at 800g. The resulting translucent eluate was used for determination of SOD activity.

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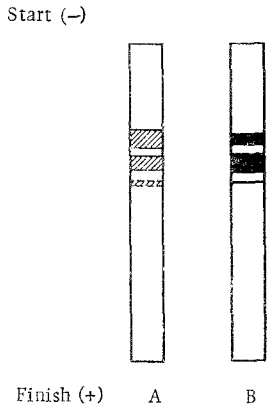

Fig. 1. Electrophoresis of dismutase in polyacrylamide gel (scheme). A) Staining for SOD activity; B) staining for protein.

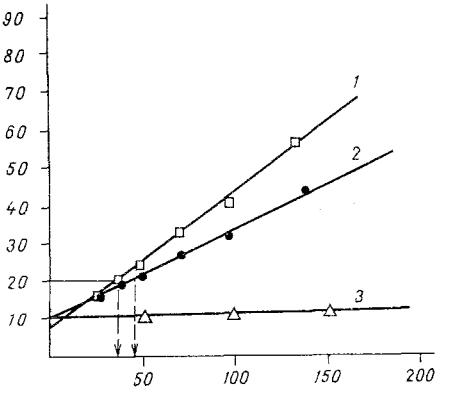

Fig. 2. Comparison of inhibitory activity of dismutase before and after electrophoresis in PAG: 1) dismutase activity after elution from gel; 2) dismutase activity without electrophoresis; 3) "inhibitory" activity of eluates of control gel. Abscissa, quantity of SOD (in μl); ordinate, reaction velocity (in $\%^{-1} \cdot 1000$).

The activity both of the original solution of SOD and of the eluates after electrophoresis was measured with a Hitachi-356 recording spectrophotometer as the degree of slowing by SOD of the rate of reduction of nitro-BT by the superoxide radical generated in the NADH–PMS system. The reaction mixture (3 ml) contained 150 nmoles nitro-BT, 234 nmoles NADH, 0.7 nmole PMS, and 0.017 M pyrophosphate buffer, pH 8.3. With these concentrations of components the change in optical density at 560 nm was 0.030 unit/min in the absence of inhibitor. After a short latent period the course of the reaction was linear for 4-5 min. Inhibition of the reaction by SOD was determined on the addition of 20, 30, 50, 75, 100, and 150 μl of the test solution, and from a graph plotted from these values the quantity of solution required to give 50% inhibition was calculated. The determination was carried out twice on each sample from one column of gel. The dilution of the sample was chosen so that 50% inhibition followed the addition of 30-60 μl of the solution, for with this level of activity the graphic constructions are most accurate and convenient. The measurements were made at 20°C. The quantity of enzyme required for 50% inhibition of the rate of nitro-BT reduction under the conditions described above was taken as the unit of SOD activity. Comparison showed that this unit of SOD was equal to 0.142 unit of enzyme activity determined by the method of McCord and Fridovich [9] with xanthine oxidase and cytochrome c. The protein content was determined by Lowry's method [7].

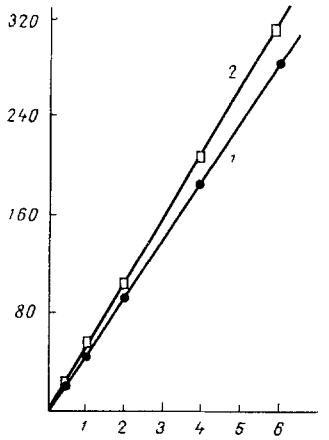

Fig. 3. Dismutase activity before and after electrophoresis in PAG as a function of quantity of enzyme applied: 1) before electrophoresis; 2) after electrophoresis. Abscissa, quantity of SOD (in μl); ordinate, SOD activity (in units).

TABLE 1. Activity of Electrophoretic Fractions of Bovine Erythrocyte SOD (M ± m)

SOD fraction	96 units applied to gel		288 units applied to gel	
	activity removed from gel, units	% of total activity	activity removed from gel, units	% of total activity
$SOD_1 + SOD_2 + SOD_3$	133,1±19,7	100	327,8±25,0	100
SOD_1	7,2±1,4	5,0±1	9,3±0,1	3±0
SOD_2	29,6±3,1	23,0±5	54,5±8,8	17±3
SOD_3	96,3±15,2	72,0±5	264,0±22,5	80±7

A partly purified preparation of SOD isolated from bovine erythrocytes by McCord and Fridovich's method [9] to the acetone precipitation stage was used as the test material. The precipitate obtained was dissolved in water and used as the original enzyme solution.

EXPERIMENTAL RESULTS

After electrophoresis in PAG and staining, three pale bands appeared on the gel columns and were described as SOD_1, SOD_2, and SOD_3 counting from the anode end of the gel. The corresponding dark bands were obtained by staining the gel for protein with Amido Black 10B (Fig. 1).

After fractionation of 44.6 units SOD on gel followed by elution of that part of the gel which contained all three bands of dismutase activity, it was found that virtually all the SOD had been removed from the gel and even the total activity of the eluate was a little higher than the activity of the SOD applied to the gel. This is partly due to the fact that eluates of the control gel (i.e., gel subjected to electrophoresis without application of SOD) has a weak inhibitory action on the reduction of nitro-BT (Fig. 2). Since the ammonium persulfate used for polymerization of the gel was found to inhibit the reduction of nitro-BT completely in a concentration of 2.5 mg/ml, the inhibitory effect of the gel could be due to trace amounts of persulfate remaining in the gel after electrophoresis and coming out with the eluates. Nevertheless, the activity of eluates containing all forms of SOD was higher, although not significantly ($\leq 15\%$), than the sum of activities of eluates of the control gel and the activity of the same quantity of SOD before application to the gel. The mechanism of this phenomenon remains unexplained. Since the ratio between the activities of the SOD applied and of the eluate was unchanged with a change in the quantity of enzyme applied to the gel (Fig. 3), this can evidently be disregarded when the ratio between the activities of the forms of SOD is determined. As Fig. 3 shows, activity of the eluate (less the activity of the control gel) is directly proportional to the quantity of enzyme applied to the gel and, consequently, the completeness of elution is unchanged by a change in the SOD concentration in the gel.

On this basis the activity of each SOD fraction was determined separately by cutting out and homogenizing the regions of the gel corresponding to the bands of activity. The results for two different SOD concentrations are given in Table 1.

The results, it must be noted, reflect the ratio between activities of SOD_1, SOD_2, and SOD_3 only in the particular sample of the enzyme, for the ratio varies for every preparation of the enzyme isolated.

During the elaboration of the method it was found that the activity of the forms of bovine erythrocyte SOD does not correlate with the intensity of staining of the bands for protein (Fig. 1). On visual estimation the SOD_2 fraction contains more protein than fraction SOD_3, which is more active as regards dismutation of oxygen anion-radicals. Several explanations of this phenomenon can be put forward: 1) Part of the staining for protein in the location of SOD_2 depends on foreign protein with electrophoretic properties very similar to those of SOD_2; 2) SOD_2 is partially inactivated during isolation; 3) the specific activity of SOD_2 is less than that of SOD_3.

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