Purification of pro- and eukaryotic superoxide dismutases by charge-controlled hydrophobic chromatography

Marlis Grunow and Wulfdieter Schöpp*

Bereich Biochemie der Sektion Biowissenschaften, Universität Leipzig, Talstrasse 33, O-7010 Leipzig (Germany)

ABSTRACT

The process of purifying superoxide dismutases was simplified using charge-controlled hydrophobic chromatography on 10-carboxydecyl Sepharose. In only one chromatographic step following ammonium sulphate precipitation, Fe-containing superoxide dismutase from *Pseudomonas putida* and Cu,Zn-containing superoxide dismutase from bovine erythrocytes were purified with an overall yield of about 70% to electrophoretic homogeneity. The specific activities of the crystalline enzyme preparations were expressed in McCord and Fridovich units and were 3000 and 3200 U/mg, respectively.

INTRODUCTION

Superoxide dismutase (SOD, EC 1.15.1.1) catalyzes the disproportionation of superoxide anion radical ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) to protect cells from oxygen toxicity caused by superoxide and other species of oxygen derived from it. This important enzyme is found in nearly all aerobic organisms as well as in some anaerobic ones. SODs are classified by their prosthetic metal into Cu, Zn-, Fe- and Mn-containing enzymes. Cu,Zn-SOD is localized mainly in the cytosol of eukaryotes and presents an independent line of descent. Different in structure from this type are the Fe- and Mn-SODs, which are present in prokaryotes; the latter are also present in mitochondria (for a review see ref. 1).

Owing to their broad distribution, SODs have been isolated and characterized from many organisms. With only a few exceptions [2–7] the described purification techniques conventionally involve ammonium sulphate fractionation, ion-exchange chromatography and gel filtration. Usually more than five steps are necessary to obtain homogeneous enzyme.

Because of the experimental and therapeutic importance, particularly of Cu,Zn-SOD, simple isolation procedures with high yields are of great practical interest.

In 1976 we reported on a special type of hydrophobic chromatography using 10-carboxydecyl Sepharose (CD-Sepharose) as a support [8]. The coupled ligand first implied that this Sepharose derivative specifically binds enzymes with hydrophobic active centers, such as alcohol dehydrogenase [8]. However, further investigations have shown that other oxidoreductases as well as enzymes of other classes, including transferases, lyases and hydrolases, can also be adsorbed to CD-Sepharose [9]. These data offer the possibility of expanding the use of this technique to the purification of pro- and eukaryotic SODs.

EXPERIMENTAL

Reagents

The sources of chemicals were as follows: xanthine oxidase, bovine serum albumin, xanthine and Coomassie brilliant blue G250, Serva (Heidelberg, Germany); cytochrome c, Biomed (Krakow, Poland); 11-aminoundecanoic acid, Fluka (Buchs, Switzerland); nitroblue tetrazolium chloride (NBT), Chemapol (Prague, Czechoslovakia); riboflavin,

Reanal (Budapest, Hungary); Sepharose 4B, Pharmacia (Uppsala, Sweden).

The chemicals for the electrophoretic experiments were products of Ferak (Berlin, Germany).

CD-Sepharose was prepared as described previously [8].

Enzyme assay and electrophoresis

SOD activity was measured by the xanthine/xanthine oxidase/cytochrome c assay of McCord and Fridovich [10] using a Beckman DK 2A spectrophotometer. Enzymatic activity is shown in McCord and Fridovich units [10].

Polyacrylamide gel electrophoresis on 8% gels was performed according to Davies [11]. Zones of protein were localized by staining with Coomassie brilliant blue G250 [12]. The detection of enzyme activity on the gels was carried out by the photochemical procedure of Beauchamp and Fridovich [13]. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Weber and Osborn [14].

Determination of protein concentration

The method of Kalb and Bernlohr [15] was employed for the determination of protein concentration. In the case of the purified enzyme solutions the protein concentration was determined according to the method of Lowry et al. [16] with bovine serum albumin as standard.

Purification procedure

Prokaryotic SOD was purified using the Fe-containing enzyme of a *Pseudomonas putida* strain [17]. Eukaryotic Cu,Zn-SOD was isolated from bovine erythrocytes.

Pseudomonas putida BL 1 was grown on *n*-hexane as a carbon source as previously reported [18]. Cells were collected by centrifugation and suspended in 0.05 M sodium phosphate buffer pH 7.0 to obtain an optical density of A_{600} (1 cm) = 20. This suspension was cooled to $-80°C$ and held at this temperature for about 4 h. Thawing was performed at 30°C. After 2 h of incubation at the same temperature cell debris was removed by centrifugation at 13 000 g for 30 min. Almost all the SOD activity was recovered in the clarified cell extract. The protein was precipitated by adding solid ammonium sulphate to reach 90% saturation. SOD was extracted from the pellet with an ammonium sulphate solution of lower concentration (depending on the composition of the protein extract between 50 and 60% saturation).

The SOD-containing fractions were directly applied to a CD-Sepharose column (10 cm × 2 cm I.D.) equilibrated with an ammonium sulphate solution. Elution of SOD activity was achieved by washing the column at a flow-rate of 0.4–0.7 ml/min with solutions of decreasing salt concentration at 20°C (fraction volume 20 ml). The SOD-containing fractions were concentrated and dialyzed against 90% saturated ammonium sulphate yielding crystalline SOD preparations.

For the isolation of the Cu,Zn-SOD, Tsuchihashi fractionation (chloroform/ethanol treatment) was performed to remove haemoglobin as described in ref. 10. The Tsuchihashi supernatant was lyophilized and dissolved in an ammonium sulphate solution of 65% saturation. Chromatography on CD-Sepharose was performed as described above.

All operations with the exception of the chromatographic steps were carried out at 4°C. The ammonium sulphate solutions used during the purification procedures contained 0.05 M disodium hydrogenphosphate.

RESULTS AND DISCUSSION

Purification of SOD from Pseudomonas putida

The cell-free extract was brought to 90% saturation with solid ammonium sulphate. After standing overnight in the cold the precipitated protein was collected by centrifugation (15 000 g, 30 min). Approximately 90% of the total SOD activity was extracted by stirring the precipitate twice for 10 min with an ammonium sulphate solution of 60% saturation. After centrifugation (15 000 g, 30 min) the two clear supernatants were pooled and diluted with 0.05 M sodium phosphate buffer pH 8.0 to reach an ammonium sulphate concentration of 50% saturation. The solution was applied to a CD-Sepharose column, previously equilibrated with an ammonium sulphate solution of the same concentration. Under these conditions SOD activity was quantitatively adsorbed onto the Sepharose derivative. A discontinuous gradient of ammonium sulphate (50% to 40% saturation) was then applied to the column, decreasing the salt concentration in
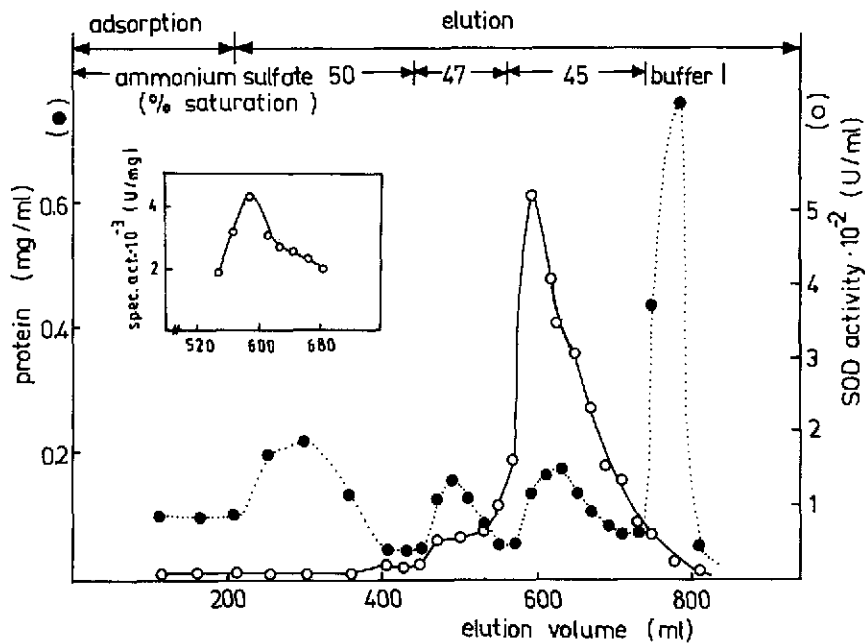
Fig. 1. CD-Sepharose chromatography of Fe-SOD from *Pseudomonas putida*. A 210-ml aliquot of the supernatant of the ammonium sulphate extract (1700 mg, 59 · 10³ U) was applied to the CD-Sepharose column.

small steps if the protein concentration in the eluates decreased below 0.06 mg/ml. SOD was eluted with an ammonium sulphate solution of 45% saturation (Fig. 1). Fractions containing more than 5% of the total SOD activity were pooled and concentrated to 10 ml on a small column (2.5 cm × 2 cm I.D.) of CD-Sepharose as follows. The pooled

fractions were brought to 55% saturation with solid ammonium sulphate and adsorbed on the equilibrated column (55% saturation). Elution was performed with 0.05 M sodium phosphate buffer pH 8.0. After discarding a volume of 5 ml the total SOD activity was removed from the column in only one fraction of 10 ml. For storage the enzyme was

TABLE I

PURIFICATION OF Fe-CONTAINING SOD FROM *PSEUDOMONAS PUTIDA*

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract[a]	615	1660	59 552	36	1	100
Ammonium sulphate extraction						
Supernatant 60% saturation 1.	110	154	35 642	231	6.4	95
2.	100	60	20 933	349	9.6	
CD-Sepharose chromatography	200	19	48 237	2486	69	81
Concentration on CD-Sepharose	10	14	41 091	2935	80	69
SOD preparation in crystalline state	5	13.5	40 500	3000	83	68

[a] Crude extract was prepared from a 7-l culture of *Pseudomonas putida* cells with an optical density of A_{600} (1 cm) = 1.7. The cells were harvested and disrupted as described in the Experimental section.
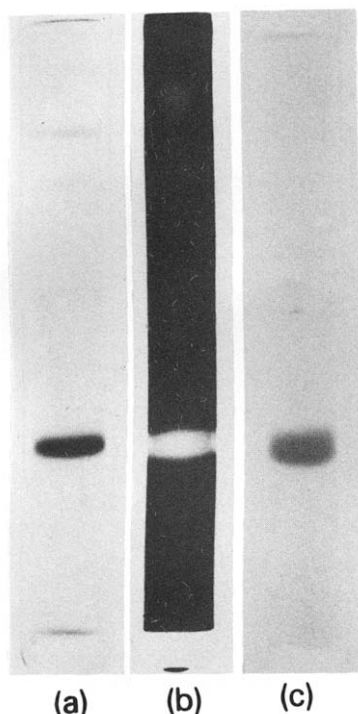
Fig. 2. Polyacrylamide gel electrophoresis of crystalline Fe-SOD from *Pseudomonas putida* in 8% gels under non-denaturing (a,b) and denaturing (c) conditions: (a) 30 μg of SOD stained for protein with Coomassie brilliant blue G250; (b) 5 μg of SOD stained for activity with the photochemical/NBT procedure; (c) 20 μg of SOD were incubated for 5 min at 95°C in the presence of 1% SDS and 1% β-mercaptoethanol and subjected to SDS-PAGE.

dialyzed against 90% saturated ammonium sulphate solution. At 4°C and a protein concentration of about 2 mg/ml no decrease of enzyme activity was observed over a period of 12 months. The re-

sults of the purification procedure are summarized in Table I. The recovery of 70% of the total SOD activity is very high in comparison with conventional purification procedures for prokaryotic SOD. The reported yields are as a rule below 40% [19–27]. Generally hydrophobic chromatography seems to be a useful technique to purify SODs. Gregory and co-workers [7,28,29], who used phenyl-Sepharose as carrier material, reported recoveries of 71–99% SOD activity for this single step. As the complete purification procedures include one or two additional chromatographic steps, the overall yields were in the range of 60%.

Because of the high specific activity of SOD in the crude extract of *Pseudomonas putida* [17], only 85-fold purification yielded homogeneous protein. Fig. 2a and b shows the electrophoretic patterns on 8% gels without SDS. A single protein band was observed, corresponding to the activity zone. The enzyme was also homogeneous as judged by SDS-PAGE (Fig. 2c). The specific activity of the purified enzyme was 3000 U/mg, which is of the same order of magnitude as described for some other Fe-containing SODs [19–27].

To obtain protein crystals, purified SOD (7 mg/ml) was dissolved in an ice-cold 53% saturated ammonium sulphate solution. Single crystals appeared after 1–2 days of storage at 25°C (Fig. 3).

Purification of SOD from bovine erythrocytes

Changing the conditions, CD-Sepharose is also suitable for purifying Cu,Zn-containing SOD. However, binding of eukaryotic SOD to the carrier required higher ammonium sulphate concentrations. The supernatant from the Tsuchihashi frac-
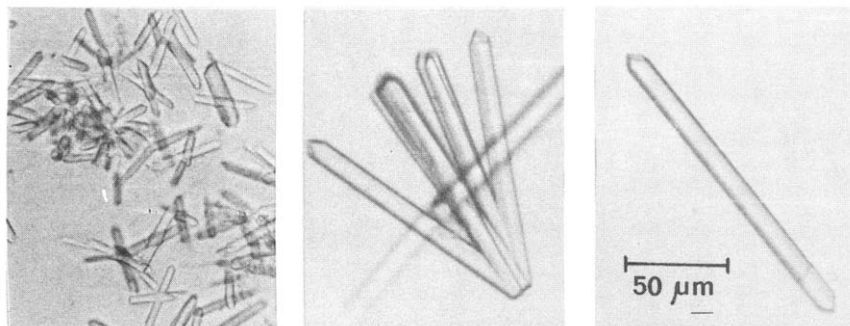

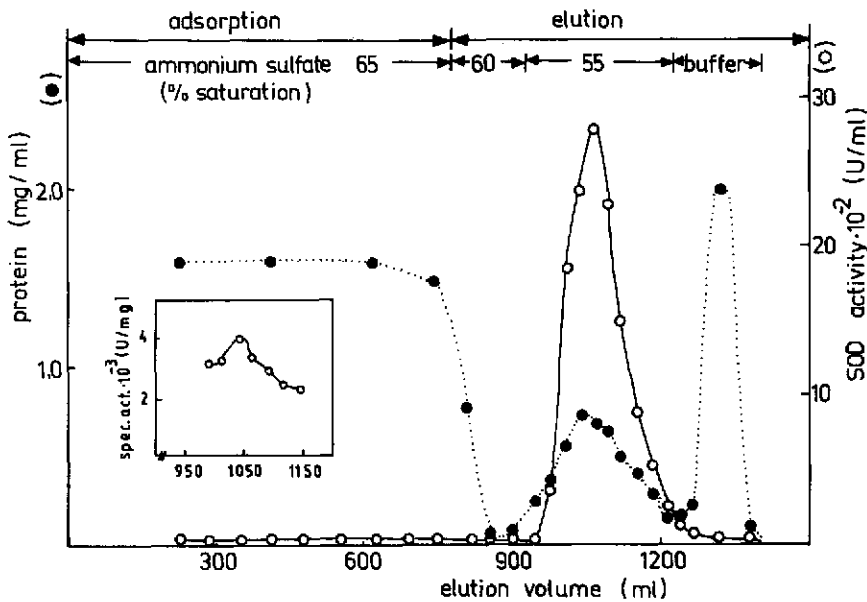Fig. 3. Microphotograph of crystalline Fe-SOD from *Pseudomonas putida*.

Fig. 4. CD-Sepharose chromatography of Cu,Zn-SOD from bovine erythrocytes. A 1300-ml aliquot of lyophilized Tsuchihashi supernatant was dissolved in 65% saturated ammonium sulphate, clarified by filtration and applied to the CD-Sepharose column (770 ml, 1900 mg, 300 · 10³ U).

tionation was lyophilized and the dried protein was redissolved in 65% saturated ammonium sulphate. The solution was percolated over filter paper and then applied to a CD-Sepharose column previously equilibrated with the same salt concentration (Fig. 4). Elution and concentration of SOD activity were done as described in the case of the Fe-SOD. The Cu,Zn-containing enzyme, however, was eluted

TABLE II

PURIFICATION OF Cu,Zn-SOD FROM BOVINE ERYTHROCYTES

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Hemolysate[a]	1830	250 000	316 235[b]	1.3[c]	1	100
Supernatant from Tsuchihashi fractionation	1300	2 080	316 235	150	115	100
Lyophilized Tsuchihashi supernatant in 65% saturated ammonium sulphate	770	1 885	300 000	159	122	94
CD-Sepharose chromatography	175	95	286 600	2950	2269	91
Concentration on CD-Sepharose	20	85	275 000	3235	2488	87
Crystalline SOD preparation	12	84	268 800	3200	2461	85

[a] Hemolysate was prepared from 1 l of packed red cells.

[b] Not estimated.

[c] Because haemoglobin interferes with the SOD test, the activity of the haemoglobin-free extract was used to calculate the specific activity.
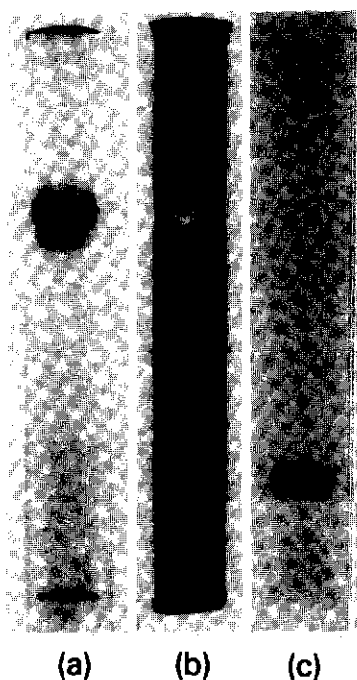
(a)   (b)   (c)

Fig. 5. Polyacrylamide gel electrophoresis of purified Cu,Zn-SOD from bovine erythrocytes in 8% gels under non-denaturing (a,b) and denaturing (c) conditions: (a) 30 µg of SOD stained for protein with Coomassie brilliant blue G250; (b) 5 µg of SOD stained for activity with the photochemical/NBT procedure; (c) 20 µg of protein were incubated for 5 min at 95°C in the presence of 1% SDS and 1% β-mercaptoethanol and subjected to SDS-PAGE.

from CD-Sepharose using 55% saturated ammonium sulphate. The results of the purification are summarized in Table II. The enzyme showed a 2400-fold purification with a yield of 85%. The specific activity of the purified enzyme was 3200 U/mg. The concentrated enzyme was stored under 90% saturated ammonium sulphate at 4°C. No loss of enzyme activity could be detected during 12 months. Homogeneity of the purified Cu,Zn-SOD was analyzed by PAGE. The electrophoretic patterns of the native enzyme show two bands, which correspond to the area of activity staining (Fig. 5a and b). These are the typical charge isomeric forms of Cu,Zn-SOD purified from erythrocytes [30]. SDS-PAGE demonstrates only one band (Fig. 5c).

CONCLUSION

In the present study we describe a special type of hydrophobic chromatography for the purification of prokaryotic as well as eukaryotic SODs. By using this technique we were able to improve the process of purification with respect to the time required, the number of purification steps and the yield. The mechanism of this chromatography was unknown till now. We suggest that conformational changes induced by variation of the ammonium sulphate concentration alter the affinity of the enzymes for the Sepharose derivative [9]. As single proteins show differences in the binding as well as elution behaviour, it is possible to separate a special enzyme from other proteins in one chromatographic step. Additionally the high concentration of structure-forming anions present at all purification steps causes stabilization of enzyme activity. The overall yields of active SOD were approximately 70%.

With the same chromatographic technique we also purified the Fe-containing SOD from *Pseudomonas testosteroni* and the Cu,Zn-containing SOD from human erythrocytes to electrophoretic homogeneity [17].

It should be pointed out that the support prepared by coupling 11-aminoundecanoic acid to cyanogen bromide-activated Sepharose was used for more than 25 preparations within 1 year with the same success. Sufficient regeneration was obtained by washing the columns with distilled water. From our experience with the preparation of other enzymes [9], the reproducibility of the purification results was influenced by the composition of the protein extract applied to the column [17].

REFERENCES

1 H. M. Steinman, in W. Oberley (Editor), *Superoxide dismutase*, Vol. 1, CRC Press, Boca Raton, FL, 1982, pp. 11–68.
2 K. Maejima, K. Miyata and K. Tomoda, *Agric. Biol. Chem.*, 47 (1983) 1537–1543.
3 R. J. Weselake, Sh. L. Chesney, A. Petkau and A. D. Friesen, *Anal. Biochem.*, 155 (1986) 193–197.
4 M. Miyata-Asano, K. Ito, H. Ikeda and S. Sekiguchi, *J. Chromatogr.*, 370 (1986) 501–507.
5 I. Bianchi, *Eur. Pat.*, EP 112 2990 A2 (1984).
6 K. Miyata, *Jpn. Pat.*, JP 589686 A (1983).
7 E. M. Gregory and Ch. H. Dapper, *Arch. Biochem. Biophys.*, 220 (1983) 293–300.

8 W. Schöpp, M. Grunow, H. Tauchert and H. Aurich, *FEBS Lett.*, 68 (1976) 198–202.

9 W. Schöpp, M. Grunow, K. Stolarski, A. Schäfer, D. Knopp and R. Lorenz, *J. Chromatogr.*, 376 (1986) 359–374.

10 J. M. McCord and I. Fridovich, *J. Biol. Chem.*, 244 (1969) 6049–6055.

11 B. J. Davies, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404–427.

12 W. Diezel, St. Liebe, G. Kopperschläger and E. Hofmann, *Acta Biol. Med. Germ.*, 28 (1972) 27–30.

13 C. Beauchamp and I. Fridovich, *Anal. Biochem.*, 44 (1971) 276–287.

14 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406–4412.

15 V. F. Kalb and R. W. Bernlohr, *Anal. Biochem.*, 28 (1977) 362–371.

16 O. H. Lowry, N. Y. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1964) 265–275.

17 M. Grunow, *Dissertation B*, Karl-Marx-Universität Leipzig, 1988.

18 S. Vorberg, W. Schöpp and H. Tauchert, *Monatsh. Chem.*, 114 (1983) 563–569.

19 F. Yost and I. Fridovich, *J. Biol. Chem.*, 248 (1973) 4905–4908.

20 F. Yamakura, *Biochim. Biophys. Acta*, 422 (1976) 280–294.

21 A. Anastasi, J. V. Bannister and W. H. Bannister, *Int. J. Biochem.*, 7 (1976) 541–546.

22 E. C. Hatchikian and Y. A. Henry, *Biochimie*, 59 (1977) 153–161.

23 J. B. Baldensperger, *Arch. Microbiol.*, 119 (1978) 237–244.

24 S. Kanematsu and K. Asada, *Arch. Biochem. Biophys.*, 185 (1978) 473–482.

25 S. Kanematsu and K. Asada, *FEBS Lett.*, 91 (1978) 94–98.

26 B. Meier, D. Barra, F. Bossa, L. Calabrese and G. Rotilio, *J. Biol. Chem.*, 257 (1982) 13977–13980.

27 E. Kusonose, K. Ichihara, Y. Noda and M. Kusonose, *J. Biochem.*, 80 (1976) 1343–1352.

28 K. B. Barkley and E. M. Gregory, *Arch. Biochem. Biophys.*, 280 (1990) 192–200.

29 E. M. Gregory, *Arch. Biochem. Biophys.*, 238 (1985) 83–89.

30 A. Gärtner and U. Weser, *Biomimetic and Bioorganic Chemistry II*, Akademie-Verlag, Berlin, 1986, pp. 1–61.