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An efficient method for purification of cuprozinc superoxide dismutase from bovine erythrocytes

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Abstract. Cuprozinc superoxide dismutase (Cu,Zn-SOD) was isolated from bovine erythrocytes by pH-controlled ammonium sulfate-methanol extraction (ASME extraction). Adjustment of the pH of a suspension of the lysed red cells in the presence of ammonium sulfate (90% saturation) to pH 5.0, followed by partition with an equal amount of methanol, resulted in isolation of the enzyme with specific activity of greater than 2000 units/mg of protein. Further purification using DEAE-cellulose column chromatography gave a highly purified Cu,Zn-SOD showing a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Using this procedure about 14 mg of pure Cu,Zn-SOD with a specific activity of 4728 units/mg of protein can be recovered from one liter of bovine blood. The enzyme was characterized and the results obtained were in agreement with earlier reports. This procedure appears, therefore, to be a convenient method for isolating the enzyme.
Key words. Cu,Zn-superoxide disumatase; purification; bovine erythrocytes.

Cuprozinc superoxide dismutase (Cu,Zn-SOD, EC 1.15.1.1), also known as erythrocuprein, is a family of metalloenzymes that catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen[1]. This enzyme is thought to be essential for the protection of cells against active oxygen species and is used experimentally or clinically as an antioxidant drug[2,3]. Efficient procedures for isolating Cu,Zn-SOD are important for clinical applications, and therefore simple isolation procedures that yield enzyme with high specific activity are of interest.

The most important step in the purification from erythrocytes is the removal of hemoglobin. Because of its stability in organic solvents,[4–7] the protein has usually been purified by the Tsuchihashi extraction, using a mixture of ethanol and chloroform, followed by salting out with K_2HPO_4, and acetone precipitation. This classical extraction procedure is efficient for removing large quantities of hemoglobin from hemolyzed samples but subsequent steps are required prior to column chromatography. Gärtner et al.[8] heated the hemolyzed sample instead of using Tsuchihashi extraction; however, the specific activity of Cu,Zn-SOD in this preparation was quite low. Isolation of SOD by a three phase partitioning method has also been reported[9], but subsequent laborious techniques were still required. In this communication, we report a convenient method that allows the enzyme to be isolated readily and selectively. To our knowledge, a Cu,Zn-SOD purified by this procedure has the highest specific activity reported to date.

Materials and methods

Chemicals. Chemicals were obtained as follows: Nitro blue tetrazolium and xanthine (sodium salt) from Sigma Chemical Co. (St. Louis, Missouri, USA); ferricytochrome *c* from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); ammonium sulfate, phenylmethylsulfonyl fluoride (PMSF), heparin and EDTA from Nacalai Tesque Inc. (Kyoto, Japan); xanthine oxidase from Oriental Yeast Co. Ltd. (Osaka, Japan); Whatman DE52 from Fisher Scientific Inc. (Pittsburgh, New Jersey, USA). All other chemicals used were of the highest grade available.

Extraction and purification of Cu,Zn-SOD. Erythrocytes were collected from fresh bovine blood (1000 ml) containing heparin (10 units/ml) by centrifugation (600 *g*, 10 min). The packed cells (430 ml) were lysed by addition of 2 volumes of cold distilled water and PMSF was added (final concentration, 10 μM). Solid ammonium sulfate was added with stirring to the hemolyzed sample to make a 90% saturated solution (662 g/1 l). The following steps were performed at room temperature unless otherwise noted. The ammonium sulfate solution was adjusted to pH 5.0 with acetic acid, and an equal volume of methanol was added slowly with stirring. The resulting mixture was vigorously stirred for 15 min. Centrifugation (3000 *g*, 10 min) of the mixture resulted in a separation of liquid and solid phases; the former contained enriched SOD activity and the latter contained denatured hemoglobin, other proteins and insoluble ammonium sulfate. The supernatant was carefully transferred and concentrated to 50 ml at 4 °C in an

Amicon Ultra filtration system with PM10 membrane. The concentrated sample was dialyzed against 10 mM potassium phosphate buffer (pH 6.5)-0.01 mM EDTA (Buffer A). The resulting concentrated sample was applied to a DE52 column (26 × 2.5 cm, i.d.) which had been carefully equilibrated with Buffer A (4 °C) according to the suggestion of West et al.[10]. The column was washed with Buffer A until unbound protein was completely eluted. The Cu,Zn-SOD was eluted with 100 ml of a 0-0.2 M KCl linear gradient in Buffer A at a flow rate of 30 ml/min. Fractions containing SOD activity were collected and stored at −20 °C.

Assay. All incubations were performed at 25 °C. SOD activity was determined by the method of McCord and Fridovich[4]. Protein content was determined by the method of Bradford[11] using bovine serum albumin as the standard. Hemoglobin concentration was determined at 555 nm according to the method of Keele et al.[12]. SDS-PAGE or native PAGE were performed according to the method of Laemmli[13] and Davis[14], respectively. SOD activity was stained with riboflavin and nitro blue tetrazolium according to the method of Beauchamp and Fridovich[15]. To compare this procedure with methods reported previously, Tsuchihashi extraction, the heat treatment and the three phase partitioning procedure were performed according to the methods of McCord and Fridovich[4], Gärtner et al.[8] and Pol et al.[9], respectively.

Results and discussion

Although solid ammonium sulfate was added up to 90% saturation, little precipitation of proteins present in red blood cells of bovine was observed. Under these conditions, the partition efficiency of Cu,Zn-SOD between organic solvent and hemolysate was dependent on the organic solvents used and on the ratio of the organic to aqueous phases. Methanol was much more effective in extracting the enzyme from the sample containing the ammonium sulfate mixture than ethanol, which recovered only 10%. For these reasons, we chose methanol as the solvent for partition of the metalloenzyme in the following experiments. Ratios (0.8 to 1.0) of lysed erythrocyte to methanol were optimal for selective isolation of the enzyme although the total activity with a ratio of 4 was about twice that with a ratio of 1. The specific activity of the enzyme tended to increase with increasing concentration of ammonium sulfate added, but the recoveries were almost the same between 30 to 90% saturation. The highest specific activities Cu,Zn-SOD were obtained at pH value of ASME extraction between 4.5 and 5.0; however, activity below pH 4.0 was low. Based on this data and the pH dependency of the yield pH 5.0 was selected as optimal.

Table 1 compares the efficiencies of Cu,Zn-SOD isolation by ASME extraction, the Tsuchihashi extraction heat treatment, and three phase partitioning, measured by several criteria. Total proteins recovered by ASME extraction, Tsuchihashi extraction, heat treatment and triphase partitioning were 0.04, 0.82, 0.20 and 2.1% of the original lysed red cells, respectively. All methods effectively removed hemoglobin. The thermal treatment of the hemolyzed sample gave a low recovery of Cu,Zn-SOD compared to other methods. The low recovery appears to be due not to inactivation but aggregation of the enzyme with other denatured proteins; Cu,Zn-SOD is relatively stable at 70 °C for even 30 min[8,16]. In fact, the recovery of Cu,Zn-SOD from the heat-treated lysed sample by ASME extraction was about 50% of that from non-treated sample (data not shown). Based on the level of hemoglobin contamination in these extracts, the proportions of proteins other than hemoglobin extracted by the procedures were 28% (ASME extract), 99% (Tsuchihashi extract), 50% (heat treatment) and 85% (three phase partitioning) of recovered protein. Except for the heat treatment, the yields of Cu,Zn-SOD from the lysed sample by the other methods were similar whereas the ASME extraction gave the highest specific activity. Thus, the selective extraction of the enzyme by the ASME procedure is superior to that of methods reported previously.

The present method was applied to the purification of Cu,Zn-SOD from bovine erythrocytes. The ASME-extractable proteins were chromatographed on a DE52 anion exchanger with the results shown in figure 1. The

Table 1. Extraction of erythrocyte Cu,Zn-SOD using different methods.

Characteristic	Method used[a]			
	ASME[b] extraction	Tsuchihashi extraction	Heat treatment	Three phase partitioning
Protein recovered (mg)	2.18	47.94	11.95	123.20
Volume (ml)	48.8	34.0	16.0	27.5
Hemoglobin contaminated (mg)	1.58	0.68	5.92	18.70
Total activity (units)	4372	5270	1199	4741
Specific activity (units/mg)	2002	111	100	38.5

[a]were determined with lysed sample (50 ml, 5869 mg of protein) under the conditions described in 'Materials and methods'.
[b]Lysed samples mixed with ammonium sulfate to make a 90% saturation, followed by adjustment to pH 5.0 were extracted with methanol (1:1, v/v).

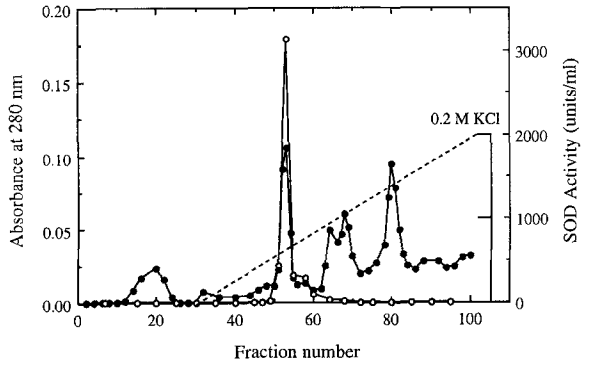

Figure 1. Separation of erythrocyte Cu,Zn-SOD by DE52 column chromatography. Open and closed circles represent SOD activity and protein elution, respectively. Dialyzed sample (63 ml) was applied to a DE52 column which had been equilibrate with Buffer A, and fractions were collected. The column was extensively washed with Buffer A and then a linear gradient of KCl ranging from 0 to 200 mM, pH 6.5, was started from fraction 31. Fractions 51–58 contained SOD activity. Proteins eluted from the column were monitored at 280 nm.

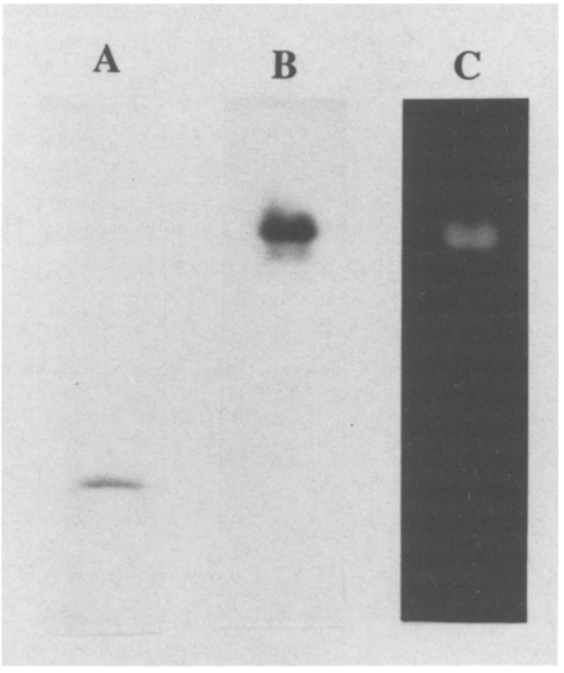

Figure 2. Polyacrylamide gel electrophoresis of purified enzyme in the presence (*A*) and absence (*B*, *C*) of sodium dodecyl sulfate. Samples of the enzyme (*A*, 1 μg; *B*, 4 μg; *C*, 0.25 μg of protein) were subjected to polyacrylamide gel electrophoresis (*A*, 12%; *B* and *C*, 9%), respectively. The electrophoresis in *A* was performed in the presence of sodium dodecyl sulfate (0.1%). The gels in *A* and *B* were stained with Coomassie Brilliant Blue G-250 and that in *C* was assayed for enzyme activity with riboflavin and nitro blue tetrazolium.

results of the purification procedure are summarized in table 2. The final yield was 14 mg per 1 liter of blood with a specific activity of 4728 units of SOD activity per milligram of protein. The purified protein exhibited a unique UV spectrum with a maximum at 259 nm based on phenylalanine residues and the absence of tryptophan and tyrosine residues[17,18]. A broad, weak visible absorption band with a peak around 680 nm was also observed. The ratio of absorption at 260 nm to that at 280 nm was 1.68. The Cu,Zn-SOD showed a single band with a molecular weight of 16.5 kDa on polyacrylamide gel in the presence of SDS (fig. 2A) whereas the electrophoretic patterns of native enzyme gave two bands (fig. 2B), which corresponded to the area of activity staining (fig. 2C). These are the typical charge isomeric forms of Cu,Zn-SOD purified from erythrocytes[19]. The results were very consistent with those reported by others.

In conclusion, a simple and reliable method for purification of Cu,Zn-SOD has been developed. The selective partition of the enzyme from red blood cells by ASME extraction seems to be due to multiple effects: solubility in methanol, insensitivity to organic solvent, salting out by ammonium sulfate, and the pH, which is close to the isoelectric point of the enzyme (pI 4.9–5.5). All steps except for the dialysis and the column chromatography can be performed at room temperature without significant loss of activity. Furthermore, addition of acetone to the ASME extract of the lysed sample resulted in precipitation of Cu,Zn-SOD with a specific activity of about 4000 units/mg of protein, suggesting that this procedure gives an enzyme preparation of a higher specific activity than commercial grade without column chromatography. Using ASME extraction and CM-cellulose column chromatography alone makes it possible to purify Cu,Zn-SOD from mammalian tissue as well (Y. Kumagai et al., unpubl. observ.), and could be used to extract it from bacteria expressing the enzyme.

Table 2. Purification of Cu,Zn-SOD from bovine erythrocytes.

Step	Volume (ml)	Protein (mg)	Total activity (units × 10^3)	Specific activity (units/mg)(%)	Yield
Red blood cells	1295	147630	n.d.	n.d.	-
ASME extract	1330	43.49	86.4	1986	100
DE52	67	13.79	65.3	4728	76

n.d., not detectable.
SOD activity was determined under the conditions described in 'Materials and methods'.

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