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Short Communication

Resolution of three forms of superoxide dismutase by immobilised metal affinity chromatography

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ABSTRACT

A new method for separation of three forms of superoxide dismutase (SOD) using immobilised metal affinity chromatography (IMAC) is reported. Fe-, Mn- and Cu/Zn-SODs were eluted sequentially from Cu^{2+}-IMAC column with an increasing gradient of a counter ion (NH^{+}_{4}) run in combination with an increasing pH gradient (6.8–7.8). The combined gradient elution method resulted in separation of SODs with high resolution, the three proteins being eluted in electrophoretically homogeneous forms. Similar preparation could not be achieved by either increasing gradient of a counter ion or decreasing pH gradients used separately. The described methodology has been succesfully applied for separation of three SODs from a protozoan parasite, indicating that this combined gradient elution system for IMAC offers new possibilities for the high-resolution separation of proteins exhibiting only minor differences in their amino acid composition and structure.

INTRODUCTION

Immobilised metal affinity chromatography (IMAC) is a commonly used method for protein purification [1,2]. In IMAC the metal, mostly chelated by iminodiacetate to the gel, interacts with amino acids on the "surface" of the protein [3–6].

Superoxide dismutases (SODs, superoxide: superoxide oxidoreductase, EC 1.15.1.1) are considered to be of major importance in protecting living cells against superoxide anion toxicity [7]. There are three main types of SOD characterised by the metal ion in the active site of the enzyme: one containing copper and zinc, one manganese and one iron [8–10]. Two new forms of the enzyme, one a hybrid containing Mn/Fe and one extracellular containing Cu/Zn have recently been reported [10]. All known SODs are soluble enzymes and can be distinguished by differential inhibition with cyanide, azide, H_2O_2 and SDS [8,11]. Cu/Zn-SOD and Mn-SOD are present in virtually all eukaryotic organisms [12], whereas all forms of the enzyme are found in prokaryotic cells [8–10,13]. Anti-oxidant enzymes and their role in immunity have recently received much attention in parasitology. Susceptibility of parasites to oxidants and the role of SODs in protecting parasites from attack are of major research interest in this laboratory [11,14,15]. Until recently SODs were purified by conventional techniques with relatively low yields and poor efficiency of purification [16]. Human [17,18], bovine and chicken [19] erythrocyte Cu/Zn-SODs have

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already been purified by IMAC. In this paper, an application of IMAC that allows resolution of Fe-, Mn- and Cu/Zn-SODs on one column is reported.

EXPERIMENTAL

Materials

The IMAC was performed using a copper-saturated chelating Superose HR 10/2 column (20 mm × 10 mm, Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was connected to a Bio-Rad high-resolution liquid chromatography (HRLC) system and activated with 0.2 *M* $CuSO_4$ in distilled water (referred to as Cu-Superose). Bovine erythrocyte Cu/Zn-SOD, *Escherichia coli* Mn- and Fe-SODs were obtained from Sigma. All other reagents were of analytical grade.

Purification procedures

The SODs were dissolved in 10 m*M* phosphate buffer, pH 7.2, containing 0.75 *M* NaCl (buffer A), filtered through a 0.45-μm filter and purified separately on the Cu-Superose column according to procedure described previously [19]. For chromatography of three SODs on one column two methods were used.

Method A (increasing NH_4^+ gradient). A mixture of three pure enzymes (0.3 mg each) was prepared in buffer A and loaded onto the Cu-Superose column. After loading the sample, the column was washed with two to three column volumes of buffer A at a flow-rate of 0.3 ml/min at ambient temperature. The sodium chloride was used to quench non-specific ion interaction between the chelating Superose and the sample. Elution of electrophoretically pure SODs was achieved by running a gradient of NH_4Cl through the column (linear gradient: 100% buffer A to 100% buffer B which was 10 m*M* potassium phosphate, pH 7.2, containing 0.75 *M* NH_4Cl). Proteins were eluted at a flow-rate of 0.3 ml/min with fractions collected every 1.5 min. Pooled fractions with SOD activity were dialysed against double-distilled water, then freeze-dried and characterised on polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS).

Method B (combined gradients of increasing NH_4^+ and pH). The superoxide dismutases (0.3 mg each) were dissolved in 10 m*M* phosphate buffer, pH 6.8, containing 0.75 *M* NaCl (buffer A). Proteins were loaded and eluted from the column in the same manner as described in method A except that the pH of eluting buffer (buffer B) was 7.8. Pooled fractions were characterised by SDS-PAGE.

SODs of a protozoan parasite

A line of *Eimeria tenella*, a protozoan parasite of poultry, was established in our laboratory from a single oocyst obtained from a field sample. The line was passaged in chickens aged between three and six weeks. Sterile, purified oocysts were prepared from the ceaca of infected birds as described previously [13]. Samples of oocysts were resuspended to a concentration of 10^7 ml^{-1} in 50 m*M* phosphate buffer, pH 7.8, containing 1 m*M* phenylmethylsulphonyl fluoride (PMSF). Oocysts were fractured by mixing with glass beads on a vortex mixer continuously for 3 min. The suspension was then examined microscopically to assure complete disruption of oocysts and centrifuged in a high-speed bench-top Eppendorf centrifuge. Supernatant which contained soluble proteins (4 mg/ml) and had SOD activity (840 U/mg protein) was chromatographed on the Cu-Superose column (method B) as described above.

Assays for SOD activity

SOD activity was measured by two methods. The first method (*in vitro* assay) was based on the inhibition of dimethyl sulphoxide (DMSO)/$K^+O_2^-$-induced reduction of nitroblue tetrazolium (NBT) (in 10 m*M* phosphate buffer, pH 7.8, containing 10 μ*M* EDTA, at 25°C) [20]. One unit of SOD activity was expressed as the amount of protein (μg) required to inhibit the reduction of NBT by 50%. SOD activity was also measured in the presence of 5 m*M* sodium cyanide or 1 m*M* H_2O_2 (inhibitors of Cu/Zn-SOD) [8,12] and/or after treatment with 2% SDS at 37°C for 30 min

(an inhibitor of Mn-SOD) [12] in order to distinguish between the various forms of the enzyme. In the second method, electrophoretic mobility of SODs was determined by PAGE. Visualisation of SOD activity on gels was peformed according to the method described previously [21]. Staining for SOD activity was also done in the presence of 5 m*M* sodium cyanide or 1 m*M* H_2O_2. PAGE was performed on a BioRad Protean II mini-gel apparatus. Sample preparation and protein detection were carried out as described previously [22].

Protein content assay

Protein content was determined by the bicinchoninic acid (BCA) method [23] using bovine serum albumin (fraction V) as a standard protein.

RESULTS AND DISCUSSION

IMAC is a useful alternative to more established forms of chromatography for protein purification [3–6]. In this method copper and zinc have been most commonly used as immobilised metal ions and Cu- or Zn-chelate columns have proved to be very effective in the isolation and characterisation of various proteins (for review see refs. 3, 6 and 24]. The binding of proteins or polypeptides is believed to be the result of the ability of electron-rich ligands, such as histidine, cysteine and tryptophan, to bind to the metal complexes. Thus, when a protein with surface-exposed amino acids interacts with a metal, which has capacity for forming additional coordination bonds, it can bind strongly by multipoint attachment. Furthermore, if the metal is a transition element, protein binding affinity will be pH-dependent and favoured at higher pH [24]. It is also suggested that the steric arrangement of the protein chain plays an important role, which means that molecules with similar properties in respect to charge, molecular size and amino acid composition, but with some differences in their secondary and tertiary structure, can be separated [6]. Recovery of most proteins from IMAC columns was found to be almost 100%. Furthermore, they remained biologically active after the purification procedures, indicating that the IMAC method does not cause any detectable damage to the proteins [24].

SODs are ubiquitous enzymes which play an important role in cell metabolism [7–13]. The IMAC method has already been shown to be effective for purification of Cu/Zn-SOD from erythrocytes, which contain only this form of the enzyme [17–19]. All three forms of the enzyme were found to coexist in many parasites [9–11]. Since a quick and relatively simple method for purification of multiple forms of SOD was in particular demand in this laboratory, the applicability of IMAC for this purpose has been researched. All SODs are soluble enzymes with relatively high histidine content, thus the choice of this chromatographic method for their purification appears advantageous. *E. coli* Fe- and Mn-SODs and bovine Cu/Zn-SOD have been studied extensively and are regarded as typical examples of three types of the enzyme. Fe- and Mn-SODs share a common polypeptide fold which is completely unlike that of Cu/Zn-SOD [25,26].

All three SODs bound to the Cu-Superose column at a pH range of 6.8–7.2 and were not removed, even with 0.75 *M* NaCl (Fig. 1). Since the elution of proteins bound to an IMAC column is usually achieved either with a decreasing pH gradient or with an increasing gradient of counter ion NH_4^+ [5,6] these methods were applied for chromatography of the three SODs on the Cu-Superose column. As expected the use of a decreasing pH gradient (pH 7.2–4.2) resulted in elution of proteins but with very poor resolution of individual peaks (data not shown). Elution with an increasing NH_4^+ gradient at pH 7.2 (Fig. 1, method A and ref. 19) resulted in poor resolution of Fe- and Mn-SODs which eluted from the column at 100 and 160 m*M* NH_4^+, respectively, whereas Cu/Zn-SOD was retained on the column and eluted at 380 m*M* NH_4^+. This can be explained by the fact that Cu/Zn-SOD contains fourteen to sixteen histidine residues per molecule [19,27] and has stronger affinity for the column relative to Fe- and Mn-SODs which contain only six and seven histidine residues respectively

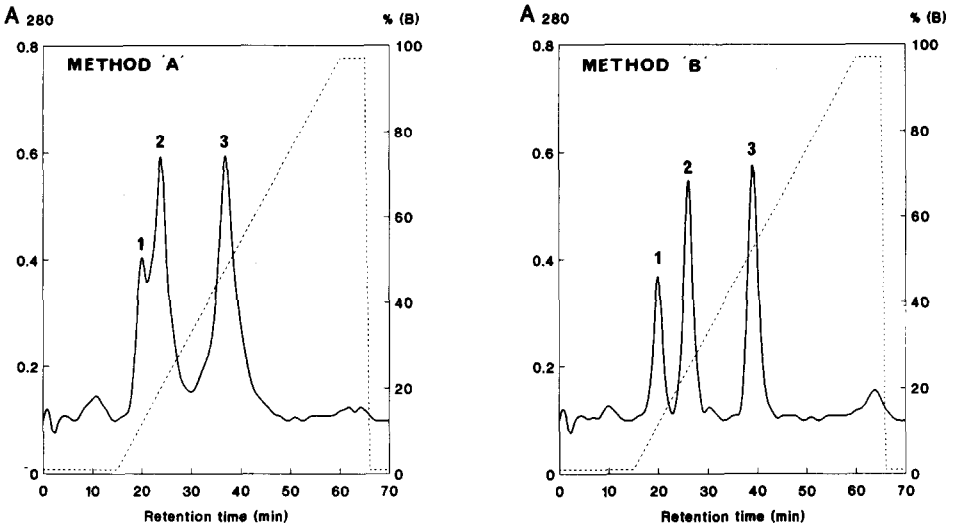

Fig. 1. Comparison of elution profiles (optical absorption at 280 nm) of Fe-SOD (peak 1), Mn-SOD (peak 2) and Cu/Zn-SOD (peak 3) chromatographed on a Cu-Superose column. Proteins were eluted from the column as described in Experimental. Method A: an increasing NH_4^+ gradient at pH 7.2; method B: an increasing NH_4^+ gradient run simultaneously with an increasing pH gradient (6.8 to 7.8). Dotted lines indicate theoretical gradient profile.

[26]. Electrophoretic analysis of SOD-active fractions obtained in method A revealed that none of the three SODs was eluted by this method in homogeneous form (Fig. 2, lanes a–d). The use of a "shallow" gradient (0–400 m*M* NH_4^+) did not visibly improve the separation of proteins (data not shown). Fe- and Mn-SODs have very similar amino acid sequences but show some surface charge differences which are distributed all along the polypeptide chain. Mn-SOD contains an additional histidine residue at position 27 and incorporates a total of eight "extra" residues between positions 42 and 56 [26]. His-27 and some other "extra" residues are surface located [26] and most likely result in a stronger affinity of this enzyme when compared to that of Fe-SOD (Fig. 1, method A). These observations were exploited in designing a new elution system which would result in complete separation of Mn- and Fe-SODs (Fig. 1, method B). Protein binding to IMAC column is pH-dependent in such a way that selectivity for histidine will be favored at a pH higher than the pK_a of histidine ($pK_a^{His} \approx 7$). Thus to achieve increased binding affinity for Mn-SOD the elution system of an increasing NH_4^+ gradient was run in combination with an increasing pH gradient. This resulted in longer retention of Mn- and Cu/Zn-SODs on the column, allowing complete separation of these proteins from the Fe-SOD (Fig. 1, method B). The SODs were eluted from the column in three well separated and defined peaks (Fig. 1), and were virtually pure as revealed by electrophoresis in the presence of SDS (Fig. 2, lanes e–g). They also retained their full catalytic activity and showed typical sensitivity to specific inhibitors (data not shown, see Experimental).

The described methodology has also been successfully applied for separation of three SODs from a protozoan parasite *E. tenella*. Unsporulated oocysts of *E. tenella* have high SOD activity and contain several electrophoretically distinct forms of the enzyme, including two forms of Cu/Zn-SOD, two forms of Fe-SOD and Mn-SOD [13]. The elution profile of *E. tenella* homogenate chromatographed on a Cu-Superose column is shown in Fig. 3. Fractions containing SOD activity were eluted from the column in three well separated peaks. Inhibitor studies revealed that SOD activities detected in peaks 1, 2 and 3 can be attributed to Fe-, Mn- and Cu/Zn-containing enzymes, respectively (data not shown, see Experi-

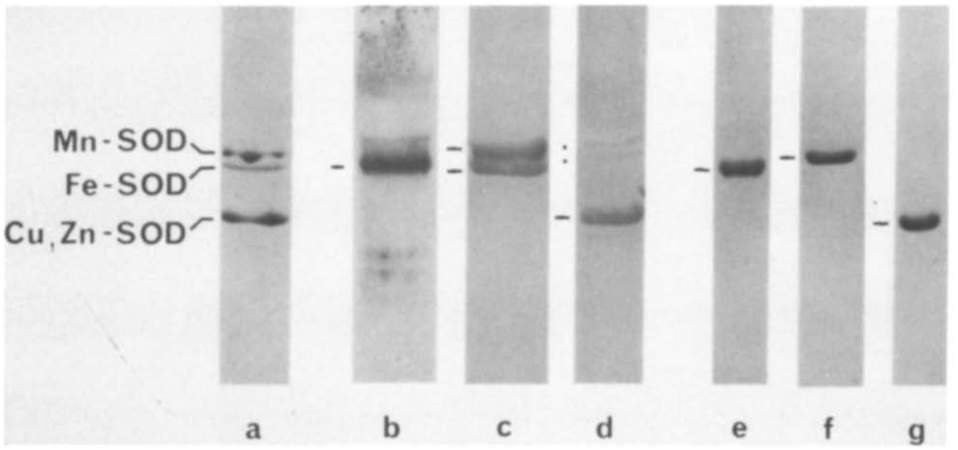

Fig. 2. SDS-PAGE electrophoretograms of SOD-active fractions from the Cu-Superose chromatography referred to in Fig. 1. Fractions corresponding to individual peaks were tested for SOD activity in the *in vitro* assay described in Experimental. Active fractions were pooled and subjected to SDS-PAGE with approximately 10 μg of protein loaded in each track. (a) Mixture of three SODs loaded on the column. Method A: (b) peak 1, fractions 11–14; (c) peak 2, fractions 15–19; (d) peak 3, fractions 21–29. Method B: (e) peak 1, fractions 12–15; (f) peak 2, fractions 16–20; (g) peak 3, fractions 25–29.

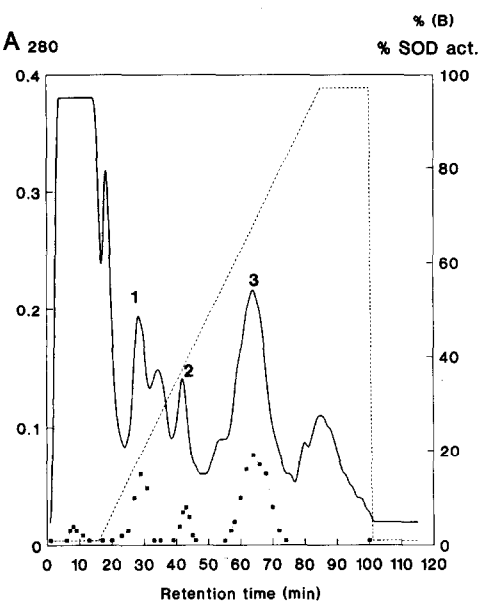

Fig. 3. Chromatography of oocyst homogenate from *E. tenella* on Cu-Superose column. Proteins were eluted from the column with an increasing NH_4^+ gradient run simultaneously with an increasing pH gradient (6.8 to 7.8). Continuous line depicts an elution profile (absorption at 280 nm) and dotted line indicates theoretical gradient profile. SOD activity (□) was detected in fractions corresponding to the three separate protein peaks (1, 2 and 3). SOD activity is expressed as a percentage of total activity applied to the column (840 U in 1 ml of homogenate).

mental). *E. tenella* oocysts contain a number of isoforms of SOD but the major forms appear to have typical features of the three main types of SOD characterised by the metal ion content [8,13]. These results indicate that the new elution system for IMAC offers new possibilities for the high resolution of proteins exhibiting only minor changes in their amino acid composition and structure.

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