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PURIFICATION OF COPPER-ZINC-SUPEROXIDE DISMUTASE AND CATALASE FROM HUMAN ERYTHROCYTES BY COPPER-CHELATE AFFINITY CHROMATOGRAPHY

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SUMMARY

A relatively simple and reproducible procedure involving copper-chelate affinity chromatography for the isolation of copper-zinc-superoxide dismutase (E.C. 1.15.1.1) and catalase (E.C. 1.11.1.6) from human erythrocytes has been developed. Using this method, the two enzymes were easily and highly purified and appeared to be homogeneous as judged by polyacrylamide gel electrophoresis.

INTRODUCTION

Copper-zinc-superoxide dismutase (SOD) and catalase are considered to be of major importance in protecting living cells against toxic oxygen derivatives. SOD catalyzes the dismutation of the superoxide anion to hydrogen peroxide, and catalase converts hydrogen peroxide into water and oxygen (see ref. 1 for a review). These two enzymes have been used experimentally or clinically as anti-inflammatory drugs. They are usually purified by a conventional technique involving precipitation by ammonium sulphate, ion-exchange chromatographies and gel filtration[2]. However, the yields and extents of purification are relatively low. By using copper-chelate affinity chromatography, preparations of the two enzymes have been highly purified. The simple isolation procedure described in this paper will make the enzymes more readily available for a variety of investigations.

EXPERIMENTAL

Materials

Xanthine oxidase was obtained from Boehringer Mannheim, xanthine from Sigma, nitroblue tetrazolium (NBT) from Nakarai Chemicals (Japan), Tonein-TP from Otsuka Assay Laboratories (Japan) and DE-52 from Whatman. Sephadex G-150 and chelating Sepharose 6B, which is basically an iminodiacetic acid agarose gel first introduced by Porath *et al.*[3], were obtained from Pharmacia Fine Chemicals.

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Enzyme assays

SOD activity was assayed using xanthine and xanthine oxidase according to Beauchamp and Fridovich[4], using a Beckman DU-50 spectrophotometer at 25°C. One unit of activity is defined as the colour intensity corresponding to half-inhibition of the development of blue formazan in 0.5 ml of the assay mixture at 25°C. The specific activity is expressed in units per mg protein. Catalase activity was assayed by the method described by Sinha[5], and the enzyme activity is expressed as $K(0)$. The specific activity is expressed as $K(0)$ per mg protein.

Protein was determined from the absorbances at 280 and 590 nm, as well as by a dye-binding method[6] using Tonein-TP. The haem concentration was determined from the absorbance at 405 nm.

Electrophoresis and isoelectric focusing

Polyacrylamide gel electrophoresis on 7.5% gels for SOD, 5% gels for catalase, was performed according to Davis[7]. The SOD preparation was applied on duplicate gels. One was stained for protein with Coomassie Brilliant Blue G-250 and the other was assayed for SOD activity with *o*-dianisidine and riboflavin[8]. The molecular weights of SOD and catalase were estimated by disc gel electrophoresis on 12% gels and slab electrophoresis on 5–15% gels, respectively, in the presence of sodium dodecyl sulphate and 1% 2-mercaptoethanol, according to Laemmli[9].

The molecular weight of SOD was also determined by high-performance liquid chromatography (HPLC) (UV-8000, Toyo Soda) on a TSK G3000SW column. Isoelectric focusing (range pH 3–10) was carried out as described[10].

Purification of SOD and catalase

All procedures were carried out at 4°C. Human blood which had been stored for 10–15 days at 4°C in a citrate–phosphate–dextrose solution was used. The erythrocytes were washed with a 0.9% sodium chloride solution by low speed centrifugation at 1500 *g* for 15 min and then haemolyzed by adding an equal volume of water to the packed red cells. The haemolysates were dialyzed against 1.5 m*M* phosphate buffer (pH 6.8) and then centrifuged at 11 600 *g* for 20 min. The supernatant was adsorbed in a batchwise manner on DE-52, which had been equilibrated with the above phosphate buffer. The DE-52 was then washed thoroughly with the same buffer and packed in the column. SOD and catalase were eluted with 1.5 volumes of 20 m*M* phosphate buffer (pH 6.8). The eluted fractions of both enzymes were applied to a column (6 cm × 1 cm) of chelating Sepharose 6B primed with a copper(II) sulphate solution, which had been equilibrated with 50 m*M* phosphate buffer containing 500 m*M* sodium chloride (pH 7.0). After the column had been washed with the same buffer, SOD was eluted with 50 m*M* Tris–HCl buffer containing 500 m*M* sodium chloride (pH 8.0) and the eluted fractions were pooled (fraction 1). Further elution was carried out with a linear gradient between 20 ml of 50 m*M* Tris–HCl buffer containing 500 m*M* sodium chloride (pH 6.0) and 20 ml of the same buffer containing 200 m*M* histidine. The eluted fractions were pooled (fraction 2).

RESULTS AND DISCUSSION

Purification of SOD and catalase

Fig. 1 shows a typical elution profile for the two enzymes in copper-chelate chromatography. After sample application the column was washed with the buffer to remove non-adsorbed proteins. Most of the SOD were recovered in fraction 1 and all the catalase activity was recovered in fraction 2. Since the catalase was eluted at the start of the gradient, a better separation might be possible by use of a shallower gradient or a solution, containing histidine or immidazole at pH 6.0. However, on Sephadex, G-150, other contaminants could be separated. Fractions 1 and 2 were dialyzed against 20 m*M* phosphate buffer (pH 6.8) in order to remove Cu^{2+} and histidine. Fraction 2 was also loaded on a column (86 cm × 2.7 cm) of Sephadex G-150 which had been equilibrated with 20 m*M* phosphate buffer (pH 6.8) (Fig. 2).

The purification steps for SOD and catalase are summarized in Table I. The specific activity of purified SOD was of the same order of magnitude as that obtained in immunoaffinity chromatography using goat-anti-SOD[11]. The specific activity of the purified catalase was also of the same order as that obtained by Mörikofer-Zwez *et al.*[12].

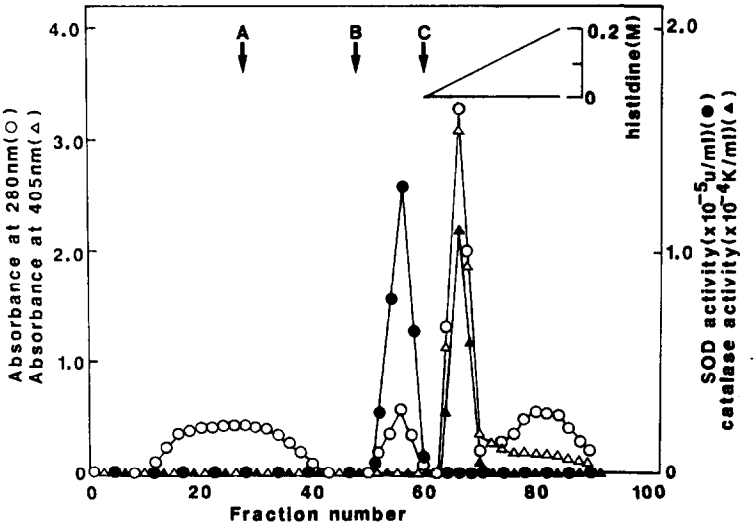

Fig. 1. Copper-chelate affinity chromatography. The fractions eluted from the DE-52 column were applied on a column (6 cm × 1 cm) of copper-chelating Sepharose 6B which had been equilibrated with 50 m*M* phosphate buffer containing 500 m*M* sodium chloride (pH 7.0). At A, the column was washed with 30 ml of the above buffer. At B, fraction 1 was eluted with 20 ml of 50 m*M* Tris–HCl buffer containing 500 m*M* sodium chloride (pH 8.0). At C, the active enzyme fraction was eluted with a linear gradient between 20 ml of 50 m*M* Tris–HCl buffer containing 500 m*M* sodium chloride (pH 6.0) and 20 ml of the same buffer containing 200 m*M* histidine (fraction 2) at a flow-rate of 10 ml/h. Fractions of 2 ml were collected. The protein concentration (○) was determined from the absorbance at 280 nm, and the haem concentration (△) from the absorbance at 405 nm. The SOD (●) and catalase (▲) activities were assayed as described in Experimental.

TABLE I

PURIFICATION OF SOD AND CATALASE

Step	*Total protein (mg)*	*SOD*				*Catalase*			
		Total activity (· 10^{-4} units)	*Specific activity (units/mg)*	*Yield (%)*	*Purification fold*	*Total activity [· 10^{-3} K(0)]*	*Specific activity [K(0)/mg]*	*Yield (%)*	*Purification fold*
Red blood cells	34 900	177	51	100	1	184	3.82	100	1
DE-52	218	98	4480	55	89	107	531	58.4	139
Copper-chelate column									
Fraction-1	5.40	60	110 500	35	2190	0			
Fraction-2	25.3					43.1	1700	23.4	445
Sephadex G-150	9.8					21.3	2160	11.6	565

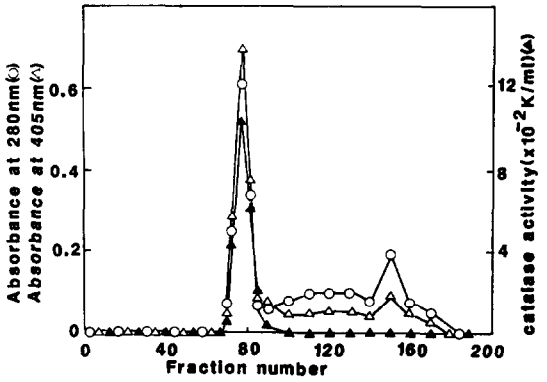

Fig. 2. Chromatography on Sephadex G-150. The pooled fractions showing catalase activity obtained from copper-chelate affinity chromatography were dialyzed against 20 m*M* phosphate buffer (pH 6.8) and then applied to a columnn (86 cm × 2.7 cm) of Sephadex G-150 which had been equilibrated with the above buffer. The column was washed with the same buffer at a flow-rate of 9 ml/h. Fractions of 3 ml were collected. ○—○, Protein concentration; △—△, haem concentration; ▲—▲, catalase activity.

Properties of the purified SOD and catalase

Figs. 3 and 4 show the electrophoretic patterns of the purified SOD and catalase. In disc electrophoresis, the SOD preparation gave two bands (Fig. 3A), which corresponded to the areas where the enzymatic activity was stained (Fig. 3B). The

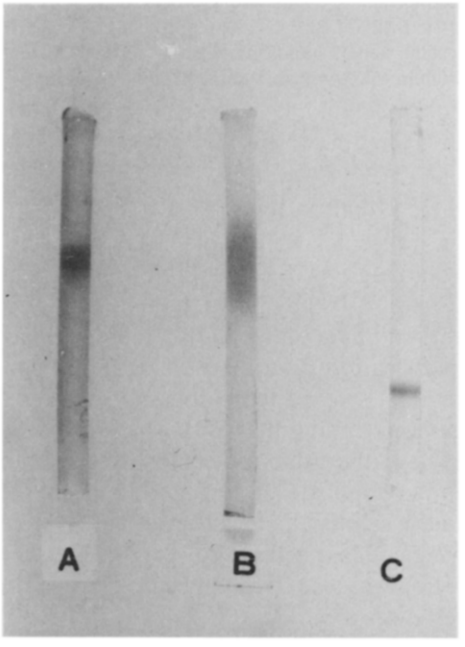

Fig. 3. Disc gel electrophoresis of the purified SOD. For lanes A and B, 7.5% gels were used. For lane C, a 12% gel in the presence of 1% sodium dodecyl sulphate was used. Lanes: A and C, protein staining with Coomassie Brilliant Blue G-250; B, SOD activity staining with *o*-dianisidine and riboflavin.

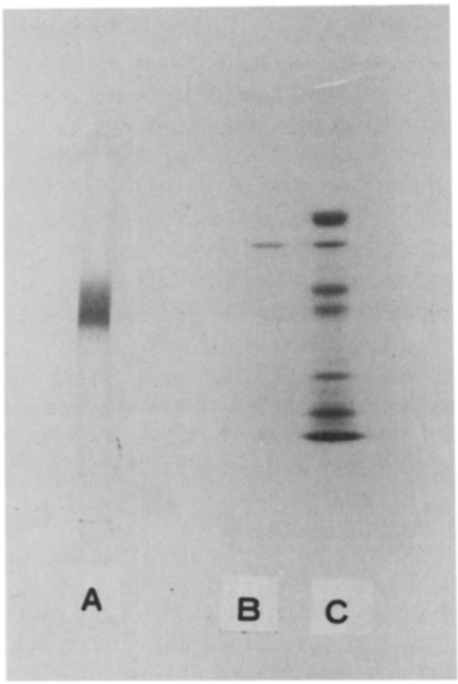

Fig. 4. Disc and slab gel electrophoresis of the purified catalase. For lane A, a 5% gel was used. For lanes B and C, 5–15% gradient gels in the presence of 1% sodium dodecyl sulphate were used. Lanes: A and B, protein staining of the purified catalase with Coomassie Brilliant Blue G-250; C, protein staining with Coomassie Brilliant Blue G-250 of the following molecular weight standards, ferritin (450000), bovine catalase (240000), aldolase (150000), bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen (25000) and cytochrome *c* (12500).

molecular weight of SOD was estimated to be 17000 ± 1000 (Fig. 3C). In HPLC the purified SOD gave a single peak with a molecular weight of 32000 (data not shown). The catalase preparation also gave a single protein band (Fig. 4A). The molecular weight of catalase was estimated to be 240000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and 1% 2-mercaptoethanol (Fig. 4B). In isoelectric focusing the SOD preparation gave three sharp bands corresponding to p*I* 4.7, 4.8 and 4.9. Therefore SOD seemed to exhibit charge heterogeneity. This result is consistent with our previous finding that SOD is antigenically cross-reactive with molecular species which have the same molecular weight[11]. In isoelectric focusing of the catalase preparation, a duplicate gel was used to locate the enzyme activity. For the detection of the catalase activity, 5-mm slices of the gel were incubated in the assay mixture. The gel showing catalase activity corresponded to the area stained for protein. The catalase preparation was also found to be heterogeneous as to p*I* 5.8 to 6.5, as reported by Mörikofer-Zwez *et al.*[13].

In the present study we used metal-chelate affinity chromatography to purify catalase as well as SOD. The affinity of a metalloenzyme to a metal-chelate column has been reported by Ohkubo *et al.*[14]. They purified nucleoside diphosphatase using

a zinc-chelate column and histidine as eluent. The binding of a metalloenzyme to a metal-chelate column is mediated by surface-located histidine residues of the metalloenzymes[15,16]. However, the affinity is dependent on the type of metalloenzyme. SOD did not bind to a zinc-chelate column, whereas catalase did (data not shown). This may also indicate that selective usage of such a column will be useful for preparing various metalloenzymes. Special care may be necessary, for metalloenzymes from which the metals, under certain conditions, can be effectively removed. In such cases, activity may be restored by the addition of the relevant metal ion. In the present study, however, such a treatment was not necessasry.

Erythrocytes contain large amounts of catalase and SOD. In contrast to the reported purification methods for enzymes, the present method involves only two or three purification steps with higher recoveries of the enzymes and no other time-consuming procedure is required. This method with a metal-chelate column will be useful for preparing metalloenzymes from human erythrocytes on a large scale. Very recently Waselake *et al.*[17] reported that SOD had been purified by use of a copper-chelate column and the result is almost consistent with our results.

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REFERENCES

1 D. A. Lewis, *Biochem. Pharmacol.*, 33 (1984) 1705.
2 M. J. Stansell and H. F. Deutsch, *J. Biol. Chem.*, 240 (1965) 4299.
3 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598.
4 C. Beauchamp and I. Fridovich, *Anal. Biochem.*, 44 (1971) 276.
5 A. K. Sinha, *Anal. Biochem.*, 47 (1972) 389.
6 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
7 B. J. Davis, *Ann. NY Acad. Sci.*, 121 (1964) 404.
8 H. P. Misra and I. Fridovich, *Arch. Biochem. Biophys.*, 183 (1977) 511.
9 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
10 M. C. Hoyle, *Int. Lab.*, 25 (1980).
11 K. Arai, S. Iizuuka, A. Makita, K. Oikawa and N. Taniguchi, *J. Immunol. Methods*, 91 (1986) 139.
12 S. Mörikofer-Zwez, M. Cantz, H. Kaufmann, J. P. Wartburg and H. Aebi, *Eur. J. Biochem.*, 11 (1969) 49.
13 S. Mörikofer-Zwez, J. P. Wartburg and H. Aebi, *Experientia*, 26 (1970) 945.
14 I. Ohkubo, T. Kondo and N. Taniguchi, *Biochim. Biophys. Acta*, 616 (1980) 89.
15 J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621.
16 E. Sulkowski, *Trends Biotechol.*, 3 (1985) 1.
17 R. J. Waselake, S. L. Chesney, A. Pethau and A. D. Friesen, *Anal. Biochem.*, 155 (1986) 193.