ISOELECTRIC FOCUSING OF SUPEROXIDE DISMUTASE ISOENZYMES

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1. Introduction

As a scavenger of superoxide radicals in biological tissues, the enzyme superoxide dismutase (SOD) ($O_2^{\cdot-}$–$O_2^{\cdot-}$ oxido-reductase, EC 1.15.1.1) is an important factor in the protection against free radical damage [1,2]. SOD catalyzes the disproportionation or dismutation of superoxide free radicals by the following reaction:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2\,H^+ \rightarrow O_2 + H_2O_2$$

This metalloenzyme has been shown to exist in two forms in animal tissues, one containing manganese as cation (Mn-SOD) and the other containing copper and zinc as cations (Cu, Zn-SOD). The activities of the two enzymes are mutually distinguishable, as Mn-SOD is insensitive to CN^-, while Cu, Zn-SOD is inhibited by CN^- [2]. The trace metal status of an animal has been found to affect the activity of SOD. Inadequate levels of copper in the diet were associated with a reduction in the activity of Cu, Zn-SOD in swine [3] and in rats [4]. Similarly, Mn-SOD activity was low in animals fed manganese-deficient diets [5]. SOD activity has also been shown to be influenced by aging [6], genetic mutation [7,8], and disease [9]. Although it is thus evident that the activity of both Mn-SOD and Cu, Zn-SOD can be influenced by several factors, the mechanism(s) of the pertubation is (are) not known. Both Mn-SOD [10] and Cu, Zn-SOD [11] have been shown to exhibit several isoenzymes, but it is not known whether the low SOD activity observed in dietary deficiencies of copper or manganese is due to a reduction in one or more of their specific isoenzymes or all of their isoenzymes together.

Electrophoresis in polyacrylamide gels followed by a specific staining technique [12] has been employed by several investigators in the study of SOD [13]. The degree of band resolution of the isoenzymes has, however, been low, probably due to the inevitable zone-spreading which occurs in gel electrophoresis. Therefore, an extensive study of SOD isoenzymes by these techniques has not been possible. An additional problem is that the comparatively low activity of the Mn-SOD in some species makes it difficult to detect this form of the enzyme by gel electrophoresis. However, its presence has been verified by spectrophotometric assay of the activity [13] as well as by isolation and characterization of the enzyme [14].

In order to study SOD isoenzymes in tissues from animals of various nutritional states, genotypes and ages, a very sensitive technique is needed. Isoelectric focusing has the advantage of concentrating the protein zones as compared to electrophoresis where zone-spreading occurs. Thus, much lower quantities of enzyme (minor bands) can be detected by isoelectric focusing and also a much higher degree of resolution is achieved. This paper describes the application of isoelectric focusing to the study of SOD isoenzymes, using tissue samples from various species, with the successful separation of isoenzyme bands.

2. Materials and methods

Male albino rats (Sprague-Dawley, 100–150 g), mice (4 way cross of C57Bl/6J; C3H/J/ AKR/J and DBA/2J, 35–40 g) and white Leghorn chickens were used. All animals had been raised on stock diets

(Purina Rat Chow; Purina Lab. Chow (fed to mice); Purina Starter Chick Ration, Ralston Purina Co., St. Louis, MO). On the day of the experiment animals were anesthetized with ether and opened along the ventral midline. In order to remove blood from the tissues, the superior vena cava was isolated and severed to allow circulatory drainage. Isotonic saline (0.9%), made with deionized–distilled water, was perfused into the left atrium until the exudate was clear. Tissues were quickly excised and weighed. Liver tissue from a cow which had died ~20 min prior to sampling was obtained from an autopsy room. There was no evidence of liver disease.

The tissues were processed immediately or stored until time of analysis at −70°C. They were homogenized in 3 vol. 0.32 M sucrose, with the aid of an Elvehjem homogenizer with a tight fitting pestle. Homogenates were sonicated for 2 min (30 s with 30 s cooling) with an Insonator Model 500 (Savant Instr., Hicksville, NY) set at maximum power. The homogenates were then centrifuged at 10 000 × *g* for 60 min to sediment unbroken cells and other tissue debris. Assays were conducted on the supernatant.

SOD activity was determined by its ability to inhibit the autooxidation of epinephrine to adrenochrome by the method in [15].

Polyacrylamide gel electrofocusing was performed according to [16], but with some modifications. The concentrated gel solution (A) contained acrylamide (40 g) and bisacrylamide (0.4 g) in water (100 ml). The polymerization solution (B) contained riboflavin (14 mg) and tetramethylethylenediamine (1 ml) in water (100 ml). Solution A (10 ml) was carefully mixed with solution B (2.7 ml) and ampholytes (1 ml). The pH-ranges of the ampholytes used were 3–10 and 4–6.5 (Pharmalyte, Pharmacia Fine Chem., Piscataway, NJ). The gel mixture (1 ml) was thereafter mixed with water or sample (2 ml) and poured into each gel tube (0.5 × 10.0 cm). The final concentration of acrylamide was 10%, and 1% of the total acrylamide concentration was bisacrylamide ($T_{10}C_1$). Water was carefully layered on top of the gel mixture. Photopolymerization with riboflavin was used since introduction of the sample prior to chemical polymerization with ammonium persulfate may cause an oxidation of histidine and tryptophan residues of protein resulting in artifacts [17]. Polymerization was established after 1 h of ultraviolet-light exposure. When samples were layered on top of gels, glycerol (10%) and ampholytes (1%) were added to the sample and this mixture was then applied to the gel surface (anodic side). The sample mixture was protected from the acidic pH by a layer of glycerol (5%) also including ampholytes (1%). In both types of sample application, 25% tissue homogenates were used. The sample volume varied (50–150 μl) as the same amount of enzyme activity was applied.

The anode solution was 0.2% sulfuric acid and the cathode solution was 0.05 M sodium hydroxide. A commercially available electrophoresis cell (Bio-Rad model 158 A, Richmond, CA) was used. A current of 2 mA/gel was used and the final voltage was 500 V. Electrofocusing was performed at 10°C until no change in current occurred (also the coinciding formation of hemoglobin bands was followed). The running time was usually 3–5 h. To estimate the actual pH-gradient in the gel, a gel containing no sample was sliced (2 mm sections), each slice was soaked in water (1 ml), and pH was measured in these fractions after 2 h. Enzyme activity in the gels was detected by the staining procedures described below.

Following focusing, gels were stained by the method in [12]. By this technique, superoxide dismutase is localized in the gel by soaking it in 2.45 mM nitro-blue tetrazolium for 20 min, followed by 15 min immersion in a solution containing 28 mM tetramethylethylenediamine, 0.028 mM riboflavin, and 36 mM potassium phosphate at pH 7.8. The gels were illuminated by ultraviolet light for ~10 min. The gels became dark blue upon illumination except at positions where SOD was localized. Cu, Zn-SOD was distinguished from Mn-SOD by running two gels in parallel and adding potassium cyanide (20 mM) to one of them. The cyanide inhibited the copper–zinc form of SOD, allowing identification of the Mn-SOD bands. Gels were stored in $CH_3OH/CH_3COOH/H_2O$ (49/1/50, by vol.).

3. Results

The results of electrofocusing in the pH-range of 3–10 are shown in fig.1. As can be seen, cow liver contained 7 Cu, Zn-SOD and 2 Mn-SOD isoenzymes. Chicken liver contained 8 Cu, Zn-SOD and 3 Mn-SOD

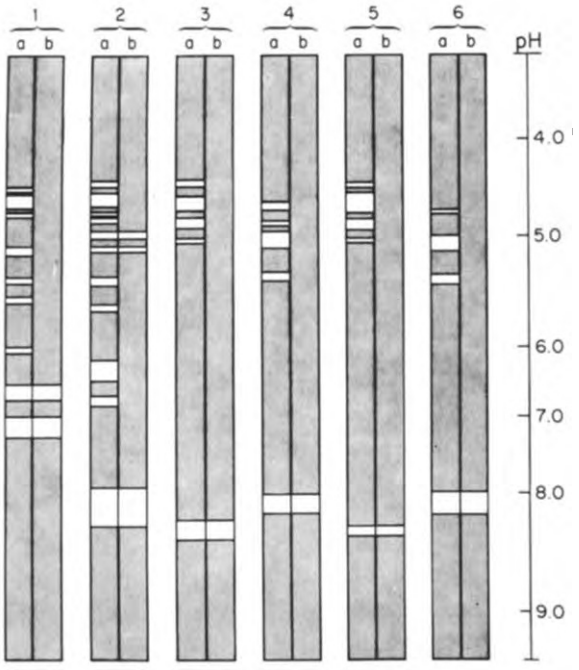

Fig.1. Polyacrylamide gel isoelectric focusing of cow liver (1), chicken liver (2), rat liver (3), rat brain (4), mouse liver (5), and mouse brain (6) homogenates. The gels, 10% acrylamide, were stained for superoxide dismutase activity in the absence (a) or presence (b) of 20 mM KCN. The pH-range of the ampholytes was 3–10.

bands. Rat and mouse tissues contained only one Mn-SOD isoenzyme, with an isoelectric point of ~8 as can be seen in fig.1. The Cu, Zn-SOD isoenzymes all had isoelectric points of 4.5–5.5, and in order to separate these bands further, focusing was performed with pH 4–6.5 ampholytes. The results of this experiment are shown in fig.2,3. The Mn-SOD band can still be seen in the bottom of the gels. A difference between tissues was obvious in both the rat and the mouse. Rat liver contained 4 and rat brain 5 Cu, Zn-SOD isoenzymes and their respective isoelectric points were different. Mouse liver contained 6 and mouse brain 3 Cu, Zn-SOD isoenzymes, and these differed in isoelectric points as well.

Direct addition of the sample to the gel mixture showed exactly the same band pattern as top loading of the gel with sample after polymerization. The direct addition method was chosen for reasons of technical simplicity.

A gel without sample was stained and showed no bands, demonstrating that SOD activity was not caused by reagents or ampholytes.

The positive gel stain described [10] was tried but proved to be less sensitive in our system than the negative staining technique in [12].

4. Discussion

A very high degree of resolution of SOD isoenzymes and a high sensitivity was obtained by the

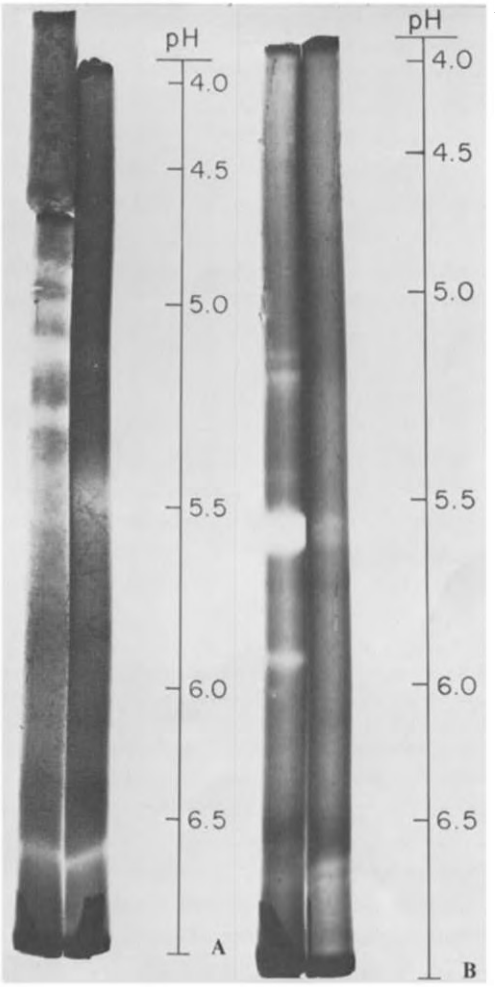

Fig.2. Polyacrylamide gel isoelectric focusing of rat liver (A) and rat brain (B) homogenates. The gels were stained for superoxide dismutase activity in the absence (left) and presence (right) of 20 mM KCN. The pH-range of the ampholytes was 4–6.5.

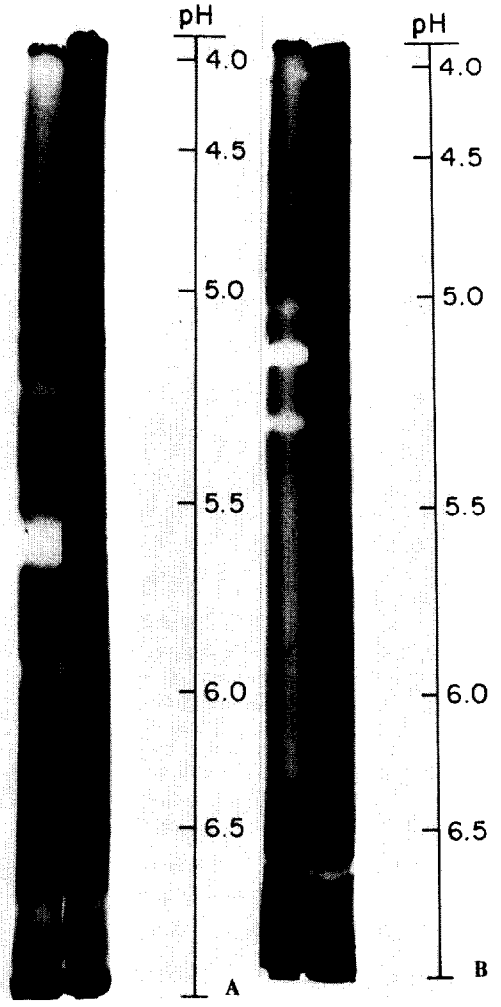

Fig.3. Polyacrylamide gel isoelectric focusing of mouse liver (A) and mouse brain (B) homogenates. The gels were stained for superoxide dismutase activity in the absence (left) and presence (right) of 20 mM KCN. The pH-range of the ampholytes was 4–6.5.

method we have described; the Mn-SOD, even with low activity, was easily detected. We were therefore able to confirm the presence of Mn-SOD in rat liver as in [14]. It can be noted that we found no difference in band position or increase in number of bands when the samples were repeatedly frozen and thawed, or left at room temperature for ⩽2 h. Mn-SOD from rat liver has been reported very sensitive and a loss of activity may easily occur [14]. We could not detect a corresponding decrease in activity by the electrofocusing technique, but as this band is one of the minor bands with respect to intensity, a substantial loss is probably needed before it is observed visually.

The isoelectric point of Cu, Zn-SOD from hog [18] and human erythrocytes [7] has been reported as 4.7, in close agreement to those we found for rat and mouse liver isoenzymes. The isoenzymes from bovine and chick liver are numerous as well as widespread in their isoelectric points, and need further characterization.

The largest of the Cu, Zn-SOD bands was sometimes not completely inhibited by cyanide. It appears that the usual concentration of CN^- was sufficient to wipe out 'normal' concentrations of the CN^--sensitive enzyme, but inadequate for a heavily concentrated major isoenzyme. When the CN^- concentration was increased, the weak 'false' Mn-SOD was wiped out while the true Mn-SOD band was still present.

With flat-bed isoelectric focusing, additional bands, shown to be artifacts, were found [19]. In our experience, this is sometimes a problem with the flat-bed technique, and a possible explanation is the free access of oxygen, which might be a potential hazard, since SOD is involved in oxygen metabolism. However, while multiple bands for the human Mn-SOD were shown [19] we found only one band in rat and mouse tissues. If multiple bands were introduced by the technique (ampholytes), or by proteolysis of sample, but only Cu, Zn-SOD isoenzymes but also Mn-SOD isoenzymes should have been detected. Furthermore, 4–6 isoenzymes were demonstrated [20] in fractionated rat liver cells, which is very similar to our findings. However, no specific staining for Mn-SOD activity was performed by these investigators.

The direct introduction of sample into the gel and the short running time of the experiment, together with the high capacity of the apparatus, provides a simple and rapid method for comparative studies. Isoelectric focusing for purified Cu, Zn-SOD from rat liver was used [6], but the method as we describe it permits the use of crude homogenates as well as simultaneous detection of both Cu, Zn-SOD and Mn-SOD isoenzymes. In summary, the high resolution and sensitivity of this method demonstrates its applicability to the study of isoenzymes, and particularly, that this method is suitable for the study of SOD-isoenzymes.

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