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PURIFICATION OF Cu, Zn-SUPEROXIDE DISMUTASE ISOENZYMES FROM FISH LIVER: APPEARANCE OF NEW ISOFORMS AS A CONSEQUENCE OF POLLUTION

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Liver cell-free extracts of fish (*Mugil sp.*) from polluted environments show new Cu,Zn-SOD isoenzymes when analyzed by polyacrylamide gel electrophoresis or isoelectrofocusing followed by *in situ* staining for SOD activity. The most active isoenzymes, with pI 6.1 and 5.1, were present both in control and problem samples while the isoenzymes of intermediate pI value showed significant differences. Fish from control areas showed three intermediate isoenzymes with pI 5.7, 5.5 and 5.4 (the last one quite faint) while polluted animals showed three bands of pI 5.9, 5.45 and 5.35, this last very intense. To further characterize their utility as biomarkers, Cu,Zn-SOD isoenzymes from polluted fish livers were purified to homogeneity. Five superoxide dismutase peaks were purified, named thereafter I (pI 6.1) to V (pI 5.1) respectively. Isoenzymes I and V displayed the highest specific activity. Upon incubation with moderate H_2O_2 concentrations, pure isoenzyme I yielded more acidic bands with pI 5.5, 5.45 and 5.35, this last being predominant. The pure isoenzyme V generated only a new band of pI 5.0. Concomitant with oxidation, the activity of peaks I and V was lost in a H_2O_2 concentration-dependent manner. The pattern of the new acidic bands generated upon the oxidixing treatment of isoenzyme I closely resembles that observed in crude extracts from polluted animals.

KEY WORDS: Cu,Zn-superoxide dismutase, *Mugil sp.*, purification, isoenzymes, pollution, oxidative stress.

INTRODUCTION

Superoxide dismutases (SOD) are a family of metalloenzymes that catalyze the disproportionation of superoxide anion into dioxygen and hydrogen peroxide, a substrate of catalase and peroxidases, according to the following reaction:[1]

$$O_2 \cdot^- + O_2 \cdot^- \rightarrow O_2 + H_2O_2$$

Eukaryotes have two major kinds of superoxide dismutase, a dimeric form ($2 \times 16\,kD$) containing 2 atoms of Cu and 2 atoms of Zn (Cu,Zn-SOD) present mainly in cytosol and nucleus,[2] recently also found in peroxisomes,[3] and a tetrameric form ($4 \times 20\,kD$) present in mitochondria which contains one Mn atom per subunit (Mn-SOD).[4]

Unless superoxide anion and hydrogen peroxide are readily removed they can generate the extremely reactive hydroxyl radical in a reaction catalyzed by iron or

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copper known as the Fenton reaction.[5] This reaction is highly plausible in the microenvironment centered around the active site of Cu,Zn-SOD because the close proximity of superoxide anion, hydrogen peroxide and Cu ions could easily form hydroxyl radicals.[6] Proteins undergo alterations in their biological activity, rupture of their polypeptide chain, or increased sensitivity to proteolytic attack.[5,7] In fact, Cu,Zn-SOD can itself be a primary target for oxidative modification by reactive oxygen species.[1]

The level of detoxifying enzymes has been extensively used as an early warning indicator of marine pollution.[8] Recently, antioxidant enzymes have also been proposed as bioindicators for environmental impact assessment,[9,10] due to the fact that both metals and certain organic xenobiotics can generate oxidative stress.[5] Increased levels of several detoxifying and antioxidative enzymes have been described in molluscs and fish from the Spanish South Atlantic littoral in response to environmental pollution. This is particularly important in the Huelva estuary, where the Tinto river brings Fe and Cu from pyrite mines and organic xenobiotics such as industrial pollutants and pesticides are released. So, molluscs and fish caught in that zone showed significant increases in SOD activity.[11,12] Instead of the total levels of detoxifying and antioxidative enzymes, the specific induction of some of their isoenzymes can be used as more sensitive biomarkers. Thus, increased levels of anionic glutathione transferases have been recently reported in fish from polluted environments.[13]

Cu,Zn-SOD shows several isoenzymic forms in tissues from different organisms, such as chicken, rat, mouse and bovine liver,[14] rat and mouse brain,[15] rat erythrocytes, and rat kidney.[15] Differential genetic expression in several tissues or differential secondary modifications which could generate intermediate forms have been proposed to explain Cu,Zn-SOD isoenzymes. An alternative explanation has recently been proposed by Yano,[16] which showed that some variants of rat liver Cu,Zn-SOD could be generated from an original protein by oxidative modification due to an attack by hydroxyl radicals. In fact, it has been shown that a number of proteins undergoes changes in structure and aminoacid composition after exposure to hydroxyl radicals.[17] In addition, the net charge of bovine erythrocyte Cu,Zn-SOD is modified by H_2O_2 in an oxidant-dependent manner which yields more negative isoforms.[18]

In the present work we show the presence of distinct isoenzymes of Cu,Zn-SOD in cell-free extracts from livers of *Mugil sp.* living in metal-contaminated environments. We have purified to homogeneity and characterized 5 of the major isoenzymes of fish-liver Cu,Zn-SOD by a procedure including FPLC ion exchange chromatography in DEAE-Sepharose and CM-Sepharose columns and FPLC chromatofocusing. Finally, the present paper reports that some isoenzymes with low specific activity arise from two main isoforms by oxidative modification.

MATERIALS AND METHODS

The marshes of the Odiel river (Huelva) were the polluted area selected since high levels of metals and organic compounds are found there. This is because the river brings down Cu ions from one of the biggest open-air copper mines of the world as well as waste from this intensive agricultural area and spills from chemical industries located nearby. As control or a less polluted area, we selected the marshes of the Guadalete river (Cádiz).

Immediately after the fish (grey mullet, *Mugil sp.*) were caught, the spinal cords

were excised, the livers extracted, immediately frozen in liquid nitrogen and stored at −80°C. The livers were ground in a mortar with liquid N_2. All operations described below were carried out at 4°C, except when indicated.

Preparation of cell-free extracts and the initial steps for purification of Cu,Zn-SOD were performed as follows. 50 grams of ground liver were mixed with of 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 5 mM GSH, 2 mM DTT and 0.5 mg/ml PMSF at a ratio of 2 ml per gram of liver and homogenized with Ultra-turrax (Janke & Kunkel) and then by sonication with three subsequent pulses of 20 sec each in a Braun Labsonic 2000 U apparatus at 28 W output. The homogenate was then cleared by ultracentrifugation at 105,000 × g for 2 h using a Beckman L8-80 M ultracentrifuge. The homogenate was dialyzed in 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA (buffer A) and this dialyzate was named the cell-free extract. The supernatant was heated at 60°C for 5 min, cooled at room temperature, and cleared by centrifugation at 12,000 × g for 15 min in a Beckman L2-21 centrifuge. The supernatant was extensively dialyzed overnight against 500 volumes of buffer A. A FPLC600 E model (Waters) was used for further purification of SOD isoenzymes (see the "Results" section).

Polyacrylamide gel electrophoresis (PAGE) of the samples in native form and in denaturing conditions was carried out by the method of Laemmli.[19] In addition, PAGE and isoelectrofocusing were carried out with Phastsystem equipment (Pharmacia). pI values of the SOD isoenzymes were determined in gels with pH gradient 4–6.5 and 3–9 using isoelectric focusing calibration kits (from Pharmacia). Staining for SOD activity was done immediately after electrophoresis using nitroblue tetrazolium, riboflavin and TEMED (from Sigma).[20] When gels for Phastsystem were used, the incubation time with NBT and RF was reduced to only 5 min because of its thinness. Proteins were stained with Coommassie brilliant blue R-250 and by silver staining,[21] according to an optimized method developed by Pharmacia for Phastsystem gels.

Protein concentration was estimated by the biuret-phenol[22] and the bicinchoninic acid methods,[23] using bovine serum albumin as the standard. SOD activity was determined spectrophotometrically by the standard xanthine/xanthine oxidase and cytochrome c assay at pH 7.8.[24] Xanthine, xanthine oxidase and cytochrome c were from Sigma. Molecular mass was determined by gel filtration on Sephadex G-100 using the following Mr standards: bovine serum albumin (67,000), ovoalbumin (45,000) and cytochrome c (12,400). Fluorescence spectra were recorded on a Kontron SFM25 spectrofluorometer. Signal intensity was calibrated against a 1.0 μM quinine hemisulfate solution in sulfuric acid.

Cu,Zn-SOD isoenzymes were treated with neuraminidase according to Jones and Masters:[25] 0.1 mg of neuraminidase (Sigma type V) was mixed with 0.1 mg of each five SOD isoenzymes (peaks I–V) in a total volume of 1 ml. The solution was buffered to pH 5.2 with 40 mM sodium acetate buffer and incubated at 37°C for 24 h and at 25°C for 72 h. For the control, the same procedure was carried out except that neuraminidase was boiled for 5 min.

RESULTS

Cu,Zn-SOD isoenzyme patterns of fish from control and polluted areas

Cell-free extracts from control and problem fish display clear differences in several SOD isoenzymes after separation by polyacrylamide gel electrophoresis and specific

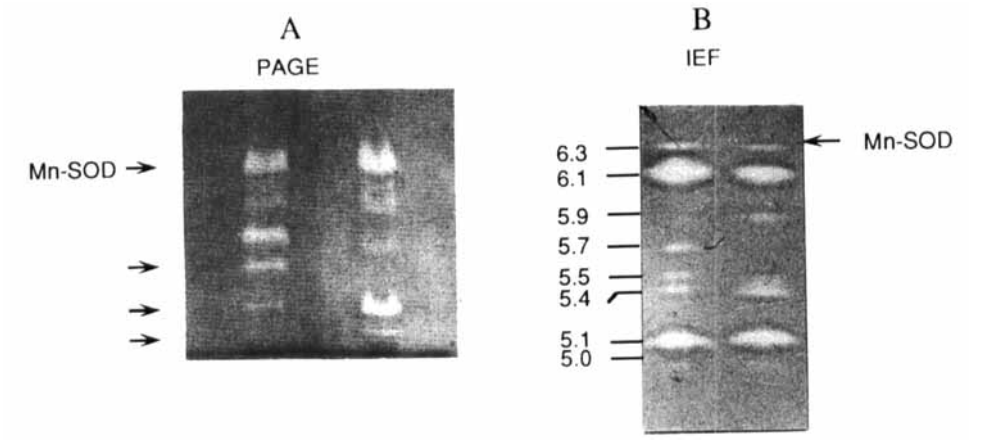

FIGURE 1 *SOD isoenzyme patterns in cell-free extracts from control (left) and polluted (right) fish.* A: Zymograms obtained after electrophoresis in 10–15% polyacylamide gradient gels. The arrows indicate differences between extracts from control (left) and polluted (right) samples. B: Zymograms obtained after separation by IEF in a 6.5 to 4.0 pH gradient. pI values were determined by using isoelectric focusing protein markers (from Pharmacia).

staining for SOD activity. As shown in Figure 1A, such differences were only appreciable in the Cu,Zn-SOD isoenzymes, while no differences were observed in the Mn-SOD after specific inhibition of the Cu,Zn-SOD isoenzymes with 1 mM potassium cyanide (data not shown). The three lower arrows in Figure 1A show the clearest differences between control and problem samples. The upper isoenzyme of this area was only present in the control, the next band increased drastically in the problem and a new band appeared in problem cell-free extracts, as indicated by the lower arrow. In addition, the position of the band with intermediate mobility changed in the problem samples. Quite different patterns of Cu,Zn-SOD isoenzymes were also observed when control and problem extracts were analyzed by isoelectrofocusing (Figure 1B). In addition to a faint Mn-SOD band with pI 6.3, seven additional Cu,Zn-SOD isoenzymes were also detected with pI ranging between 6.1 and 5.0. No differences were appreciable between control and problem samples with respect to the two most active isoenzymes with pI 6.1 and 5.1, respectively. Cu,Zn-SOD isoenzymes of intermediate pI values were clearly different in control and problem samples. Thus, while control animals showed three isoenzymes with pI 5.7, 5.5 and 5.4, the last band being quite faint, animals living in polluted environments showed a very intense band with pI 5.35 and two additional bands with pI 5.9 and 5.45.

To explore the possibility of new Cu,Zn-SOD isoenzymes being formed during the initial steps of homogenization, we compared the Ultraturrax plus sonication procedure decribed above with a much milder homogenization performed with a glass-teflon Potter Elvehjem, both in control and problem samples. The same SOD bands at exactly the same positions were observed with both homogenization procedures upon analysis by isoelectrofocusing.

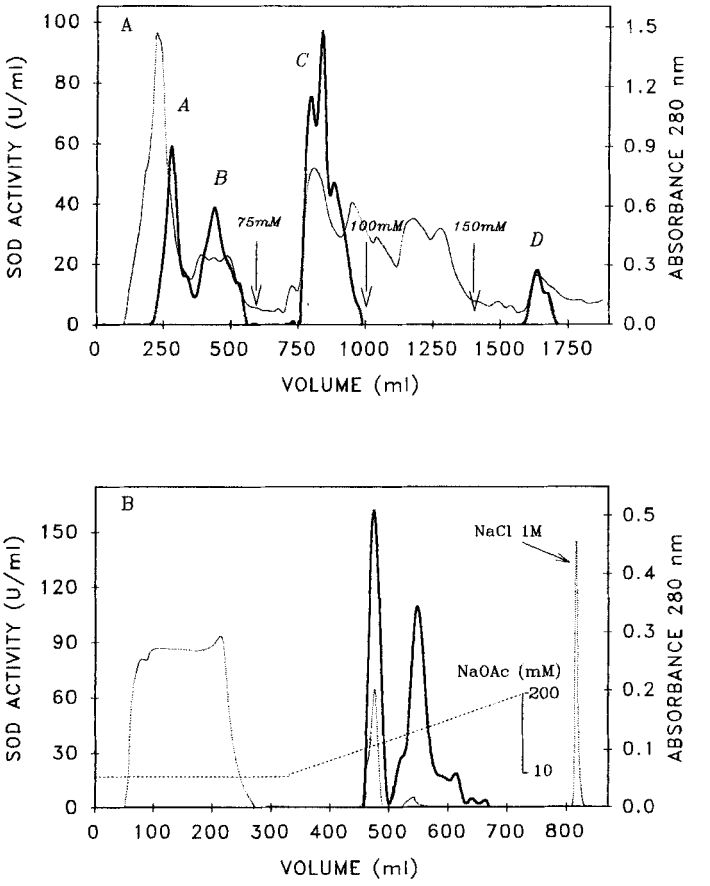

FIGURE 2 *Ion-exchange chromatography of SOD activity.* A: 39,000 U of SOD were loaded onto a DEAE-Sepharose fast flow. After washing with 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, the retained activity was eluted with three pulses of 75, 100 and 150 mM Tris-HCl pH 8.0, 0.1 mM EDTA. Peaks were named A to D according to their elution profile. B: 30,000 U of Cu,Zn-SOD were loaded onto a CM-Sepharose fast flow. After washing with 10 mM sodium acetate pH 5.0, 0.1 mM EDTA, the retained activity was eluted with a 10–200 mM sodium acetate gradient pH 5.0, 0.1 mM EDTA. An additional protein peak eluted with a 1 M NaCl pulse.

Purification of SOD isoenzymes

The existence of several SOD isoenzymes with different pI led us to carry out their purification in order to study the basis of such diversity. Thus purification of Cu,Zn-SOD from livers of fishes living in contaminated areas was undertaken.

After preparation of cell-free extracts, heating, centrifugation, and extensive dialysis (see Materials and Methods), the dialyzate was subsequently purified using three further steps by means of a FPLC system. The first step was an ion-exchange FPLC chromatography in a fast-flow DEAE-Sepharose column (21 × 2.5 cm) equilibrated with buffer A at a flow of 120 ml/h (Figure 2A). 39,000 SOD units were loaded into the column, which was then washed with 600 ml of buffer A and with

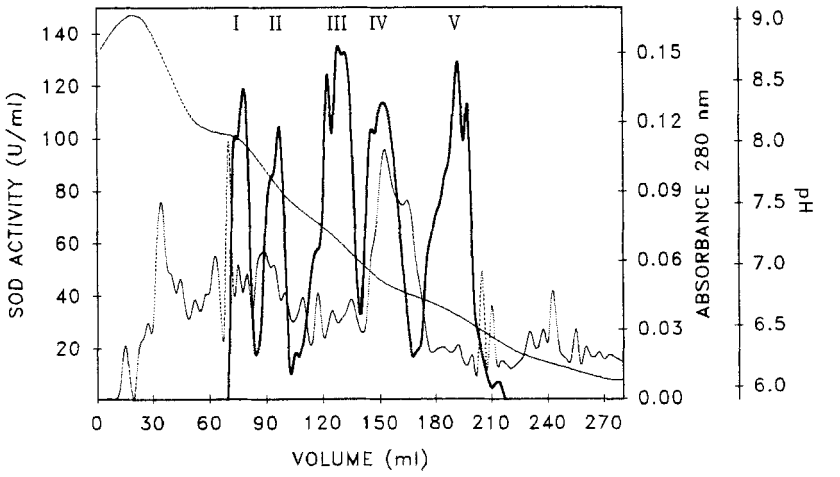

FIGURE 3 *Chromatofocusing of Cu,Zn-SOD activity.* 17,500 U of Cu,Zn-SOD were loaded onto PBE-94 previously equilibrated with 25 mM ethanolamine-CH_3COOH buffer, pH 8.3 and the column eluted with a mixture of polybuffer 96 (1:10): acetic acid pH 6.0, 0.1 mM EDTA. Peaks were named I to V according to their elution profile.

3 pulses of 400 ml each of Tris-HCl buffer pH 8.0 containing 0.1 mM EDTA, at concentrations 75 mM, 100 mM, and 150 mM, respectively. The Cu,Zn-SOD activity eluted in three distinct peaks (A,B,C) with some shoulders and smaller peaks during the initial washing with 10 mM Tris-HCl and with the 75 mM Tris-HCl pulse. On the contrary, the Mn-SOD activity was only eluted from the DEAE-Sepharose column after washing with 150 mM Tris-HCl, in a last and smallest peak (o). Fractions from peaks A,B and C containing Cu,Zn-SOD activity were combined, concentrated in SM 13200 ultrafiltration collodion bags (Sartorius) with a molecular exclusion range of 10,000 daltons and extensively dialyzed overnight against 10 mM sodium acetate buffer, pH 5.0, containing 0.1 mM EDTA (buffer B).

The dialyzate was then purified by ion-exchange FPLC chromatography in a fast-flow CM-Sepharose column (17.5 × 2.0 cm) equilibrated with buffer B at a flow of 120 ml/h (Figure 2B). 30,000 SOD units were loaded into the column which was eluted with a 10–200 mM linear gradient of sodium acetate buffer pH 5.0, containing 0.1 mM EDTA. The Cu,Zn-SOD activity eluted in two activity peaks which were pooled, concentrated by ultrafiltration and dialyzed for 8 hours with two changes of 200 volumes of 25 mM ethanolamine/acetic acid buffer pH 9.4 containing 0.1 mM EDTA (buffer C).

Finally, the last purification step consisted in FPLC chromatofocusing using a PBE-94 column (27 × 1 cm) equilibrated with buffer C and developed according to Pharmacia protocols with a mixture of polybuffer 96 (1:10)/acetic acid, pH 6.0. Figure 3 shows the Cu,Zn-SOD elution profile. The activity eluted in 5 distinct peaks I, II, III, IV and V. The purity of these five isoenzymes of Cu,Zn-SOD was analyzed by isolectrofocusing (Figure 4). Peak I consisted of a single isoenzyme of pI 6.1 which showed a single protein band upon protein staining. Peak II of Cu,Zn-SOD was a mixture of three protein bands, the one present in peak I plus two additional bands, with pI 5.45 and 5.1 also present in peak III. It should be mentioned, however,

TABLE I
Purification steps of Cu,Zn-SOD

Step	Volume (ml)	Protein (mg)	Units	Spec. activ. (U/mg)	Yield (6)	Purification (-fold)
Homogenate	84	2,882	50,643	17.6	100	1.0
Heat at 60°C	64	571	39,767	69.6	79	4.0
DEAE-Sepharose	168	99.70	30,478	305.7	60	17.4
CM-Sepharose	12.8	10.40	17,739	1,705.7	35	96.9
Peak I	11.5	1.41	2,231	1,582.2	4.4	89.9
Peak II	12.0	2.40	1,961	817.1	3.9	46.4
Peak III	14.6	2.76	2,777	1,006.0	5.5	57.2
Peak IV	22.0	1.41	3,850	2,730.5	7.6	155.1
Peak V	25.0	1.55	3,300	2,129.0	6.5	121.0

that both peaks II and III were pure since the same bands were observed upon staining for SOD activity and for protein. Peaks IV and V of Cu,Zn-SOD consisted of a single isoenzyme of pI 5.1, highly homogeneous according to protein staining. It should be noted that although the position of bands IV and V were coincident after isoelectrofocusing, they were differenciated by gel electrophoresis under non-denaturing conditions (results not shown).

Table I summarizes the purification of Cu,Zn-SOD from fish liver. Ion-exchange in DEAE-Sepharose purified Cu,Zn-SOD 4.4-fold while chromatography on CM-Sepharose produced an additional 5.5-fold purification. The crucial step on purification was chromatofocusing which allowed the separation of the five different forms whose specific activity ranged from 817 U/mg (46-fold purification) for peak II, to 2,730 U/mg (155-fold purification) for peak IV.

Characteristics of Cu,Zn-SOD isoenzymes from Mugil sp.

After the different Cu,Zn-SOD isoenzymes had been purified to homogeneity and separated by chromatofocusing, further investigations were undertaken to establish the basis of such heterogeneity and the relationship between certain specific isoenzymes as well as the exposure of the fish to environmental contaminants.

The different Cu,Zn-SOD isoenzymes, initially separated by their charge to mass ratio upon gradient polyacrylamide gel electrophoresis (Figure 1A), displayed identical molecular weight, 31 kD and subunit molecular mass, 15.9 kD, when analyzed respectively by gel filtration on Sephadex G-100 and PAGE under denaturing conditions (results not shown). Thus their separation by PAGE or IEF in cell-free extracts (Figures 1A and B) should be due to their charge properties because several activity peaks were separated by DEAE-and CM-Sepharose (Figure 2) and upon chromatofocusing (Figure 3). Isoelectrofocusing of peaks I to V finally confirmed that they were formed by combinations of three molecular entities of pI 6.1, 5.35 and 5.1, respectively (Figure 4).

The charge differences of Cu,Zn-SOD isoenzymes were not due to the presence of sialic acid residues since extensive incubation with neuraminidase did not alter their respective charges when analyzed by IEF (not shown). On the other hand, the emission fluorescence spectra of identical concentrations of peaks I to V revealed clear differences (Figure 5). Peak I displayed the highest quantum yield while peak

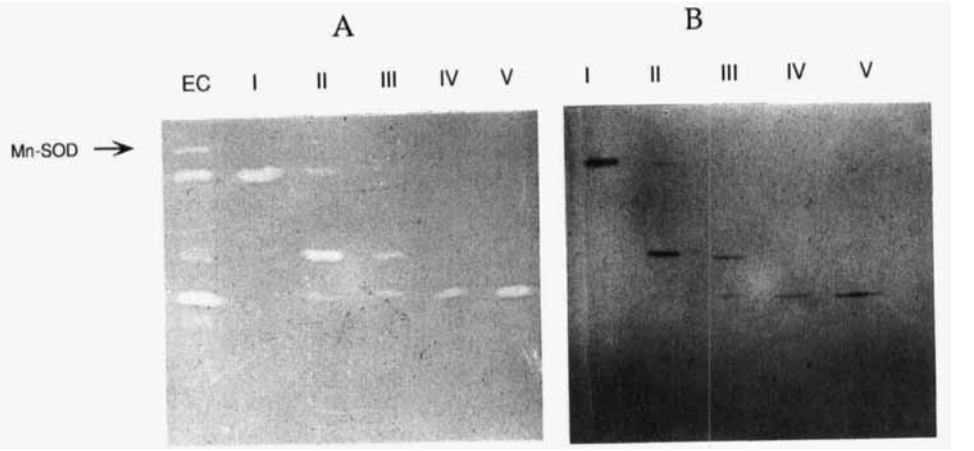

FIGURE 4 *Analysis by isoelectrofocusing of the Cu,Zn-SOD isoenzymes.* Peaks I to V obtained after FPLC chromatofocusing were analyzed by IEF using the PhastSystem gels of Pharmacia with a pH gradient 4 to 6.5. In A, the gel was stained for activity (EC, crude extract, and peaks I to V), and in B, for protein using the silver staining.

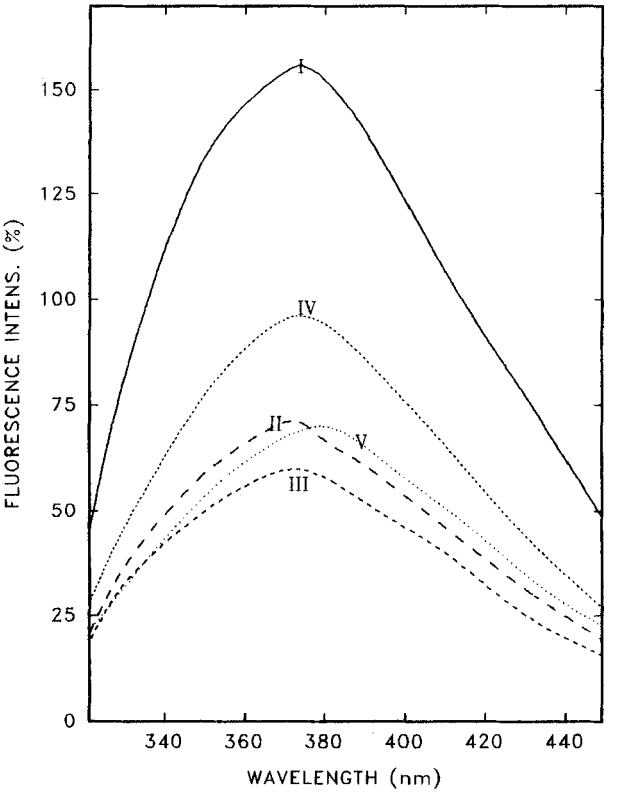

FIGURE 5 *Fluorescence spectra of peaks I to V Cu,Zn-SOD.* An emission spectrum of each peak was carried out with 38 μg/ml protein concentration. For all spectra, samples were excited at 280 nm wavelength.

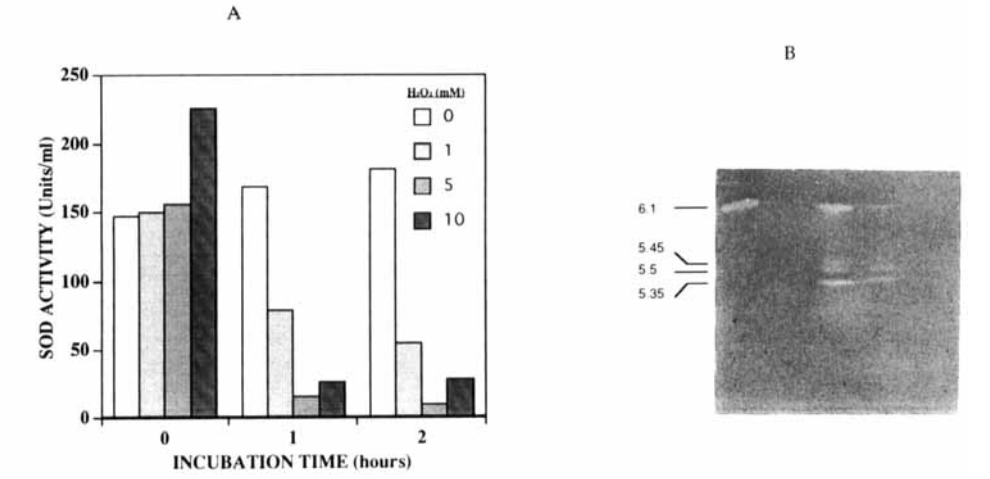

FIGURE 6 *Effect of incubation with H_2O_2 on the activity and isoelectrofocusing zymogram of pure peak I Cu,Zn-SOD.* 3 μg of pure peak I Cu,Zn-SOD was incubated either in the absence or the presence of 1 to 10 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA at 25°C, in 0.05 ml final volume. A: The SOD acfivity was determined at the times indicated. B: Aliquots were analyzed after 1 hour of incubation by means of IEF with a pH gradient 4 to 6.5. The left lane was incubated without H_2O_2, the next three lanes with 1, 5, and 10 mM H_2O_2, respectively. The gel was subsequently stained for activity. pI values were determined as in Figure 1.

III showed the lowest one. In addition, while peak I showed maximum emission at 374 nm, the maximum for peaks II, III and IV shifted to shorter wavelengths (370, 372 and 372 nm, respectively) and for peak V to longer ones (378 nm), suggesting that their fluorophores should be surrounded by a more hydrophobic (for peak II) or hydrophilic (for peak V) environment compared to the other peaks. Such divergences suggested different conformations of the five isoenzymes which would affect the environments of the fluorophores in each protein.

Effect of H_2O_2 on isoenzymes I and V of Cu,Zn-SOD

The possibility of some of the SOD isoforms deriving from others by oxidative modification was then investigated. Figure 6 summarizes the results obtained. Incubation of pure peak I SOD isoenzyme with different H_2O_2 concentrations led to a progressive loss of activity which was concentration-dependent. Thus the enzymatic activity declined to 45% and 8% after 1 h incubation in the presence of 1 mM and 5 mM H_2O_2, respectively, and lower activities were determined after 2 h incubation (Figure 6A). The pure peak I isoenzyme showed a single activity band of pI 6.1 after IEF (Figure 6B). However 1 hour exposure to different H_2O_2 concentrations promoted a decrease in the band of pI 6.1 concomitant with the appearance of new more acidic bands with pI 5.5, 5.45 and 5.35 (the two last bands presented an intense staining of SOD activity) with a pattern very similar to that previously observed in zymograms from cell-free extracts of polluted animals. It

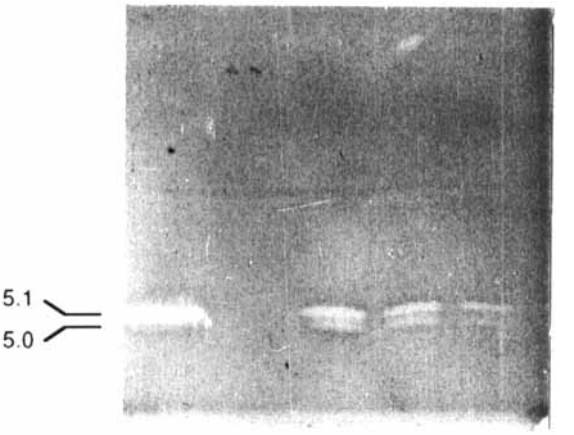

FIGURE 7 *Effect of incubation with H_2O_2 of the activity and isoelectrofocusing zymogram of pure peak V Cu,Zn-SOD.* 1.55 μg of pure peak V Cu,Zn-SOD isoenzyme was incubated either in the absence (left lane) or in the presence of 0.5, 1, 1.5 and 2 mM H_2O_2 in 50 mM potasium phosphate buffer, pH 7.8, 0.1 mM EDTA at 25°C in 0.05 ml final volume. Aliquots were removed after 2.5 hours incubation and analyzed by IEF with pH gradient ranging from 3.0 to 9.0. The IEF gel was then stained for activity. pI values were determined as in Figure 1.

should be noted that the low pI bands did not form when peak I SOD was incubated with H_2O_2 at concentrations below 0.3 mM. The bands with pI 5.1 and 5.0 never formed from peak I SOD isoenzyme. When 10 mM H_2O_2 was used, the band of pI 6.1 disappeared completely and only two faint bands of pI 5.45 and 5.35 were still visible (Figure 6B). The effect of H_2O_2 on pure peak V SOD isoenzyme was also investigated (Figure 7). The single activity band with pI 5.1 present in peak V yielded a new more acidic band of pI 5.0 upon incubation under oxidizing conditions. A parallel decrease in activity was observed in both bands after H_2O_2 incubation, thus the activity after 2.5 hours in the presence of 2.0 mM H_2O_2 was only 20% of the untreated control. Bands with pI over than 5.1 never formed from peak V.

DISCUSSION

The present work started with the initial observation that fish living in contaminated environments displayed distinct Cu,Zn-SOD isoenzyme patterns, a fact that could be used for the biomonitoring of environmental pollution, as recently reported for glutathione transferase.[13] Thus, our present efforts have been focused on the purification and characterization of five Cu,Zn-SOD isoenzymes present in red mullet livers (*Mugil sp.*).

Isoenzymes from Cu,Zn-SOD have been previously described in various tissues of chicken, rat, mouse and bovine origin. In all cases the isoenzymes showed identical molecular mass, differing only in charge.[14,15] We have succeeded in purifying to homogeneity the major Cu,Zn-SOD isoenzymes present in *Mugil sp.* cell-free extracts by a combination of heat treatment, ion-exchange FPLC chromatographies on

DEAE-Sepharose and CM-Seharose, respectively, and a final chromatofocusing step which allowed a clear-cut separation of 5 different peaks of activity. The Mn-SOD from *Mugil sp.* was separated by the DEAE-Sepharose chromatography. It is interesting to note that Mn-SOD was tightly bound to the DEAE-Sepharose column at pH 8.0, although its isoelectric point was 6.3 and should have been the least retained by the anion-exchange column. Similar chromatographic behaviour has been described for Mn-SOD of glyoxysomes from watermelon cotyledons.[26] The 5 Cu,Zn-SOD isoenzymes were homogeneous when identical IEF gels were stained for activity and protein. The isoenzymes isolated as peaks I, IV and V after chromatofocusing contained one single band (pI 6.1, 5.1 and 5.1, respectively) and displayed high specific activities, 1,582, 2,730 and 2,129 U/mg, respectively. In addition to their behaviour in chromatofocusing, peaks IV and V could be distinguished by their different mobility in PAGE under non-denaturing conditions, thus indicating that they were not identical molecular entities. The isoenzymes II and III, with the lowest specific activities (817 and 1,006 U/mg, respectively), contained an additional protein band of pI 5.35. The five Cu,Zn-SOD isoenzymes also diverged in their fluorescence properties, with peaks I and V showing high quantum yields and the fluorescence maximum of peak V shifted to longer wavelengths, suggesting either distinct conformations or different foldings around their fluorophores. Considered all together, the properties of peaks I to V showed that the protein bands with pI 6.1 and 5.1 were associated with highly active isoenzymes, while the band of pI 5.35 characterized the less-active isoenzymes.

It is well established that proteins exposed to hydroxyl radicals undergo oxidative modifications leading to both fragmentation and aggregation.[17] It has been recently shown that Cu,Zn-SOD from bovine erythrocytes is a target for oxidative modifications and losses in enzymatic activity in a concentration dependent manner when exposed to H_2O_2, the product of the reaction that it catalyzes.[18] Recently, it has been shown that Cu,Zn-SOD catalyzes the formation of hydroxyl radical from hydrogen peroxide.[6] So, the two high activity Cu,Zn-SOD isoenzymes from *Mugil sp.*, peaks I and V, lost activity when incubated with moderate H_2O_2 concentrations in a time- and concentration-dependent process. In parallel with this inactivation, new protein bands with more acidic pI were formed in both cases similar to those characteristic of cell-free extracts from animals living in polluted environments. It could be argued that an apparent contradiction could exist between the relative increase in Cu,Zn-SOD observed in fishes from metal-polluted sites[11] and the decrease in Cu,Zn-SOD activity produced *in vitro* by H_2O_2. It should be noticed however that the *in vitro* inactivation of SOD, since it is time-dependent (as shown in Figure 6A), is only observed at very high H_2O_2 concentrations, ranging from 0.3 to 10 mM. Such hydrogen peroxide concentrations are much higher than the intracellular physiological H_2O_2 concentrations, in the order of 10^{-8} M in rat liver[27]. For this reason, the above mentioned contradiction is only apparent, since oxidative stress can increase SOD levels as an overall response of the organism, and at the same time induce the appearance of lower pI isoenzymes, the same which can be reproduced quite rapidly during *in vitro* incubation with H_2O_2 at higher concentrations.

It has been proposed that inactivation of Cu,Zn-SOD by H_2O_2 involves the reduction of the enzyme bound Cu^{+2} to Cu^{+}, followed by a Fenton-type reaction of this Cu^{+} ion with H_2O_2; this would generate the Cu^{+2}-OH· complex. In fact, by using spin traps, Yim *et al.*[6] have demonstrated that Cu,Zn-SOD catalyzes the formation of hydroxyl radical from H_2O_2. So, that radical could oxidatively attack

an adjacent histidine residue thus inactivating the enzyme.[28] Recent work has established that Cu,Zn-SOD also catalyzes the nitration of one of its own tyrosine residue from peroxynitrite, yielding more negatively charged superoxide dismutase variants.[29] It has been clearly established that glutamine syntethase is oxidatively inactivated by H_2O_2 through modification of a histidine residue which is converted to carbonyl derivative, resulting in the loss of one positive charge,[7] thus yielding a more negative protein. A similar mechanism could be responsible for the intermediate pI forms observed in cell-free extracts from fishes living in contaminated environments.

Liver of fishes from polluted areas contain 3.7 and 1.5-fold higher Cu and Fe concentration than those from cleaner areas.[11] Since Cu and Fe catalyze the generation of oxygen active species, animals living in metal-polluted sites would be subjected to oxidative stress. One of these oxygen-active species, H_2O_2, inactivates and possibly oxidizes Cu,Zn-SOD. Recently, Cu,Zn-SOD has been demonstrated to be a peroxisomal enzyme by immunofluorescence using monoclonal antibodies. Therefore, the enzyme is presumably located close to sources of superoxide anion, which it converts into H_2O_2. In addition, the enzyme can suffer from the deleterious effect of the hydroxyl radicals that it itself generates from hydrogen peroxide.[6] In summary, we suggest that the Cu,Zn-SOD isoenzymes of intermediate pI can be useful biomarkers for metals or organic xenobiotics able to generate oxidative stress.

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References

1. I. Fridovich (1975) Superoxide dismutases. *Annual Review of Biochemistry*, **44**, 147–159.
2. L.-Y. Chang, J.W. Slot, H.J. Geuze and J.D. Crapo (1988) Molecular immunocytochemistry of the Cu,Zn-superoxide dismutase in rat hepatocytes. *Journal of Cell Biology*, **107**, 2169–2179.
3. G.-A. Keller, T.G. Warner, K.S. Steimer and R.A. Hallewell (1991) Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. *Proceedings of the National Acadamy of Sciences of USA*, **88**, 7381–7385.
4. J.W. Slot, H.J. Geuze, B.A. Freeman and J.D. Crapo (1986) Intracellular localization of the copper-zinc and manganese superoxide dismutases in rat liver parenchymal cells. *Laboratory Investigation*, **55**, 363–371.
5. H. Sies (1986) Biochemistry of oxidative stress. *Angewandte Chemie International Edition in English*, **25**, 1058–1071.
6. M.B. Yim, P.B. Chock and E.R. Stadtman (1990) Copper-zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide. *Proceedings of the National Academy of Sciences of USA*, **87**, 5006–5010.
7. R.L. Levine (1983) Oxidative modification of glutamine sinthetase. *Journal of Biological Chemistry*, **258**, 11823–11827.
8. D.R. Buhler and D.E. Williams (1988) The role of biotransformation in the toxicity of chemicals. *Aquatic Toxicology*, **11**, 19–28.
9. D.R. Livingstone (1991) Towards a specific index of impact by organic pollution for marine invertebrates. *Comparative Biochemistry and Physiology*, **100C**, 151–155.
10. G.W. Winston and R.T. Di Giulio (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology*, **19**, 137–161.
11. A. Rodriguez-Ariza, N. Abril, J.I. Navas, G. Dorado, J. López-Barea and C. Pueyo (1992) Metal,

mutagenicity, and biochemical studies on bivalve molluscs from Spanish coasts. *Environmental Molecular Mutagenesis*, **19**, 112–124.
12. A. Rodriguez-Ariza, G. Dorado, J. Peinado, C. Pueyo and J. López-Barea (1991) Biochemical effects of environmental pollution in fishes from Spanish South-Atlantic littoral. *Biochemical Society Transactions*, **19**, 301S.
13. E. Martínez-Lara, P. Pascual, F. Toribio, J. López-Barea and J.A. Bárcena (1992) A rapid method for the quantification of glutathione transferase isoenzymes in crude extracts. *Journal of Chromatography*, **609**, 141–146.
14. B. Lönnerdal, C.L. Kee and L.S. Hurley (1979) Isoelectric focusing of superoxide dismutase isoenzymes. *FEBS Letters*, **108**, 51–55.
15. N. Crosti and P. Sausa (1980) Evidence for tissue-specific electromorphs of Cu/Zn-SOD unrelated to genetic control. *Biochemical Genetics*, **18**, 693–697.
16. S. Yano (1990) Multiple isoelectric variants of copper, zinc-superoxide dismutase from rat liver. *Archives of Biochemistry and Biophysics*, **279**, 60–69.
17. K.J.A. Davies (1987) Protein damage and degradation by oxygen radicals. *Journal of Biological Chemistry*, **262**, 9895–9901.
18. D.C. Salo, R.E. Pacifici, S.W. Lin, C. Giulivi and K.J.A. Davies (1990) Superoxide dismutase undergoes proteolysis and fragmentation following oxidative modification and inactivation. *Journal of Biological Chemistry*, **265**, 11919–11927.
19. U.K. Laemmli (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
20. C.O. Beauchamp and I. Fridovich (1971) Superoxide dismutase improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*, **44**, 276–287.
21. J. Heukeshoven and R. Dernick (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis*, **6**, 103–112.
22. O.H. Lowry, M.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the Folin phenol reagents. *Journal of Biological Chemistry*, **193**, 265–275.
23. P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, **150**, 76–85.
24. J.M. McCord and I. Fridovich (1969) Superoxide dismutase: an enzymic function for erythrocuprein. *Journal of Biological Chemistry*, **244**, 6049–6055.
25. G.L. Jones and C.J. Masters (1975) On the nature and characteristics of the multiple forms of catalase in mouse liver. *Archives of Biochemistry and Biophysics*, **169**, 7–21.
26. P. Bueno and L.A. del Río (1992) Purification and properties of glyoxysomal cuprozinc superoxide dismutase from watermelon cotyledons (Citrullus vulgaris Schrad). *Plant Physiology*, **98**, 331–336.
27. B. Chance, A. Boveris, Y. Nakase and H. Sies (1978) Hydroperoxide metabolism: an overview, in *Functions of glutathione in liver and kidney* (ed. H. Sies and A. Wendel) Springer-Verlag, Berlin Heidelberg, pp. 95–106.
28. E.K. Hodgson and I. Fridovich (1975) The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme. *Biochemistry*, **14**, 5294–5303.
29. H. Ischiropoulos, L. Zhu, J. Chen, M. Tsai, J.C. Martin, C.D. Smith and J.S. Beckman (1992) Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Archives of Biochemistry and Biophysics*, **298**, 431–437.

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