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# APPLICATION OF A NEW ION EXCHANGER TSK-GEL DEAE-5PW, TO THE PURIFICATION OF Cu,Zn-SUPEROXIDE DISMUTASE OF BOVINE ERYTHROCYTES

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## SUMMARY

We have developed an effective method for the purification of Cu,Zn-superoxide dismutase [E.C. 1.15.1.1] from bovine erythrocytes. This enzyme functions as a scavenger of superoxide radical, and it seems to be a key enzyme in the metabolism of active oxygen species. Application of this enzyme as a drug has been considered, and for this purpose a highly purified preparation is necessary.

The first purification of this enzyme was reported by McCord and Fridovich [*J. Biol. Chem.*, 244 (1969) 6049]. The limiting step in their method is the removal of the small amount of contaminating protein from the acetone-precipitated crude preparation (3000 units/mg protein). We found that a new, high-performance ion exchanger, TSK-GEL DEAE-5PW, improves this step and makes possible the large-scale preparation of pure protein. Optimal conditions for a TSK-GEL DEAE-5PW preparative column (150 × 21.5 mm I.D.) were established. The acetone-precipitated crude enzyme was dissolved in 20 m*M* Tris–HCl buffer (pH 7.5), protein concentration 11.0 mg/ml, applied to the column, and eluted with a linear gradient of sodium chloride from 0 to 0.3 *M*. The flow-rate and sample volume were set to 4.0 ml/min and 8.0 ml, respectively. Under these conditions, the fractionated Cu,Zn-superoxide dismutase showed a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and ion exchange high-performance liquid chromatography, and a specific activity of 3800 units/mg protein. The recovery of activity was 75%, and the cycle time was 120 min. A yield of 80–85 mg of purified enzyme was obtained per cycle. The preparation thus isolated has the highest purity and activity so far reported. We conclude that TSK-GEL DEAE-5PW is the practical choice for large-scale purification of Cu,Zn-superoxide dismutase.

## INTRODUCTION

Superoxide dismutase [E.C. 1.15.1.1] catalyzes the dismutation of the superox-

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ide radical ($O_2^{\cdot-}$) to molecular oxygen and hydrogen peroxide[1]. The superoxide radical is the first product of the univalent reduction of oxygen, and it is known to be involved and produced in various biological processes[2–7]. Its toxicity is a consequence of its involvement in a metal-catalyzed Haber–Weiss reaction or Fenton reaction, the final product of which is the highly reactive hydroxyl radical ($^{\cdot}OH$). The first line of defense against oxygen toxicity is therefore the removal of superoxide radicals. In fact, superoxide dismutases are found in all oxygen-utilizing organisms so far examined[8], and these enzymes have been implicated in protection against various types of damage[9–12].

Superoxide dismutase is a metalloprotein with a redox metal at the active site. Three different forms, Cu,Zn-, Mn-, and Fe-forms, have been isolated. The cytoplasm of eukaryotic organisms contains the Cu,Zn-enzyme, which is a dimer with a subunit molecular weight of *ca.* 16 000. The amino acid sequence[13] and the X-ray structure at 2.0 Å resolution[14] for the bovine enzyme have been determined.

Huber and co-workers discovered an anti-inflammatory protein, Orgotein, from bovine liver and erythrocytes [British Patent Specification No. 1,160,151 (Nov. 26, 1969)] later determined to be Cu,Zn-superoxide dismutase[1–17]. This enzyme has been considered for application in rheumatoid arthritis therapy and against side-effects of cancer chemo- and radio-therapies[15,18,19]. A highly purified preparation is required for this purpose. The first purification of this enzyme from bovine erythrocytes was reported in 1969 by McCord and Fridovich[1]. The limiting step in the preparation of the highly purified enzyme is the removal of the small amount of contaminating proteins from the acetone-precipitated crude preparation. We will show in this paper that ion-exchange high-performance liquid chromatography (HPLC) on TSK-GEL DEAE-5PW is a practical procedure for the large-scale purification of Cu,Zn-superoxide dismutase.

## EXPERIMENTAL

### *Crude Cu,Zn-superoxide dismutase preparation*

The purification procedure was the same, in principle, as that of McCord and Fridovich[1]. Fresh bovine blood (a gift from a local slaughterhouse) was preserved with acid/citrate/dextrose solution. Cells were collected from 10 l of blood by centrifugation, resuspended and washed in 0.9% sodium chloride by centrifugation. The packed cells (5 l) were lysed by addition of an equal volume of water. The total superoxide dismutase activity of the hemolysate was $4.0 \cdot 10^6$ units. Hemoglobin was precipitated from the 10 l of hemolysate by the Tsuchihashi chloroform–ethanol treatment. With stirring, 2.5 l of ethanol and 1.5 l of chloroform were added at 0°C. This mixture was stirred until it became very thick (*ca.* 20 min). The precipitate was removed by centrifugation. To the supernatant (6.7 l), solid dipotassium hydrogen phosphate (2.0 kg) was added, resulting in the separation of two phases. All the superoxide dismutase activity was recovered from the upper phase (1.8 l). Cold acetone (1.35 l) was added to this solution to precipitate the enzyme. This precipitate was collected by centrifugation, dissolved in water, and dialyzed against the starting buffer of the ion-exchange HPLC. This solution is subsequently referred to as crude enzyme solution, in which superoxide dismutase activity of $2.1 \cdot 10^6$ units was recovered. The specific activity of the crude enzyme solution was estimated to be 3000 units/mg protein, and the protein concentration was 11.0 mg/ml.

*Superoxide dismutase assay*

The superoxide dismutase activity was assayed according to the method of McCord and Fridovich[1]. Xanthine, bovine milk xanthine oxidase, and horse heart cytochrome *c* were purchased from Sigma (St. Louis, MO, U.S.A.).

*Protein concentration*

Protein concentrations were determined by the Lowry method, using serum albumin as standard[20].

*Gel electrophoresis*

Polyacrylamide gel (12.5%) electrophoresis (PAGE) in the absence and presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol were conducted according to Davis[21] and Weber and Osborn[22], respectively, using a slab gel system (Hoefer, San Francisco, CA, U.S.A.). A constant current of 25 mA was applied for 6 h. Proteins were stained by Coomasie brilliant blue G-250. For molecular weight estimation in SDS–PAGE, hen-egg lysozyme (Sigma, Lot 129C8760) and bovine milk $\beta$-lactoglobulin (Sigma, Lot 13F8780) were used as standard proteins. Their molecular weights are $1.43 \cdot 10^4$ and $1.84 \cdot 10^4$, respectively. Bovine Cu,Zn-superoxide dismutase is a dimer with a subunit molecular weight of 16 400[13].

*Spectrophotometry*

All spectrophotometric measurements were carried out with a Union Giken spectrophotometer SM 401 (Osaka, Japan) at 25°C.

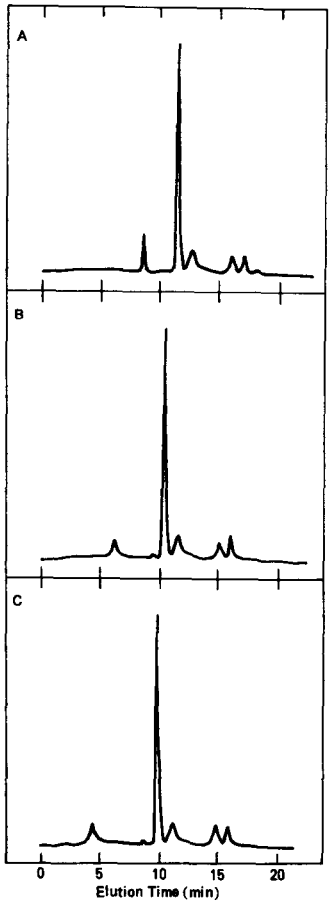
*Ion-exchange HPLC*

Ion-exchange HPLC measurements were carried out on an analytical column (75 × 7.5 mm I.D.) and a preparative column (150 × 21.5 mm I.D.), with TSK-GEL DEAE-5PW purchased from Toyo Soda (Tokyo, Japan)[23]. The column was connected to a high-speed liquid chromatograph, HLC-803C, equipped with a gradient generator GE-2 and a variable-wavelength UV detector UV-8 (all from Toyo Soda). The sample solution was injected into the column, equilibrated with the starting buffer and, simultaneously, the linear salt gradient elution was started at 25°C. Flow-rates of 1.0 and 4.0 ml/min were selected for the analytical and preparative columns, respectively. The elution was monitored by absorption at 280 nm. In the preparative ion-exchange HPLC, the eluent was fractionated for further analyses.

## RESULTS AND DISCUSSION

*Effects of pH on the separation of Cu,Zn-superoxide dismutase*

The effects of pH on the separation of the enzyme were examined by using a TSK-GEL DEAE-5PW analytical column in 20 m*M* Tris–HCl buffer at pH 8.5, 8.0 and 7.5. A 30-$\mu$l volume of a crude enzyme solution (protein concentration, 7.5 mg/ml; specific activity, 3000 units/mg protein) was injected into the column, equilibrated with the starting buffer. A linear gradient (30 min) of sodium chloride from 0 to 0.3 *M* in equilibrating buffer was begun simultaneously with sample injection. As shown in Fig. 1, the enzyme (main band) showed a tendency to be eluted faster with decreasing pH, and the separation from contaminating proteins became better.



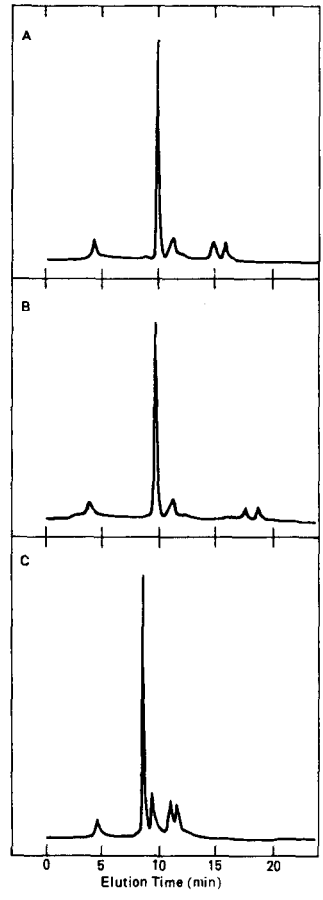




Fig. 1. Effects of pH on the separation of Cu,Zn-superoxide dismutase. A 30-$\mu$l volume of the crude enzyme solution (7.5 mg protein/ml; 3000 units/mg protein) was injected into a TSK-GEL DEAE-5PW ion-exchange HPLC column (75 × 7.5 mm I.D.) equilibrated with 20 m*M* Tris–HCl buffer at (A) pH 8.5, (B) pH 8.0, and (C) pH 7.5. A linear gradient elution for 30 min was started at the same time as sample injection (time, 0 min) and went from the starting buffer to the buffer containing 0.3 *M* sodium chloride at a flow-rate of 1.0 ml/min. The best separation was obtained at pH 7.5.

Fig. 2. Effects of salt species on the separation of Cu,Zn-superoxide dismutase. A 30-$\mu$l volume of the crude enzyme solution (7.5 mg protein/ml; 3000 units/mg protein) was injected into a TSK-GEL DEAE-5PW ion-exchange column (75 × 7.5 mm I.D.) equilibrated with a 20 m*M* (A) Tris–HCl, (B) Tris–acetate, and (C) Tris–$HClO_4$ buffer at pH 7.5. A linear gradient elution for 30 min was started at the same time as sample injection (time, 0 min) and went from the starting buffer to the buffer containing 0.3 *M* (A) sodium chloride, (B) sodium acetate, and (C) sodium perchlorate at a flow-rate of 1.0 ml/min. The best separation was obtained in the Tris–HCl–sodium chloride buffer system.

The separation was also tested in 20 m*M* potassium phosphate buffer at pH 7.0 and 6.5. However, the elution of the proteins was faster and the separation was poor. This may be due to the higher ionic strength of the buffer and/or the smaller difference between the pH examined and the isoelectric point of the enzyme (p*I* 4.9–5.5). Accordingly, the Tris–HCl buffer of pH 7.5 was used in ion-exchange HPLC.

*Effects of salt species on the separation of Cu,Zn-superoxide dismutase*

The effects of salt species on the separation of the enzyme were examined. The TSK-GEL DEAE-5PW analytical column was equilibrated with 20 m*M* Tris–HCl buffer, Tris-acetate buffer, or Tris–$HClO_4$ buffer at pH 7.5. A 30-$\mu$l volume of crude enzyme solution was injected, and the 30-min linear gradient elution was carried out from each starting buffer to the buffer containing 0.3 *M* sodium chloride, sodium acetate or sodium perchlorate, respectively (Fig. 2). The enzyme was well separated in the Tris–HCl–sodium chloride buffer system. The separation from the small amount of protein eluting just before the enzyme band was especially good. Thus, this buffer system was the best of the three systems examined.

*Effects of gradient time and sample volume on the separation of Cu,Zn-superoxide dismutase*

The effects of gradient time and sample volume of crude enzyme solution (protein concentration, 11.0 mg/ml) were examined for ion-exchange HPLC on a TSK-GEL DEAE-5PW preparative column, in 20 m*M* Tris–HCl–sodium chloride buffer (pH 7.5). A linear gradient elution for 120 min from 0 to 0.3 *M* sodium chloride and a sample volume of 8.0 ml were selected optimally at a flow-rate of 4.0 ml/min. Under these conditions, the enzyme solution eluted between 24.0 min and 27.6 min was fractionated; 75 ± 3% of the total activity injected onto the column was recovered in the fractionated solution, and the specific activity was 3800 ± 100 units/mg protein. The purity of the fractionated enzyme was checked by an analytical ion-exchange HPLC under the same conditions as those used in Fig. 1C. The chromatogram showed a single band, indicating that the enzyme solution was highly purified and homogeneous.

*Electrophoresis of the Cu,Zn-superoxide dismutase, fractionated in the preparative ion-exchange HPLC*

Fig. 3A shows the polyacrylamide gel electrophoresis of the Cu,Zn-superoxide dismutase, fractionated by ion-exchange HPLC. One major and one minor band were detected in the fractionated enzyme (Lanes 2–5), whereas the crude enzyme showed at least seven bands (Lane 1). Fig. 3B shows the SDS–PAGE of the fractionated enzyme. The samples were denatured by the incubation for 3 min at 100°C in 10 m*M* sodium phosphate buffer (pH 7.2), containing 1.0% SDS and 2% 2-mercaptoethanol. In the crude enzyme solution (Lane 1), several bands are observed, whereas in the enzyme solutions fractionated by ion-exchange HPLC, shown in Lanes 2–4, only one band was observed. The enzyme in Lane 5 was purchased from Toyo Soda and purified by ion-exchange chromatography on TSK-GEL DEAE-Toyopearl 650M. The specific activity of this preparation is 3300 units/mg protein, and it also shows a single band in SDS–PAGE (Lane 5). The molecular weight of the band in Lanes 2–5 was estimated to be 16 400, which corresponds to the molecular weight of the subunit of Cu,Zn-superoxide dismutase[13]. Therefore, the fractionated enzyme solution is believed to be highly homogeneous. Although the protein concentration and the sample volume applied to the electrophoresis shown in Fig. 3A and B were the same, the minor band was detected in the electrophoresis in the native conditions (Fig. 3A), but not in the denatured conditions (Fig. 3B). The minor band did not appear in analytical ion-exchange chromatography. This minor band detected in Fig.

3A might be due to the apoenzyme lacking Cu and/or Zn ions, or to the monomeric form of the enzyme, if it exists in the low protein concentration (*ca.* 1 mg/ml) used for the electrophoresis in non-denaturing conditions. The enzyme preparation purified on TSK-GEL DEAE-Toyopearl (Fig. 3B, Lane 5) showed only one band in the

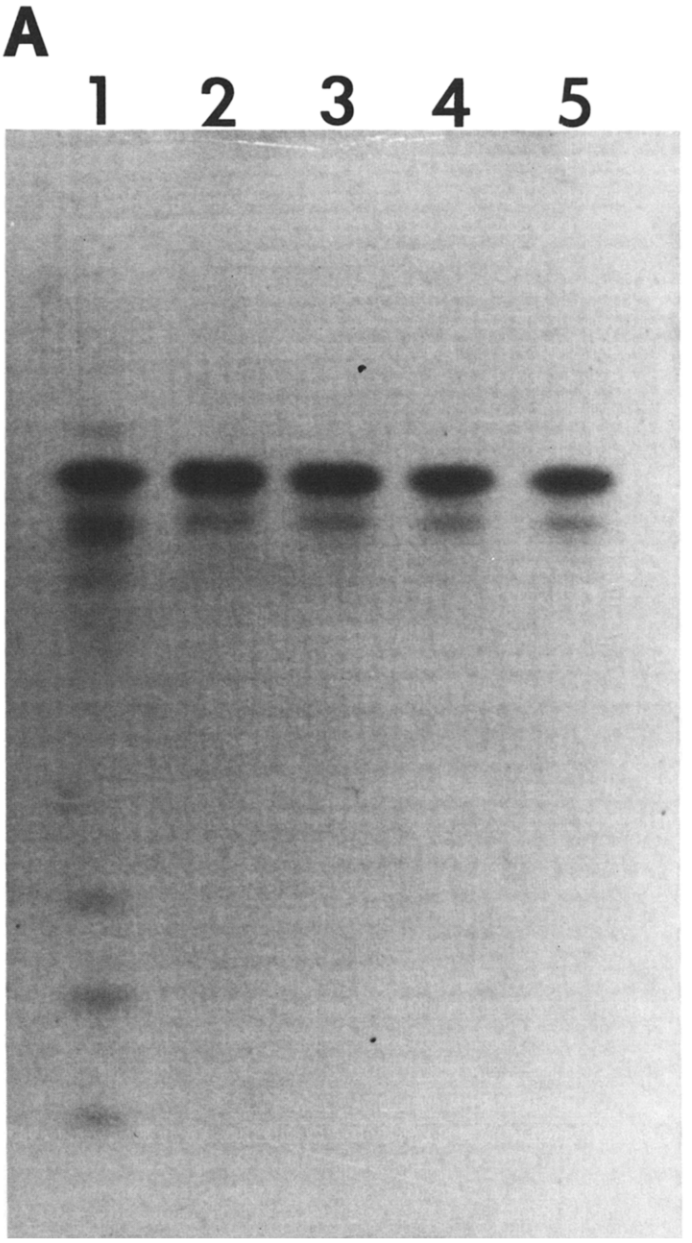



Fig. 3.

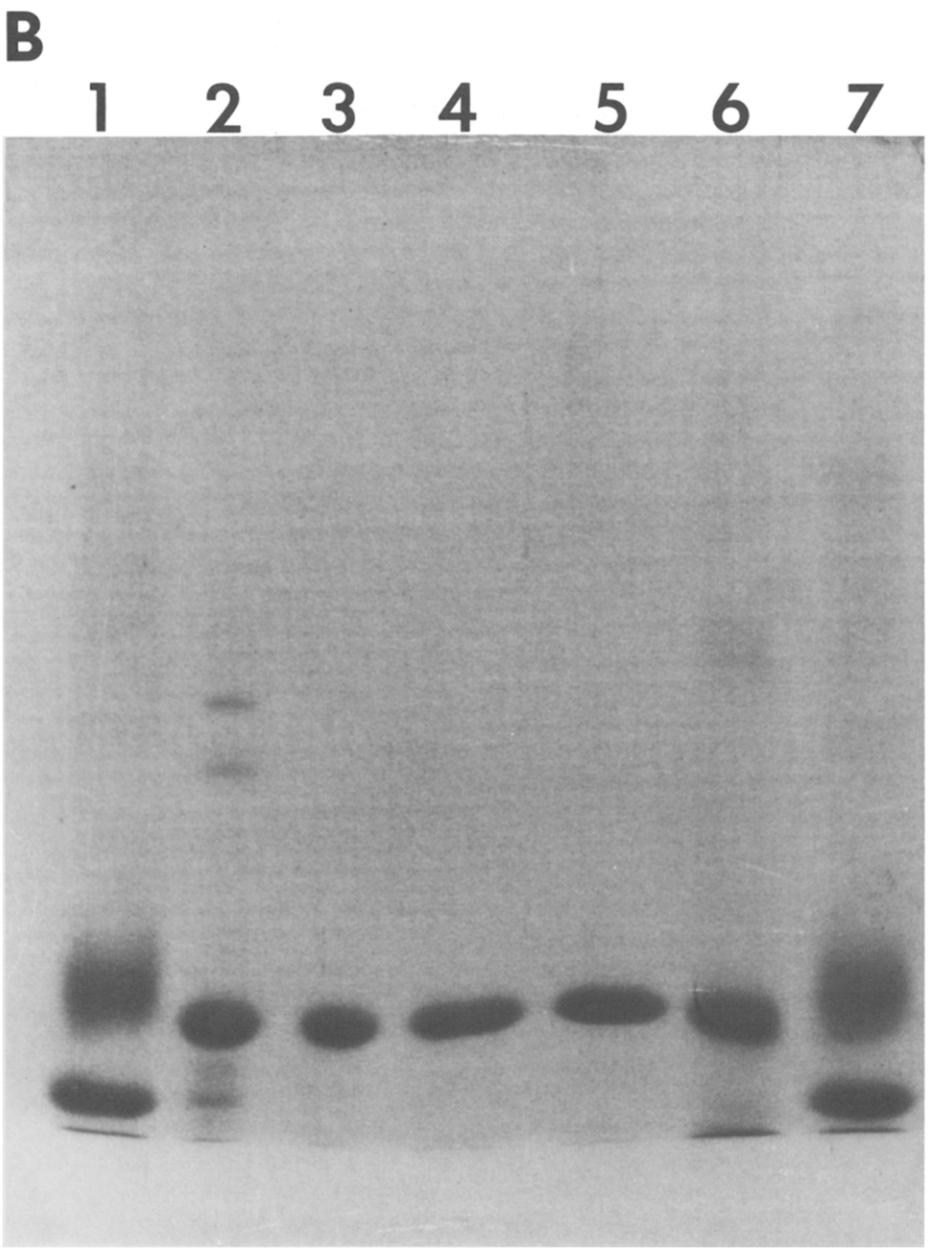



Fig. 3. Electrophoresis of the Cu,Zn-superoxide dismutase fractionated by ion-exchange HPLC. (A) Polyacrylamide gel electrophoresis at pH 9.5. (B) SDS–polyacrylamide gel electrophoresis. A 10-$\mu$l volume of sample solution containing 10 $\mu$g of protein was applied to each lane. (A) Lane 1, crude enzyme subjected to the ion-exchange HPLC; Lanes 2, 3, and 4, enzyme fractionated in ion-exchange HPLC with the injection of sample volume of 2.0, 4.0, and 8.0 ml, respectively; Lane 5, the enzyme purchased from Toyo Soda (specific activity, 3300 units/mg protein). (B) Lanes 1 and 7, standard proteins for molecular weight (MW) estimation, hen-egg lysozyme (MW 14 300) and bovine milk $\beta$-lactoglobulin (MW 18 400). Lane 2, the same as (A) Lane 1; Lanes 3, 4, and 5, the same as (A) Lanes 2, 3, and 4; Lane 6, the same as (A) Lane 5.

denatured state, like the preparation purified on TSK-GEL DEAE-5PW, though its specific activity is 3300 units/mg, suggesting that both the preparations were purified to homogeneity. Thus, the preparation purified on DEAE-Toyopearl (this is accepted as a medium-performance liquid chromatography gel) may contain more apoprotein than does that purified by DEAE-5PW. (The purification on TSK-GEL DEAE-Toyopearl has been reported by Inouye[26]).

*Ultraviolet absorption spectra of Cu,Zn-superoxide dismutase*

UV absorption spectra of the crude enzyme solution and the fractionated enzyme solution in the TSK-GEL DEAE-5PW ion-exchange HPLC are shown in Fig. 4. Fig. 4A shows the UV absorption spectrum of a five-fold diluted solution of the crude enzyme solution subjected to ion-exchange HPLC. The protein concentration and the specific activity of this diluted solution were 2.2 mg/ml and 3000 units/mg, respectively. Fig. 4B shows the spectrum of the fractionated enzyme solution (protein concentration, 3.8 mg/ml; specific activity, 3800 units/mg). The absorption at *ca.* 280 nm is greatly decreased in the fractionated enzyme compared with that in the crude enzyme. It is known that bovine Cu,Zn-superoxide dismutase does not contain tryptophyl residues[13], and the UV absorption spectrum should be essentially due to the absorption of phenylalanyl and tyrosyl residues and Cu- and Zn-complexes. Consequently, the UV absorption is different from those of common proteins; the maximum is observed at *ca.* 260 nm, and not *ca.* 280 nm. The contaminating proteins, if any, would be expected to contribute to a large extent to the absorption *ca.* 280 nm. In fact, the ratio of the absorption at 260 nm to that at 280 nm can be a useful purification index. The values are 1.30 for the crude enzyme and 1.66 for the fractionated enzyme. And this index was actually used practically to

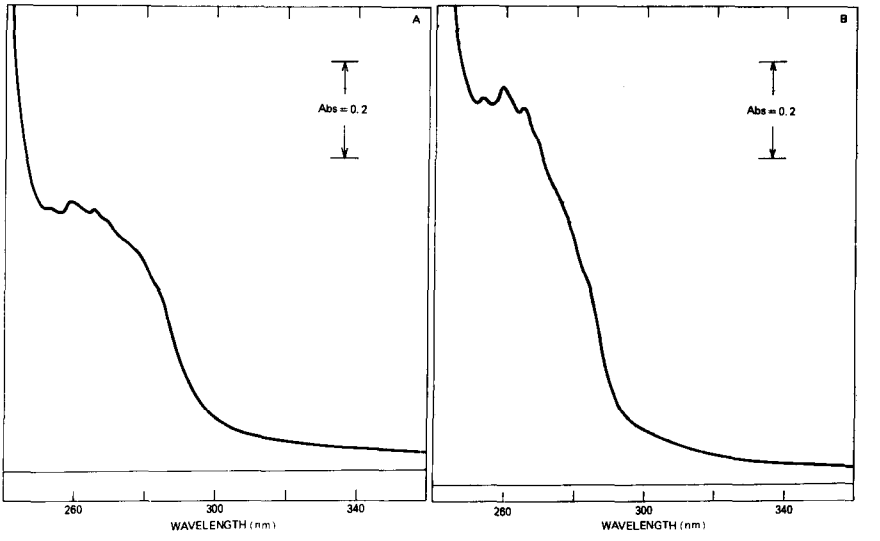



Fig. 4. Absorption spectra of Cu,Zn-superoxide dismutase. (A) Five-fold diluted crude enzyme solution subjected to the ion-exchange HPLC. Protein concentration, 2.2 mg/ml; specific activity, 3000 units/mg protein. (B) Enzyme solution fractionated by ion-exchange HPLC. Protein concentration, 3.8 mg/ml; specific activity, 3800 units/mg protein.

check the purity of the enzyme solutions in the purification procedures. Here, it should be noted that the absorption spectrum of the crude enzyme (Fig. 4A) is very similar to that reported by McCord and Fridovich for the Cu,Zn-superoxide dismutase (specific activity, 3300 units/mg), which suggests that the enzyme first purified by them was not homogeneous.

*Comparison of commercially available Cu,Zn-superoxide dismutase preparations*

The ion-exchange HPLC patterns of commercially available preparations of bovine Cu,Zn-superoxide dismutase were compared (Fig. 5). Ion-exchange HPLC was carried out on the TSK-GEL DEAE-5PW analytical column in 20 m*M* Tris–HCl buffer (pH 7.5). A 30-$\mu$l aliquot of protein solution was injected, and at the same

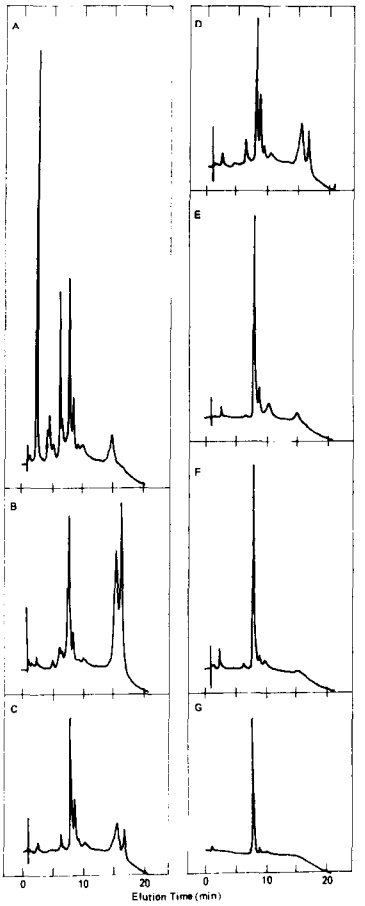



Fig. 5. Comparison of HPLC patterns of commercially available Cu,Zn-superoxide dismutase preparations. Ion-exchange HPLC was carried out on a TSK-GEL DEAE-5PW column (75 × 7.5 mm I.D.) equilibrated with 20 m*M* Tris–HCl buffer at pH 7.5. A 30-$\mu$l volume of enzyme solution was injected into the column, and a linear gradient elution for 60 min was started at the same time from the starting buffer without sodium chloride to the buffer containing 0.5 *M* sodium chloride. The elution was followed by absorption detection at 280 nm. (A) Sigma, Lot 72F9365; (B) Calbiochem, Lot 202044; (C) Miles, Lot 7017; (D) Boehringer Mannheim, Lot 1112203; (E) Toyobo; (F) Diagnostic Data; (G) Toyo Soda.

time the 60-min linear gradient elution was begun. The starting buffer contained no sodium chloride, the final buffer contained 0.5 *M* sodium chloride. The preparations purchased from Sigma, Calbiochem, Miles, and Boehringer Mannheim were shown to contain significant amounts of contaminating proteins. On the other hand, the preparations from Toyobo (Osaka, Japan), Diagnostic Data (Mountain View, CA, U.S.A.) and Toyo Soda appeared to be nearly homogeneous, especially the Toyo Soda preparation (specific activity, 3300 units/mg protein). The latter is purified on TSK-GEL DEAE-Toyopearl 650M and although this chromatography is time-consuming in comparison with that on TSK-GEL DEAE-5PW, it was the best of the preparations examined. The enzyme preparation, purified by the method presented in this paper, shows only one band under analytical ion-exchange HPLC conditions.

The SDS–PAGE of commercially available Cu,Zn-superoxide dismutase preparations was also examined (Fig. not shown). Taken together with the results shown in Fig. 5, this indicated that the preparations from Toyobo, Diagnostic Data, and Toyo Soda are the purest, although one or two minor bands are still detectable. The SDS–PAGE pattern of the enzyme purified by the method presented in this paper was shown in Fig. 3B to contain a single band.

These lines of evidence indicate that our Cu,Zn-superoxide dismutase preparation is the most highly purified. Preparative ion-exchange HPLC on TSK-GEL DEAE-5PW is the most effective and practical method of obtaining the highly purified enzyme.

To sum up our purification method for Cu,Zn-superoxide dismutase, 8 ml of the crude enzyme solution (protein concentration, 11.0 mg/ml; specific activity, 3000 units/mg) was subjected to preparative ion-exchange HPLC on TSK-GEL DEAE-5PW. In one gradient elution for 120 min from 0 to 0.3 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 7.5), the enzyme solution containing 80–85 mg of protein was fractionated to yield a single band in the analysis by analytical ion-exchange HPLC and SDS–PAGE. It had a specific activity of 3800 units/mg protein. The Cu,Zn-superoxide dismutase preparation isolated by this method showed the highest purity and the specific activity so far reported. Furthermore, the outstanding feature of this method is its applicability to large-scale preparation. Ion-exchange HPLC can be operated repeatedly by a computerized automated system. Therefore, we conclude that ion-exchange HPLC on TSK-GEL DEAE-5PW is a practical method for the large-scale purification of the enzyme from the acetone-precipitated crude enzyme solution.

There has been a recent focus on enzymes as therapeutic agents. Examples are enzyme replacement in genetic disorders, nutrient or metabolite depletion in cancer therapy, enzymatic alteration of cell surface for immuno-therapy, management of autoimmune and other non-infectious inflammatory diseases, use of enzymes and proteins to promote or inhibit blood-clotting, and enzyme replacement for digestive disorders[24]. The application of other macromolecules such as peptide hormones, interferons, and antibodies as drugs have also been studied. The use of these biological macromolecules as drugs depends on the fact that they have unique advantages in their reaction specificity, catalytic efficiency, and capacity to operate under physiological conditions. Nevertheless, they have some unique disadvantages as drugs. One of them is that they must be exhaustively purified to eliminate contaminating materials, especially for parenteral administration. Since its introduction, HPLC has been

developed to a high degree and is used widely in analytical chemistry and biochemistry[25]. We applied HPLC to the purification of Cu,Zn-superoxide dismutase from bovine erythrocytes, and we are convinced that it has great potential and advantages in the large-scale purification of biological substances.

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