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Review

Chromatographic and electrophoretic methods for analysis of superoxide dismutases[1]

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Abstract

A brief overview of the family of superoxide dismutase (SOD) enzymes and their biomedical significance is presented. Methodology for the purification and electrophoretic analysis of superoxide dismutases is reviewed and discussed, with emphasis on the specific problems raised by the separation of individual superoxide dismutase isoenzymes. Purification methods and their performance, as reported in the literature, are summarised in table form. Generally used methods for measuring SOD activity in vitro and SOD visualisation after electrophoresis are outlined, particularly those relevant to the monitoring of progress of SOD purification.

Keywords: Reviews; Superoxide dismutases; Enzymes

Contents

[1]This paper is dedicated to the memory of late Emmeritus Professor D.J.D. Nicholas of Waite Agricultural Research Institute, University of Adelaide, Australia.

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1. Introduction

1.1. Superoxide dismutases

Superoxide radical-anion ($O_2^{\cdot-}$), as a reactive intermediate of the reduction of oxygen, is produced in all aerobically respiring cells by a number of biological reactions and can result in many deleterious physiological effects [1–5]. It can be present in only minuscule amounts at any instant and the reaction of its disproportionation or dismutation proceeds at a rapid rate. Yet, an enzyme, namely superoxide dismutase (SOD, EC 1.15.1.1.) is required for protecting living cells against superoxide anion toxicity (reactions 1–3)

$$O_2^{\cdot-} + M^{2+} \rightarrow O_2 + M^{1+} \quad (1)$$

$$O_2^{\cdot-} + M^{1+} + 2H^+ \rightarrow H_2O_2 + M^{2+} \quad (2)$$

The sum of these reactions is:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

Aerobic and microaerobic organisms contain relatively high levels of SOD, consistent with their niche in the biota. Anaerobic bacteria in general are devoid of the enzyme, but the ability of some anaerobes to synthesise SOD greatly improves their resistance to oxygen toxicity [6]. SODs are characterised by a metal ion in the active site of the enzyme: ie, copper and zinc, manganese and iron [3–5]. Two new forms of the enzyme, one a hybrid containing Mn/Fe and the other an extracellular one containing Cu and Zn have recently been reported [3]. Cu/Zn SOD and Mn SOD are present in virtually all eukaryotic organisms [3,4], whereas all forms of the enzyme are found in prokaryotic cells [3,4,7]. The metal content of SOD isoenzymes distinguishes different forms of SOD and is usually determined by differential inhibition, in that a Cu/Zn SOD is cyanide- and H_2O_2-sensitive, whereas Fe SOD and Mn SOD are inhibited by H_2O_2 and SDS, respectively [3,8]. Literature on the role and characterisation of various SODs has been reviewed regularly. For the most recent review see Ref. [9] and citations therein. Contents and activities of individual SODs in various tissues and organisms are given in Table 1. These data are intended only to provide reference for practical purposes and a certain degree of variability may be expected, depending on the material and methods employed.

1.1.1. Cytosolic Cu/Zn SOD

Although the nature of the enzymatic activity of Cu/Zn SOD was discovered over two decades ago, the protein had been known much earlier. It was first isolated as a copper protein from bovine erythrocyte (erythrocuprein) and liver (hepatocuprein) in 1938 [10]. This was followed by the discovery of a whole family of copper-containing proteins that were present in various organs [11]. Much later, as a the result of various observations carried out by McCord and Fridovich [2] on the reduction of cytochrome *c* by xanthine oxidase reaction, erythrocuprein was identified as copper/zinc superoxide dismutase. Cu/Zn SOD is a soluble enzyme found mainly in the cytosol of eukaryotic cells. It has a molecular mass of 32 000 and it consists of two identical subunits. Each subunit contains one Cu^{2+} and one Zn^{2+} in the active site. Cu/Zn SODs are generally very stable enzymes, tolerating exposure to organic solvents and retaining activity in 8.0 *M* urea [12] or in 2% SDS [13]. Cu/Zn SODs from some sources are not as stable in the presence of these denaturants [14,156]. In general, the properties and structure of Cu/Zn SOD have been remarkably resistant to evolutionary modifications, and enzymes obtained from plants, fungi, birds and mammals are very similar [1,3–6,9,15].

1.1.2. Periplasmic SODs

Cu/Zn SOD has also been isolated from several bacterial species, including *Escherichia coli* [15–20], and its presence has been indicated in some protozoan parasites [7]. Bacterial Cu/Zn SODs are most likely periplasmic [18–22] and have properties similar to those of eukaryotic Cu/Zn SOD [17,23].

Table 1
Content and activity of individual SODs in various organisms and tissues

Source of enzyme	EC SOD	Cu/Zn SOD	Mn SOD	Fe SOD	References
Commercial (Sigma)[1]		2000–6000	2500–5000	3000–6000	
Human[2]					[83,84]
Brain		118.0	80.0	–	
Lung		74.7	15.2	–	
Heart		34.4	111.1	–	
Kidney		128.0	67.5	–	
Liver		639.0	231.0	–	
Placenta		42.2	19.9	–	
Erythrocytes[3]		1.43±0.11	–	–	[61]
Lymphocytes[3]		4.57±0.16	1.10± 0.31	–	[61]
Granulocytes[3]		1.09±0.16	0.13± 0.05	–	[61]
Serum[4]	0.02±0.006	–	–	–	
Rat[3]					[61]
Brain		94.4±16.5	39.0±26.0	–	
Lung		84.8±10.2	30.8±16.7	–	
Heart		106.2±20.8	85.0±18.6	–	
Kidney		413.0±60.5	103.0±53.2	–	
Liver		603.2±158.8	216.0±51.0	–	
Plasma[4]					[35,37,131]
Rabbit	7.50±1.06	–	0.65±0.1	–	
Rat	3.13±0.65	–	0.46±0.08	–	
Pig	0.75±0.18	–	0.43±0.18	–	
Dog	0.02±0.01	–	0.29±0.04	–	
Bacteria (E. coli)[5]	–	–	1–3	1–4	[159]
Higher plants (pea leaves)[6]		–	1–2	–	[102,105–107]

Notes: [1] specific activity of purified enzymes expressed in units/mg of protein; [2] μg enzyme/g of tissue; [3] μg enzyme/10^5 cells; [4] μg enzyme/ml of plasma, recalculated from refs. [35–37] assuming one unit of EC SOD corresponds to 8.3 ng of enzyme and one unit of Mn SOD corresponds to 64 ng of enzyme [83,84]; [5] μg enzyme/mg of protein, recalculated from ref. [159], assuming one unit of Mn/Fe SOD corresponds to 64 ng of enzyme [83,84]; [6] μg enzyme/g of green leaf tissue.

In *E. coli*, Cu/Zn SOD seems to be important for aerobic growth in mutants that cannot produce Mn SOD and Fe SOD [20]. It remains to be elucidated whether there are sources of $O_2^{\cdot-}$ within the periplasm or whether the periplasmic SOD protects against extracellular sources of $O_2^{\cdot-}$ [9].

1.1.3. Mn SOD

Manganese-containing SOD was first isolated from *E. coli* [24] and has since been isolated from a wide range of bacteria [25,26]. The bacterial enzyme contains manganese in its active site (one Mn^{3+} per subunit, in the resting state), usually has a molecular mass of 40 000 to 46 000, and is a dimer made up of identical subunits. High molecular mass tetrameric Mn SODs have been identified in several species of bacteria with molecular masses of 110 000 to 140 000 [27–29]. Mn SOD has also been obtained from mitochondria. The mitochondrial enzyme is strikingly similar to the Mn SOD from prokaryotes, with the notable difference that it is always tetrameric [3,25,26]. Mn SODs are much more susceptible to denaturation by heat or chemicals (including solvents and detergents) than are Cu/Zn SODs [30].

1.1.4. Fe SOD

Fe-containing SOD was first isolated from *E. coli* and subsequently from many other bacteria [1,15]. No animal tissues have been found to contain Fe SOD. Of 43 plant families examined, Fe SOD was found in only three [31]. All known Fe SODs are

very similar to Mn SOD, and with few exceptions are dimeric with a subunit molecular mass of 23 000 and with one atom of metal per subunit [1,3–6,15]. Like mitochondrial Mn SOD, some bacterial Fe SODs were found to be tetrameric [32]. Some bacteria contain both Mn SOD and Fe SOD, whereas others contain only one type of the enzyme. In some bacterial strains, the type of SOD expressed appears to depend on the availability of a metal in the growth medium [1,33]. Mn SOD and Fe SOD have very similar amino acid sequences and share a common polypeptide fold, which is completely unlike that of Cu/Zn SOD. It has been concluded that Mn SOD and Fe SOD are structural analogues. Yet these enzymes from *E. coli* exhibit substantial differences in their solution properties and absolute metal co-factor specificity [34].

1.1.5. Extracellular SOD

Extracellular superoxide dismutase (EC SOD) is a secretory, tetrameric copper- and zinc-containing glycoprotein, with a subunit mass of about 30 000. EC SOD is the major SOD isozyme in extracellular fluids, such as plasma, lymph and synovial fluid [9,35,36], but it can also be found in small amounts in other tissues [37]. The amino acid sequence of the human EC SOD indicates the presence of an 18 amino acid signal peptide, characteristic of secreted proteins [38]. Residues 96–193 show strong homology to the cytosolic Cu/Zn SOD, whereas residues 1–95 do not. The amino acids that compose the active site are highly conserved in all of the Cu/Zn SOD family, including EC SOD. It is therefore expected that the conformation of EC SOD around the active site would be very similar to that of cytosolic Cu/Zn SOD [38]. EC SOD exhibits affinity for heparin and other acidic glucosamino-glucans. The enzyme in plasma of man and other mammals is heterogeneous with regard to affinity to heparin–Sepharose. The binding to heparin is of an electrostatic nature, and interaction with the strongly negatively charged heparin appears to be mediated by the very hydrophilic carboxy-terminal of EC SOD [39]. In vivo, the apparent explanation for the enzymes affinity for heparin is that its binding to haparan sulfate proteoglycan in the glycocalyx of the cell surface [63].

1.2. Oxygen toxicity and biomedical significance of SODs

Toxicity of oxygen is expressed and amplified under a variety of conditions. Although molecular oxygen is not toxic, it can produce metabolic by-products whose reactivity might pose significant potential for cellular damage. The superoxide radical-anion is the first product formed by the univalent reduction of oxygen. It is believed to be only mildly reactive and controversy surrounds its direct role in oxygen toxicity [40]. However, increased $O_2^{\cdot-}$ production increases the formation of the more reactive oxidants such as H_2O_2 (reactions 1–3) and hydroxyl radical ($OH^{\cdot}$), through the metal-catalysed Haber–Weiss reaction (reactions 4–6). Many believe this reaction to be one of the underlying mechanisms for oxygen toxicity [41–43].

$$O_2^{\cdot-} + M^{3+} \rightarrow O_2 + M^{2+} \quad (4)$$

$$M^{2+} + H_2O_2 \rightarrow M^{3+} + OH^{\cdot} + OH^{-} \quad (5)$$

The sum of the reactions is:

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + O_2 \quad (6)$$

On the other hand, proponents of the chemical inertia of $O_2^{\cdot-}$ claim that SODs do not serve a significant biological function, as the $O_2^{\cdot-}$ dismutation accelerates the generation of H_2O_2. Whatever importance SOD may have, it is related to that of $O_2^{\cdot-}$. A renewed interest in SOD research in recent years provided strong arguments in support of the notion that $O_2^{\cdot-}$ acts directly and that it could possess a toxicity of its own. The question of singlet oxygen ($^1O_2$; excited state of O_2) generation during spontaneous dismutation of superoxides is still awaiting a definitive answer, as there is no definitive proof that SOD is able to inhibit $^1O_2$ formation [40,44].

Superoxide dismutases have been found in virtually all oxygen-tolerant organisms and there are many published results supporting the notion of the role SOD plays in oxygen and superoxide toxicity. With few exceptions, adaptation to hyperoxia is closely correlated with an increase of SOD, both in prokaryotes and eukaryotes [45,46].

The many potential sources of $O_2^{\cdot-}$ in living systems, together with the variety of reactions by

which the radical is converted to more reactive species, suggest that as a scavenger of $O_2^{\cdot-}$, SOD has a pleiotropic effect. Indeed, it has been shown that SOD can alleviate a wide range of effects produced by exposure to $O_2^{\cdot-}$-generating systems and it has an anti-inflammatory property which was discovered well before the protein was identified as an enzyme [47]. Orgotein is the generic name adopted for drug versions of highly purified Cu/Zn SOD and was reported to have anti-inflammatory and anti-viral activities [48]. SOD administered intravenously along with catalase, was found to protect against oxygen toxicity and reduced inflammation caused by irradiation. Intra-articular injections of SOD aid in the treatment of joint diseases. Other therapeutic uses of SOD, and its role in human pathology, have been reviewed extensively [49,157,158].

In recent years, the most significant progress in SOD therapy has been made in two areas; the treatment of ischaemia–reperfusion and the practice of graft and transplantation. SOD, together with catalase, is currently administered to counter the $O_2^{\cdot-}$-mediated destructive phenomena of ischaemia–reperfusion observed in transplantation [50]. It has also been postulated that SOD is beneficial when used as an anti-oxygen agent in organ preservation and transplantation [51].

2. Assays for SOD activity

2.1. Principle

Since the discovery of enzymatic activity of SOD, a variety of methods for quantitation of the enzyme activity have been developed and employed. The instability of the substrate (superoxide radical-anion) makes it difficult to assay SOD activity directly. Direct methods such as viewing of $O_2^{\cdot-}$ in the far-UV, electron spin resonance using spin trapping, ^{19}F NMR or stop flow spectroscopy are not practical with crude enzyme preparations and at SOD levels found in biological systems [52]. The most commonly used and the most accessible assays are the indirect methods. Due to the nature of these indirect assays, the amount of superoxide dismutase giving 50% inhibition (SOD_{50}) of an $O_2^{\cdot-}$-dependent reaction is defined as one unit of SOD activity [47,58].

2.2. Assays

In the indirect methods of SOD assay, $O_2^{\cdot-}$ is generated enzymatically or non-enzymatically in the reaction mixture, which also contains measurable indicator reacting with $O_2^{\cdot-}$. An overview of the most commonly used systems is given in Table 2. The application of various methods often leads to erroneous determination of SOD activity and care should be exercised to determine the influence of, and, if possible, to avoid interfering substances, particularly when used for the determination of enzyme activity in crude enzyme preparations [52]. Selection of an appropriate method is usually influenced by particular experimental need, but it is often, due to a large number of similar methods, made on the basis of the personal preference of a researcher. In our laboratory, SOD activity is routinely measured by two relatively simple and sensitive methods; the inhibition of the xanthine/xanthine oxidase-induced reduction of cytochrome *c* [47] and the inhibition of $DMSO/K^+O_2^{\cdot-}$-induced reduction of nitroblue tetrazolium (NBT) [53]. These are particularly suitable for monitoring the enzyme activity during purification procedures. In the latter method, it is important that the DMSO used for dissolving potassium superoxide be absolutely anhydrous and stored over a suitable drying agent (e.g., molecular sieves). The activity and content of the EC SOD is low, both in extracellular fluids and in other tissues, and a highly sensitive enzyme assay is required. It has been reported that the direct spectrophotometric assay for $O_2^{\cdot-}$ obtained from $K^+O_2^{\cdot-}$ was suitable for analysis of this enzyme and was 40 times more sensitive than the xanthine oxidase/cytochrome assay [63]. Many tissues and cell preparations often contain more than one form of SOD, and various inhibitors capable of distinguishing among these enzymes should be included in activity assays. It is accepted that cyanide and ethyldithiocarbamate selectively inhibit Cu/Zn SOD [54,55] and that treatment with 2% SDS for 30 min effectively inhibits Mn SOD [55,56], whereas Fe SOD and Cu/Zn SOD are reported to be inhibited by $EDTA/H_2O_2$ [57]. Fe SODs are more sensitive to inhibition by azide than are Mn SODs, while the Cu/Zn SODs are least sensitive. This test can be applied to the SODs in crude extracts for the purpose of deciding to which class they belong [58–61].

Table 2
Methods used for assaying SOD activity[a]

Source of superoxide anion	Detector of superoxide radical-anion	Reaction measured and references
Xanthine or hypoxanthine/ xanthine oxidase	Cytochrome *c*	Reduction, ΔA (also available as automated assay for SODs in blood) [64]
	NBT	Reduction, ΔA
	Luminol	Light-emission
	Luciferin analogue	Light-emission [61]
	Adrenaline	Oxidation, ΔA
	Hydroxylamine	Nitrite formation (colorimetric method)
	Hydroxylamine derivative	Oxidation to nitroxide, detected by ESR
Autoxidation reactions	Adrenaline	Oxidation, ΔA
	Sulphite	O_2 uptake
	Pyrogallol	O_2 uptake or ΔA
	6-Hydroxydopamine	Oxidation, ΔA
	Hydroxylamine	Oxidation, ΔA
	Hematoxylin	Oxidation, ΔA ('positive' assay) [62]
Directly added $K^+O_2^-$	–	Loss of $O_2^{\cdot-}$, ΔA in UV (used for EC SOD assay) [63]
	NBT	Reduction, ΔA
	Cytochrome *c*	Reduction, ΔA
	Tetranitromethane	Reduction, ΔA
Illuminated flavins	NBT	Reduction, ΔA O_2 uptake (SOD accelerates)
	Dianisidine	Oxidation, ΔA ('positive' assay)
	Hydroxylamine	Nitrite formation (applicable to whole bacterial cell SOD assay) [52]
NADH + PMS	NBT	Reduction, ΔA (applicable to whole bacterial cell SOD assay) [52]

[a] Compiled table from Refs. [1] and [60], supplemented with additional references as stated.

3. Chromatographic procedures for purification and characterisation of SODs

3.1. Cytosolic Cu/Zn SOD

Cu/Zn SOD is a stable protein that is resistant to a number of chemical treatments. The list of procedures employed for the purification of this enzyme from a variety of organisms is given in Table 3. For animal tissues, Cu/Zn SOD isolation can be either started with organic solvents in the classical manner [30,65,66,68,79] or the organic solvent step can be omitted [30,67,74,76,78–85,91,92,95]. There is a consensus that organic solvent procedures do not inactivate the enzyme or change its properties. The use of solvents precludes co-purification of Mn SOD, which, like Fe SOD, is not resistant to organic solvents [30]. Thus, for the simultaneous purification of both Cu/Zn SOD and Mn SOD from whole tissues, like liver, the procedure does not involve the use of solvents. The use of an ethanol–chloroform mixture in the early stages of purification of erythrocytes' Cu/Zn SOD removes haemoglobin almost completely and facilitates further purification. The haemoglobin-free extract is typically fractionated by anion-exchange chromatography and the enzyme can finally be purified to homogeneity by size exclusion chromatography and/or preparative polyacrylamide gel electrophoresis (PAGE) or isoelectric focusing (IEF). From whole tissues, Cu/Zn SOD can be extracted with or without organic solvents, in both cases producing extracts that are very rich in proteins. It is recommended that these extracts are fractionated by either $(NH_4)_2SO_4$ precipitation or heat treatment (Cu/Zn SOD remains stable in temperature up to 60°C) prior to chromatography [78,79,81]. The purification on anion-exchange resins typically gives the best results, but it often

requires additional steps involving cation-exchange, chromatofocusing or hydroxyapatite chromatography, and/or preparative electrophoresis, before the enzyme is finally purified by size exclusion chromatography. The application of chromatofocusing and preparative IEF allows detection and isolation of various isoforms of Cu/Zn SOD [70,73,76,77]. Human Cu/Zn SOD has been cloned and expressed in *E. coli* [132,133] and has been highly purified using the classical method. This enzyme consists mainly of three isoforms which were isolated by DEAE–Toyopearl chromatography [134]. A procedure for the large scale purification of recombinant human Cu/Zn SOD has been successfully applied to the production of gram quantities of the enzyme from yeast homogenate [135].

The development of immobilised metal affinity chromatography (IMAC) [136–138] has offered a new approach to enzyme purification. In IMAC, the metal, chelated to an activated column, interacts with amino acids on the "surface" of the protein. The steric arrangement of the protein chain also plays an important role, which means that molecules with similar properties with respect to charge, molecular size and amino acid composition, but with some differences in their secondary and tertiary structure, can be separated. Application of the IMAC technology has resulted in the purification of human [91,92], bovine and chicken erythrocyte Cu/Zn SOD [95]. It is critical that the enzyme extract be partially purified prior to the application of IMAC, for the column becomes easily saturated, particularly in the presence of a major protein, such as haemoglobin. Human and bovine SODs were eluted from a copper chelate-affinity column with a pH gradient. For Cu/Zn SOD from erythrocytes, the enzyme was eluted with either an increasing gradient of a counter ion (NH_4^+, 0 to 1 *M*) or decreasing pH gradients (8.0 to 6.0). The IMAC procedure resulted in an increase in specific activity of approximately 30-fold with an 80–90% recovery of activity [95]. An IMAC method that allows resolution of Fe-, Mn- and Cu/Zn-SODs on one column has recently been reported [96]. In this case, SODs were eluted sequentially from a Cu^{2+}–IMAC column with an increasing gradient of a counter ion run in combination with an increasing pH gradient (Fig. 1). The above methodology has been applied to the separation of three SODs from a protozoan parasite *Eimeria tenella* (Fig. 2). The purification of commercial Fe, Mn and Cu/Zn SODs for raising specific antibodies was also achieved using this method [96].

3.2. Mn SOD

Mn SOD is more susceptible to denaturation by organic solvents and SDS than Cu/Zn SOD is. The majority of methods for the enzyme purification from solid animal and plant tissues employed a variety of lengthy series of ammonium sulphate precipitation and ion-exchange chromatographic steps, resulting in a relatively low yield (Table 3). Mitochondrial Mn SOD can be successfully co-purified with Cu/Zn SOD from liver tissue, provided the initial homogenisation and enzyme extraction is performed using mild treatment at a pH above 7 [97,98]. Exploitation of the differing isoelectric points of the two enzymes permits their separation using anion-exchange chromatography [98]. SODs can be further purified separately using cation-exchange and size exclusion chromatography [97]. It is important to note at this point that activity of both enzymes could be monitored using the cyanide sensitivity test, but the use of electrophoresis with activity staining gives a more definitive distinction between the two enzymes. Purification of bacterial Mn SOD appears to be less demanding and a combination of ammonium sulphate fractionation with various chromatographic techniques usually results in good yield of the enzyme with a high specific activity (Table 3). Mn SOD, like Cu/Zn SOD, can be purified using IMAC. A relatively simple and reproducible procedure involving Cu^{2+}–IMAC has enabled the purification of homogeneous Mn SOD from *Corynebacterium glutamicum* [110] and partial purification of the enzyme from *E. tenella* [7,96].

3.3. Fe SOD

Several methods have been reported for the successful purification of Fe SOD from plants and bacteria (Table 3). Some of these, however, were quite lengthy and resulted in low yields of enzyme. In general, fractionation of homogenates with ammonium sulphate followed by anion-exchange chromatography gives good results as starting steps of

Table 3
Summary of procedures used for the purification of SODs

Superoxide dismutase	Purification procedure	References and comments
Cu/Zn SOD		
Animals		
Mammalian erythrocytes	AExC/preparative PAGE	[76] two isoforms isolated
	Organic solvents/AExC/SEC	[65,66]
	Phase partitioning/AExC/SEC	[74]
	AExC/preparative IEF	[67]
Human erythrocytes	AExC/IMAC	[91,92] requires complete removal of haemoglobin by AExC
Chicken erythrocytes	AExC/IMAC/SEC	[95] fast method; requires complete removal of haemoglobin by AExC
Retina	AExC/SEC	
Placenta	Immunoaffinity chromatography on anti-hSOD–Sepharose	[83] partial loss of Zn but reconstituted with $ZnCl_2$ dialysis
Bovine liver	Buffer extraction/heat treatment/AExC	
Rat/rabbit liver	Organic solvents/$(NH_4)_2SO_4$ precipitation/chromatofocusing	[79]
Fish liver	$(NH_4)_2SO_4$ precipitation/heat treatment/AExC	[78,81] prepared from acetone powder
Plants		
Mung bean	$(NH_4)_2SO_4$ precipitation/AExC/SEC/preparative PAGE	[70] two isoforms isolated
Brussels sprouts	$(NH_4)_2SO_4$ precipitation/preparative IEF/SEC	[71] two isoforms isolated
Green algae, ferns, maize	$(NH_4)_2SO_4$ precipitation/AExC/SEC/hydroxyapatite	[72,75] chloroplast and cytosolic isoforms isolated
Yeast		[69]
Insects		
Delia antiqua	$(NH_4)_2SO_4$ precipitation/AExC/HIC/SEC	[93]
Protozoa		
Eimeria tenella	IMAC	[7,96] partial purification
Dirofilaria imminis	AExC/SEC/preparative PAGE	[73]
	Organic solvents/AExC/SEC	[82] large-scale purification
Taenia taeniaeformis	AExC/SEC/chromatofocusing	[77]
Schistosoma mansoni	Detergent extraction/$(NH_4)_2SO_4$ precipitation/AExC/SEC	[86] four isoforms detected
Bacteria		
Pseudomonas spp.	Clarified homogenate	[87] crude preparation
Caulobacter crescentus	$(NH_4)_2SO_4$ precipitation/AExC/CExC/SEC	[88,90] periplasmic enzyme
Haemophilus influenzae	Clarified homogenate	[89] crude preparation, secretory enzyme
Oncocerca volvulus	AExC/HIC	[94] recombinant form expressed in *E. coli*

Mn SOD		
Animals		
Liver mitochondria	Buffer extraction/CExC	[30]
Mammalian liver	$(NH_4)_2SO_4$ precipitation/AExC/AExC/SEC	[30,117] two AExC steps required to separate from Cu/Zn SOD
Plants		
Pea	Heat treatment/$(NH_4)_2SO_4$ precipitation/AExC/SEC/preparative PAGE	[105,106]
Spinach	AExC/$(NH_4)_2SO_4$ precipitation/AExC/SEC/HIC/AExC	[107] chloroplast thylakoid-bound; low yield
Groundnut	Heat treatment/$(NH_4)_2SO_4$ precipitation/AExC/SEC/preparative PAGE	[101] very low yield
Mung bean	$(NH_4)_2SO_4$ precipitation/AExC/SEC/hydroxyapatite/preparative PAGE	[102] low specific activity and yield
Norway spruce	$(NH_4)_2SO_4$ precipitation/AExC/HIC/chromatofocusing/SEC	[112]
Scots pine	$(NH_4)_2SO_4$ precipitation/AExC/HIC/chromatofocusing	[113]
Blue–green algae		
Plectonema boryanum	$(NH_4)_2SO_4$ precipitation/AExC/SEC	[111] repetitive $(NH_4)_2SO_4$ precipitation steps; co-purifies with Fe SOD
Yeast		
Saccharomyces cerevisiae	Ultrafilter concentration/AExC/AExC/crystalization	[104] good specific activity and yield
Protozoa		
Eimeria tenella	IMAC	[7,96] partial purification
Bacteria		
Bacillus sp.	$(NH_4)_2SO_4$ precipitation/AExC/SEC	[97,99]
Lactobacillus acidophilus	Buffer extraction/$(NH_4)_2SO_4$ precipitation/AExC/SEC	[100] Molecular mass of native enzyme 160 000; octamer?
Seratia marcencens	$(NH_4)_2SO_4$ precipitation/dye-ligand chromatography	[103] simple, fast method; good yield
Actinomyces sp.	Protamine precipitation/$(NH_4)_2SO_4$ precipitation/HIC/AExC/SEC	[108] good yield
Halobacterium cutirubrum	$(NH_4)_2SO_4$ precipitation/AExC/hydroxyapatite/SEC	[109] good yield
Corynebacterium glutamicum	IMAC/SEC	[110] simple, fast method, good specific activity and yield
Fe SOD		
Plants		
Water lily	Buffer extraction/$(NH_4)_2SO_4$ precipitation/AExC/CExC/SEC	[114]
Tomato leaves		[115]
Cyanobacteria		
Anabaena cylindrica	$(NH_4)_2SO_4$ precipitation/AExC/SEC	[111,116] repetitive $(NH_4)_2SO_4$ precipitation steps
Protozoa		
Eimeria tenella	IMAC	[7,96] partial purification
Tetrahymena pyriformis	$(NH_4)_2SO_4$ precipitation/CExC/chromatofocusing/	[118]
Babesia hylomysci	$(NH_4)_2SO_4$ precipitation/CExC	[119–125]

(*Continued on p. 68*)

Table 3 (Continued)

Superoxide dismutase	Purification procedure	References and comments
Bacteria		
E. coli	Streptomycin treatment/$(NH_4)_2SO_4$ precipitation/CExC/AExC	[98]
Streptococcus mutans	Heat treatment/$(NH_4)_2SO_4$ precipitation/chromatofocusing	[120,121] active with either Fe and Mn
Bacteroides spp.	Protamine treatment/HIC/AExC–salt gradient/AExC-pH gradient/chromatofocusing	[122–124] induced by O_2; two isoforms isolated
Helicobacter pylori	SEC/AExC-pH 7.5/AExC-pH 9.5	[118] very low yield
Methanobacterium thermoautotrophcum	$(NH_4)_2SO_4$ precipitation/AExC/SEC	[125] recombinant form expressed in *E. coli*, tetramer
Aerobacter aerogenes	$(NH_4)_2SO_4$ precipitation/AExC/SEC/HIC/hydroxyapatite	[126] dimer
Azotobacter vinelandii	AExC/SEC/hydroxyapatite	[127,128] one of four isoforms purified
Extracellular SOD		
Small scale procedures		
Plasma (pig, cat, rabbit, guinea pig, mouse)	SEC/Heparin–Sepharose/Concavalin A–Sepharose	[35,37]
Human lung	AExC-pH 5.5/AExC-pH 8.4/HIC/Concavalin A	[36] three forms with different affinity to Heparin (EC SOD A, B, C)
	Sepharose/SEC/Wheat germ lectin–Sepharose/ Blue–Sepharose/Heparin–Sepharose/SEC	
Human umbilical cord	Affinity chromatography on anti-EC SOD IgG–Sepharose/AExC/Heparin–Sepharose	[129] various forms, heterogeneous to Heparin affinity
Rat C_6 glioma cells (secreted)	Hydroxyapatite/Heparin–Sepharose/HIC/C_4 RPC	[130] rat EC SOD B appears different from that of human
Large scale procedures		
Calf serum	Repetitive (3–4×) 80% ethanol extraction and borate crystallization /Concavalin A–Agarose	[131]
Bovine milk	A; 80% ethanol extraction and borate crystallization/Concavalin A Agarose	[131]
	B; KBr–acetate (pH 5.5) extraction/crystallization/Concavalin A–Agarose	[131] KBr interferes with spectrophotometric enzyme assay
Yeast	Repetitive (2×) 80% ethanol extraction and borate crystallization/ Concavalin A–Agarose	[131] sixteen strains surveyed as rich source of EC SOD
Wheat germ	Repetitive 80% ethanol extraction and borate crystallization/ centrifugation	[131]
Bacillus subtilis	80% ethanol extraction and borate crystallization/Concavalin A agarose	[131]
E. coli	As for *B. subtilis*	[131] there is doubt that *E. coli* contains EC SOD
Recombinant EC SOD		
Human EC SOD C	Affinity chromatography on anti-EC SOD IgG–Sepharose/AExC/ Heparin–Sepharose	[129] expressed in Chinese Hamster Ovary cells

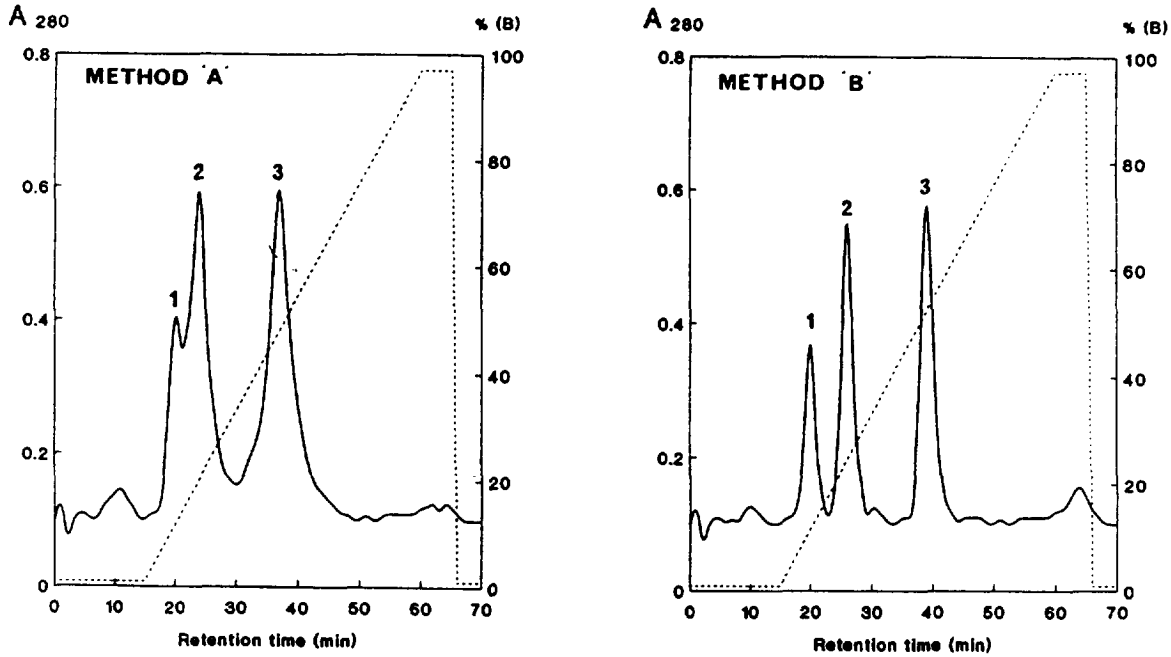

Fig. 1. Comparison of elution profiles (optical absorption at 280 nm) of Fe SOD (peak 1), Mn SOD (peak 2) and Cu/Zn SOD (peak 3) separated by IMAC on a Cu–Superose column. Proteins were eluted from the column as follows: method A, an increasing NH_4^+ gradient at pH 7.2 or method B, an increasing NH_4^+ gradient run simultaneously with an increasing pH gradient (6.8 to 7.8). Dotted lines indicate theoretical gradient profile. (from Ref. [96] with permission).

enzyme purification. Further purification can then be achieved using chromatofocusing and hydroxyapatite chromatography [118,120–124,126,127]. In bacteria, Fe SOD often co-exists with Mn SOD, thus, it is essential that enzyme activity is determined using inhibitor sensitivity tests (see Section 2.2). Again it appears advantageous to employ electrophoresis with activity staining, together with an in vitro activity assay, for monitoring the enzyme's purification. Finally, metal content analysis should be performed for the purpose of definitive differentiation between the two enzymes. The usefulness of the IMAC technology for purification of Fe SOD is yet to be unequivocally demonstrated, as only a partially purified preparation of the enzyme was obtained using this method [96].

3.4. EC SOD

Although SODs were studied extensively for decades, the family of EC SOD has only been known since the 1980's. EC SOD, a glycoprotein enzyme, has been purified for the first time from human lungs with a lengthy procedure including repetitive ion exchange and heparin and lectin affinity chromatography [35–38,63]. A major obstacle in the purification of EC SOD is the contamination with other SODs. EC SOD is, like Cu/Zn SOD but unlike Mn SOD and Fe SOD, very sensitive to inhibition by cyanide, azide, H_2O_2 and arginine-specific reagents. Monoclonal and polyclonal antibodies raised against these enzymes help to distinguish the enzymes to some degree, but often anti-EC SOD antibody reacts poorly with the enzyme from other related species [63,129]. A prominent and unique feature of EC SOD is its affinity for heparin. By chromatography on heparin–Sepharose, EC SODs from mammalian plasma can be divided into three types: A, without affinity; B, with weak affinity and C, with relatively strong affinity. The enzyme from tissues (other than extracellular fluids) is mainly composed of isoforms with high affinity, whereas in rat plasma, only EC

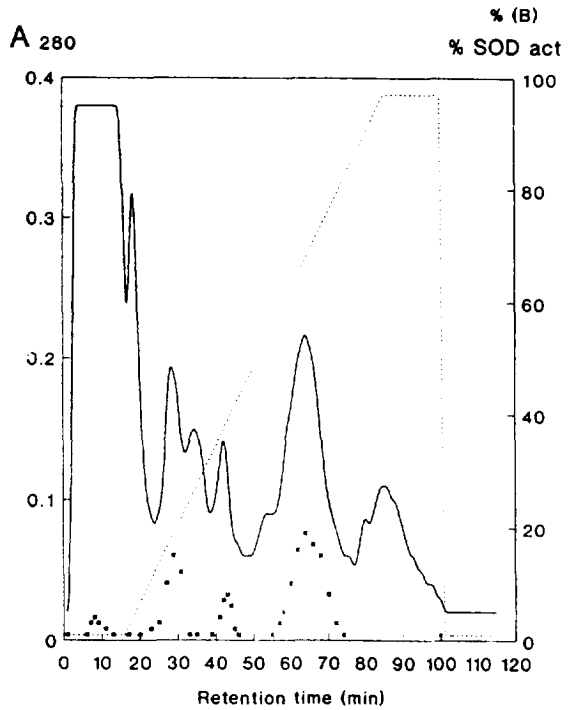

Fig. 2. Immobilised metal affinity chromatography of oocyst homogenate from *Eimeria tenella* on a Cu–Superose column. Proteins were eluted as described in Fig. 1, method B. The continuous line depicts an elution profile (absorption at 280 nm) and the dotted line indicates the theoretical gradient profile. SOD activity is expressed as a percentage of the total activity applied to the column. (from Ref. [96] with permission).

SOD types A and B are found [37,130]. EC SOD is, unlike other SODs, a glycoprotein and binds to lectins recognising mannose- and glucose-containing glycans, such as concanavalin-A and lentil and wheat germ lectins. Affinity chromatography on these lectins immobilised to Sepharose provides a useful method for distinguishing EC SOD from other SODs and proves to be an effective procedure for enzyme purification [39,63]. In recombinant human EC SOD type C, the carbohydrate moiety is indistinguishable from that of native enzyme of the same type, which facilitates its efficient purification [39].

A novel, large scale purification procedure has been designed for EC SODs from milk, calf serum, wheat germ and yeast [131]. The method exploits features of EC SODs that are similar to those of many large glycosylated proteins. The enzymes are, unlike ordinary proteins, soluble and active in 80–95% ethanol and can be extracted in large scale using this solvent. Ethanol-extracted EC SOD is then precipitated with tetraborate, since the enzyme–tetraborate complex co-crystallises with free tetraborate. After extensive dialysis, milligram quantities of EC SODs can be purified by lectin chromatography. This method produces excellent recovery of enzyme activity and the initial ethanol extraction alone enriches the specific activity by up to 400-fold with respect to biomass (Table 3).

4. Electrophoretic methods for the analysis of SODs

4.1. Identification and quantitative analysis

SODs are soluble enzymes and can be easily separated by gel electrophoresis under non-denaturing conditions and by IEF. For the separation of various SODs, several media have been used, including agarose, starch and polyacrylamide [60,139–144]. SOD activity can be visualised on gels following separation using methods in which $O_2^{\cdot-}$ generated photochemically causes reduction (change of colour) of a suitable acceptor. Detection of SOD activity following electrophoresis is usually achieved by two methods. On polyacrylamide gels (non-denaturing PAGE and IEF), it is usually done by a system containing NBT and riboflavin [139,140]. Flavins can be photochemically reduced in the presence of an oxidisable substance (TEMED) and will generate superoxide radical-anions when reoxidised in air. Superoxide, in turn, reduces NBT to an insoluble formazan. Achromatic zones indicate where the deficiency of superoxide radicals, due to the SOD activity, prevented the reduction of NBT. This relatively simple method is widely used and it is considered to be the most reliable and reproducible method [1,57,60]. The NBT/riboflavin can be effectively applied to the analysis of SOD patterns on IEF gels and after separation by rocket immunoelectrophoresis [60]. Developed polyacrylamide gels transferred to distilled water will not develop further and can be stored indefinitely in the presence of a preserving agent. SODs separated on starch and

agarose gels can be visualised by the above method or by using an overlay technique. In this, in principal, similar technique, a buffered solution of another tetrazolium salt (MTT) and phenazine methosulphate (PMS) is mixed with agar and applied on the surface of processed starch gels. Agar-overlayed gels are then exposed for several minutes to daylight and are further incubated at 37°C until achromatic zones appear on a blue background, indicating the presence of SOD activity [144]. To study sensitivity of SODs to inhibitors, gels are stained using the above methods, except that various inhibitors (1–5 m*M* KCN, 1–5 m*M* NaN_3 or 2–5 m*M* H_2O_2) are included in the solutions. These methods are widely used for the screening of SOD patterns following electrophoresis of crude homogenates, but they may produce erroneous results. The presence of phenolic compounds and peroxidases will mimic SOD activity. It is recommended that, in order to be specific, the SOD techniques have to be combined with other means of SOD recognition, such as immunodetection [60]. SODs belonging to the same family but derived from various sources migrate differently and cannot always be compared by electrophoresis. For example, liver mitochondria of rat, mouse and chicken contain a fast-migrating, cyanide-insensitive SOD. Liver mitochondria, as well as whole homogenates from every tissue of mouse and chicken, also have two additional slow-migrating cyanide-insensitive SODs. It has been reported that the NBT technique was unsuitable for the detection of the fast form of SOD on agarose gels [145].

Detection limits of both methods have been reported to be at the low nanogram level, and at least two densitometric methods have been described for the quantitative determination of SODs [60,146,147]. Generally, however, these methods are considered to be more of a qualitative test, used for the fast identification of SODs.

4.2. Analysis of isozymes

About half of all enzymes investigated so far exist in multiple molecular forms, which usually differ in electrophoretic mobility. Studies of isozyme variants and their linkages with other isozymes and morphological traits can be useful in qualitative genetic studies, gene mapping and regulation, and perhaps in plant breeding [1,57,148–150]. The development of the electrophoretic SOD staining technique provided a valuable tool for studies of SOD isozymes and a large number of papers have been published showing the existence of numerous SOD variants. Identification of primary SOD isoenzymes, representing the four enzyme families, is useful in studies of the phylogenetic relationship among organisms and the evolutionary origin of cell organelles [57,151]. Although non-denaturing PAGE generally provides good separation of SOD variants, much better resolution can be achieved using the IEF method. Indeed, new isoforms have recently been detected in plants, algae and an insect using this technique [150–155]. When analysing crude tissue extracts, caution must be exercised in attributing multiple SOD bands to the presence of SOD isoenzymes, since they might arise from the activity of proteolytic enzymes present in the extract [1]. However, unequivocal evidence for the identification of a SOD isozyme variant can only be achieved after the enzyme is purified and its amino acid sequence compared with those of other isoforms.

5. Conclusions

There has been a renewed interest in SOD research in recent years and studies demonstrating the importance of SOD in protecting microorganisms and grafts and transplantations strengthened the argument in support of the notion of $O_2^{\cdot-}$ toxicity. It is now widely accepted that SODs are able to weaken, or eliminate altogether, a wide range of toxic effects produced by the exposure to $O_2^{\cdot-}$-generating systems. These studies have also been of value in extending the role of SODs and free radical processes to many pathophysiological states, such as tumour initiation and ischaemic/reperfusion injury, and it appears that the struggle against superoxide radical-anion became a pharmaceutical necessity. As these additional applications for SOD emerge, new technologies will be required to produce and purify the enzyme (most likely as recombinant proteins) in a way that would be acceptable for wide pharmaceutical use. Until recently, SODs were purified by

conventional techniques that gave relatively low yields. The development of immobilised metal affinity chromatography is one example of such technology that has already offered a new approach to the enzyme purification.

6. List of abbreviations

AExC	Anion-exchange chromatography
CExC	Cation-exchange chromatography
Cu/Zn SOD	Copper-, zinc-containing superoxide dismutase
DMSO	Dimethyl sulphoxide
EC SOD	Extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
ESR	Electron spin resonance
Fe SOD	Iron-containing superoxide dismutase
HIC	Hydrophobic-interaction chromatography
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IMAC	Immobilised metal affinity chromatography
M^{n+}	Metal ion
Mn SOD	Manganese-containing superoxide dismutase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	2,2′-Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(dimethoxy-4,4′-diphenylene)ditetrazolium chloride
NMR	Nucleic magnetic resonance
OH·	Hydroxyl radical
$O_2^{\cdot}$	Superoxide radical-anion
PAGE	Polyacrylamide gel electrophoresis
PMS	Phenazine methosulphate
PMSF	Phenylsulphonyl fluoride
RPC	Reversed-phase chromatography
SDS	Sodium dodecylsulphate
SEC	Size-exclusion chromatography
SOD(s)	Superoxide dismutase(s)
TEMED	N,N,N′,N′-Tetramethylethylenediamine

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