Studies on cytosolic superoxide dismutase from intestinal mucosa

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CuZn superoxide dismutase from monkey (Macaca radiata) intestinal mucosa was purified to homogeneity. The enzyme showed a subunit molecular weight of 16000. The enzyme preparation from intestinal mucosa of rat, rabbit, guinea-pig and monkey was distinctly different in electrophoretic mobility and in elution profile on ion-exchange chromatography, possibly due to their difference in charge. The difference may not be due to glycosylation, since the enzyme was not stained for glycoprotein. Polyclonal antibody against purified monkey enzyme inhibited the activity of intestinal CuZn superoxide dismutase from rat, rabbit and guinea-pig. Thus it appears that intestinal CuZn superoxide dismutases from different sources, despite being similar in immunological and other properties, differ in certain amino acids and hence in charge.

In recent years, free radical-induced lipid peroxidation has been implicated as a possible pathological mechanism in a number of disease conditions such as respiratory distress syndrome, stroke, reperfusion injury of a variety of organs, diabetes, and oncogenesis4–6. Free radicals alter the activity of certain key membrane enzymes which influence cell division5. It has been pointed out that peroxidation damage to cell leads to carcinogenesis6. Recently, there has been an increasing interest in the possible protective role of antioxidants in various disease conditions. Antioxidants such as α-tocopherol and the enzymes superoxide dismutase, catalase and glutathione peroxidase offer protection to the cell from peroxidation damage.

Although several studies have been carried out on lipid peroxidation in different tissues7–10, very little is known about peroxidation and the role of antioxidant enzymes in the gastrointestinal tract. This tissue is vulnerable to peroxidation due to presence of inducers of peroxidation such as bacterial metabolites11, the dietary peroxidised lipids and iron in the lumen of the intestine and accumulation of fat droplets inside the epithelial cells, as seen in certain pathological conditions12. In addition, the mucosal cells of the intestine contain high xanthine oxidase activity which could generate free radicals13. Our previous studies on peroxidation of gastrointestinal mucosa have shown that free monounsaturated fatty acids present in this membrane prevent the cells from peroxidation14,15. We have earlier shown the presence of α-tocopherol and antioxidant enzymes in the gastrointestinal mucosa16,17.

Superoxide dismutase is one of the antioxidant enzymes which protect the cell against ischemic injury and haemorrhagic lesions in the small intestine18,19. Although a comparative study on structure-function relationship of superoxide dismutase has been carried out in other tissues20, very little is known about this enzyme in the gastrointestinal mucosa. We describe here purification to homogeneity of CuZn superoxide dismutase from monkey intestinal mucosa and a comparative study of this enzyme from the small intestinal mucosa of commonly used laboratory animals, such as rat (Rattus norvegicus), rabbit (Oritolagus cuniculus), guinea-pig (Cavia porcellus), and monkey (Macaca radiata).

Materials and Methods

Chemicals—Nitro blue tetrazolium, Tris, DEAE-cellulose, bovine erythrocyte superoxide dismutase and Sephadex G-75 were obtained from Sigma Chemical Co. Chemicals for polyacrylamide gel electrophoresis were purchased from Eastman Kodak Co. All other chemicals were of analytical grade.

Purification of monkey intestinal CuZn superoxide dismutase—All purification steps were carried out at 4°C. Intestine was removed from 24-hr-fasted monkey, washed with 1.15% KCl and mucosa scraped after opening longitudinally. This was homogenised in 4 vols of 10mM phosphate buffer (pH 8.0) containing 0.1 mM EDTA in a teflon glass homogenizer. The homogenate was centrifuged at 50,000 × g for 1 hr and the supernatant was subjected

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to \((\text{NH}_4)_2\text{SO}_4\) precipitation. The precipitate obtained between 35-85% saturation was dissolved in 10mM phosphate buffer (pH 8.0) containing 0.1mM EDTA and dialysed against the same buffer. The dialysed enzyme was treated with chloroform-ethanol mixture (0.05:0.025, v/v) as described\(^{21}\). The precipitate obtained was removed, dissolved in minimum volume of 10 mM phosphate buffer (pH 8.0) and dialysed against the same buffer. This was passed through a DEAE-cellulose column (5 x 20 cm) equilibrated with phosphate buffer in which most of the other proteins were bound to the column. Unbound enzyme was concentrated by lyophilization and dialysed against 10 mM phosphate buffer (pH 8.0). This was subjected to gel filtration on Sephadex G-75 (2 x 50 cm) pre-equilibrated with 10 mM phosphate buffer. Active fractions from this column were pooled, lyophilised and subjected to a final purification by preparative tube gel electrophoresis (2 x 15 cm). The enzyme was identified by staining a portion of the gel with specific CuZn superoxide dismutase stain and rest of the enzyme eluted with 10 mM phosphate buffer (pH 8.0). This enzyme showed a single protein band on SDS-gel electrophoresis and was used for preparation of antibody. SDS polyacrylamide gel electrophoresis was done in 10% gel containing 0.1% SDS according to Laemmli\(^{22}\) and glycoprotein staining was done as described\(^{23}\). Protein was estimated according to Lowry et al.\(^{24}\), using crystalline bovine serum albumin as standard.

**Antibody preparation**—Antibody against CuZn superoxide dismutase from monkey intestinal mucosa was raised in rabbit. Protein (200 µg) of purified enzyme was mixed with an equal volume of Freund's complete adjuvant and injected intradermally. A booster dose of 100 µg of the enzyme in incomplete adjuvant was injected on 21st day. A week later blood was collected and serum separated. The serum was saturated with \((\text{NH}_4)_2\text{SO}_4\) to 50% and the precipitated protein was collected by centrifugation at 12,000 x g for 30 min. This was dissolved in 20 mM phosphate buffer (pH 6.8) containing 2 mM mercaptoethanol. The purified antibody was free of any detectable CuZn superoxide dismutase activity.

**Enzyme assay**—Superoxide dismutase was assayed according to the method of Beauchamp and Fridovich\(^{25}\) and 1.25 mM KCN was used in this assay to discriminate the CN-insensitive, Mn superoxide dismutase from CN-sensitive CuZn superoxide dismutase. Activity that produces 50% of maximum inhibition in the colour production of nitroblue tetrazolium was taken as one unit of superoxide dismutase. Maximum inhibition in the assay was calculated using purified bovine erythrocyte superoxide dismutase.

**Comparative Studies**

**Enzyme preparation**—Cytosolic fractions, used for comparative studies, were obtained from 24-hr-fasted rat, rabbit and guinea-pig intestinal mucosa and were dialysed against 10 mM phosphate buffer (pH 8.0).

**Gel electrophoresis**—Electrophoretic analysis was performed in 5%, 7.5% and 10% polyacrylamide gels according to Davis\(^{26}\) using 50 mM Tris-glycine buffer (pH 8.3) at a constant current of 3 mA/tube. The gels were stained for CuZn superoxide dismutase activity as described\(^{25}\).

**DEAE-cellulose chromatography**—The dialysed mucosal cytosol mixture (2 ml) containing equal units of enzyme activity from rat, rabbit, guinea-pig and monkey was loaded on to a DEAE-cellulose column (1 x 6 cm) pre-equilibrated with 10 mM phosphate buffer (pH 8.0). The column was washed with the same buffer and eluted with a gradient of 0 to 200 mM NaCl in the same buffer. Fractions of 2 ml were collected and subjected to protein as well as superoxide dismutase activity assays. Peaks with enzyme activity were pooled separately, concentrated by lyophilisation, dialysed against 10 mM phosphate buffer (pH 8.0) and used for electrophoresis.

**Immuno-diffusion**—This was performed on 1% agarose gel made in phosphate-buffered saline containing 0.02% sodium azide as described\(^{27}\). Loaded gels were developed in a moist chamber at 4°C, washed with phosphate buffered saline and dried. The immunoprecipitate was stained for protein with coomassie brilliant blue R.

**Immuno-electrophoresis**—This was performed in 1% agarose gels using 20 mM barbitone buffer (pH 8.6) for 3 hr at constant current of 4 mA per slide. Purified antibody was applied in a trough cut in middle of the slide and the gels were developed as for immunodiffusion.

**Immuno-inhibition**—A constant amount of CuZn superoxide dismutase (cytosolic fraction) from rat, rabbit, guinea-pig and monkey in 100 µl of 10 mM phosphate buffer (pH 8.0) was mixed with an equal volume of varying concentration of antibody (IgG) in the same buffer and incubated for 24 hr at 4°C in presence of 4% polyethylene glycol. After incubation, 50 µL aliquots were drawn for CuZn superoxide dismutase assay to measure the inhibition of enzyme activity by antibody. To the rest of incubation mixture, 20 µl of bacterial suspension of
Staphylococcus aureus (Cowan 1) was added and incubated for 1 hr at 4°C (ref. 28). After centrifugation at 12,000 \times g for 30 min, the enzyme activity was assayed in the supernatant. The CuZn superoxide dismutase activity assayed after centrifugation represents the activity of the free enzyme present in supernatant and not of the enzyme antibody complex. Two types of control experiments were carried out. In the first control, the enzyme was treated with 10 mM phosphate buffer instead of the antibody and bacterial suspension. In the second control, the antibody was replaced by equal amounts of rabbit non-immune IgG and buffer. Equal amounts of CuZn superoxide dismutase from all three sources were used for immunoinhibition.

Results

The CuZn superoxide dismutase was purified to apparent homogeneity from monkey intestinal mucosa. The 50,000 \times g supernatant of intestinal mucosa was subjected to precipitation by ammonium sulphate and ethanol-chloroform mixture, separated by DEAE-cellulose and Sephadex G-75, and finally purified by preparative gel electrophoresis. Treatment with chloroform-ethanol modified its binding ability towards DEAE-cellulose, since the untreated cytosolic enzyme was found to bind to DEAE-cellulose. A similar observation has been made with erythrocyte superoxide dismutase\(^9\). The enzyme was purified 181-fold and showed a single band in sodium dodecyl sulphate polyacrylamide gel electrophoresis corresponding to a molecular weight of 16,000 (Fig. 1). The enzyme is thermally stable and requires copper and zinc for its activity (Data not shown). Polyclonal antibody was raised against the purified enzyme. The specificity of antisemum to superoxide dismutase was tested by immunoelectrophoresis with purified and crude enzyme. As shown in Fig. 2, a single, precipitin line was observed in both cases, thereby indicating the specificity of antiserum. Control serum did not show any precipitin line.

The results of our study on the relative mobility of superoxide dismutase from intestinal mucosa of rat, rabbit, guinea-pig and monkey on 7.50% polyacrylamide gel are presented in Fig. 3. It is evident that the enzyme from different sources exhibits different electrophoretic mobility, the enzyme from rat showing maximum and that from monkey the minimum mobility. In view of the above observation, these enzyme preparations were further analysed using ion-exchange chromatography. A mixture of equal units of enzyme from the cytosolic fractions of the four species, when separated on

![Fig. 1](image1.png)

![Fig. 2](image2.png)
DE-52, resolved into three activity peaks (Fig. 4), each peak being identified by activity staining after polyacrylamide gel electrophoresis (Fig. 4, inset).

Specificity of binding of antibodies to superoxide dismutase from different species was examined by inhibition of enzyme activity by antisera. As shown in Fig. 5, the pattern of inhibition of enzyme activity in the four species was identical and the control serum was devoid of any effect on the enzyme activity. The purified enzyme preparation from monkey was also inhibited by the antisera similar to the crude enzyme. The observation was confirmed by immunodiffusion (Fig. 5, inset) when CuZn superoxide dismutase from rat, rabbit and guinea-pig cross-reacted with antisera to purified monkey enzyme.

**Discussion**

Several lines of evidence indicate that oxygen-free radicals are produced during normal oxidative metabolism. It has been reported that superoxide radicals are the cause of ischemic injury in intestinal mucosa which can be prevented by superoxide dismutase 10. This enzyme plays an important role in defense against free radical damage. Its catalytic dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide makes it an unique enzyme in defense against oxygen centered radicals.

Superoxide dismutase has been isolated and characterised from different prokaryotic and eukaryotic cells10,20,31. It is known to be present in all oxygen utilising organisms. CuZn superoxide dismutase has been isolated from different
mammalian species and similarities in physicochemical properties were observed between different species indicating conservation of structure and function during evolution. CuZn superoxide dismutase has been shown to have a molecular weight of 32,000 made up of two identical subunits and each subunit has one Cu and one Zn atom in its structure. This enzyme shows considerable resistance to denaturing agents and heat. The CuZn superoxide dismutase purified from monkey intestinal mucosa is similar to the enzyme reported from other sources and it showed a subunit molecular weight of 16,000. Monkey intestinal enzyme was thermostable and copper and zinc were found to be essential for its activity.

However on comparison of the intestinal enzyme from different species, certain interesting observations have emerged. Electrophoretic mobilities of this enzyme from four different species were found to be different. Each species gave a single band on enzyme staining and mobilities were distinctly different. Difference in electrophoretic mobility may not be due to difference in charge contributed by the carbohydrate, since the purified enzyme could not be stained for glycoprotein. It is known that superoxide dismutase from other sources is also not a glycoprotein. Elution profile from DEAE-cellulose was different, suggesting that charge on the protein may contribute to difference in the electrophoretic mobility. There are earlier reports suggesting multiple forms of isomorphs. Three electromorphs from bovine erythrocyte and four from chicken liver on PAGE have been reported. Two of the electromorphs from bovine erythrocyte failed to show any difference in molecular weight, metal content, antigenic reactivity and optical spectra. Immunological studies described here have shown that the intestinal enzyme from all four species has same antigenic property.

It has been suggested that oxidation of some of the amino acids in the structure might change total charge of the protein. However, since all experiments were carried out under similar conditions, it is unlikely that the enzyme from one or two sources is oxidised. The difference in electrophoretic mobility may be due to charge difference, possibly due to difference in amino acid composition or due to posttranslational modification. Amino acid composition of CuZn superoxide dismutase from yeast, bovine, horse and human is known.

Comparison of the amino acid sequence between these species shows a homology of 74.5% and there is a hypervariable region between residues 17 to 30.

Beckman et al. have proposed the existence of two allelic gene SOD-A1 SOD-A2 with the phenotypes SOD-A1, SOD-A2, and SOD-A2 in human. The rare phenotype SOD-A2 shows slower anodic mobility than common type SOD-A1. It has been isolated from the erythrocytes of homoygotes. The present study has shown that the intestinal superoxide dismutase from four commonly used laboratory animals possibly differ in their charge, which is evident from the difference in electrophoretic mobility and DEAE elution profile. This may be due to the difference in amino acid composition or posttranslational modification.

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