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# POLYMERS OF Cu/Zn SUPEROXIDE DISMUTASE PRODUCED BY CROSS-LINKING WITH GLUTARALDEHYDE

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Soluble polymers of bovine Cu/Zn superoxide dismutase (EC 1.15.1.1) have been prepared using the homobifunctional cross-linking reagent, glutaraldehyde. A form of the enzyme, a tetramer, with a molecular weight of 64, 000 has been purified by gel filtration. The functional properties of the tetramer have been investigated. Reconstitution with copper and zinc was required for full activity. After metal reconstitution, the specific activity of the tetramer was shown to be close to 90% that of the native dimeric enzyme.

The serum half-life of the tetramer in rats was found to be increased by a factor of six when compared with native superoxide dismutase. The tissue distribution of the two forms was also found to be different with the tetramer accumulating predominantly in the liver.

KEY WORDS: Superoxide dismutase (SOD), glutaraldehyde, serum half-life, superoxide.

## INTRODUCTION

Superoxide ions are believed to be mediators of cellular and tisse damage in a number of clinical conditions.[1,2] The removal of superoxide ions by administration of SOD would therefore be expected to have a protective effect on cells and tissues in relation to free radical damage. SOD has, in fact, been shown to be moderately effective in alleviating some of the symptoms of such conditions.[3-7]

There is however a major drawback to the use of SOD as a therapeutic agent. Cu/Zn SOD is an enzyme of molecular weight 32,000, just below the "cut off" point for filtration and removal by the kidneys. The half-life of the enzyme, when injected, is therefore very low, in the order of a few minutes, impairing the effectiveness of treatment.[8]

Several groups have attempted, with some success, to increase the circulating time of SOD in the body by linking it to macromolecules such as serum albumin,[9] polyethylene glycol (PEG)[10] or Ficoll[11] thereby increasing its apparent molecular weight. PEG-SOD is currently undergoing clinical testing for use in kidney transplantation, burns and trauma.[12] More recently a report has appeared where genetically engineered Cu/Zn SOD polymers have been isolated.[13] Two subunits of human Cu/Zn SOD were linked by a 19 amino acid human immunoglobulin IgA1 hinge sequence. The circulating times of polymers isolated were increased.

The purpose of the work presented here was to prepare a polymer of Cu/Zn SOD

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by chemical cross-linking. A tetramer form of SOD has been prepared and isolated. Activity is retained and circulating time is increased.

## MATERIALS AND METHODS

### *Materials*

Bovine kidney SOD was a generous gift from CALZYME Laboratories Inc., California, U.S.A. Xanthine oxidase, from cow milk, was purchased from BCL, East Sussex, U.K. Xanthine was purchased from BDH Chemicals Ltd., Poole, U.K. UltroGel AcA gel filtration media were purchased from I.B.F. biotechnics, through Life Science Laboratories Ltd., Luton, U.K. Glutaradehyde (25% w/v aqueous solution) and all other chemical were purchased from Sigma Chemical Co., Poole, U.K. Glutaraldehyde was used as received, without any further purification.

### *Cross-linking and Isolation of Active Polymeric SOD*

A solution of bovine kidney SOD (16 mg ml$^{-1}$) in Na-phosphate buffer (0.1 M, pH 7.2) was prepared and placed in an ice bath. Glutaraldehyde solution (1.0% w/v in Na-phosphate buffer, 0.1 M, pH 7.2) was added to the protein solution dropwise, with stirring, to a final glutaraldehyde concentration of 0.1% w/v. After thirty minutes the reaction was stopped by addition of acetic acid (10 mM) to lower the pH to pH 5.0. Any precipitate formed was removed by centrifugation for 2 minutes in a Haemicrofuge.

2.5 ml of supernatant were loaded onto and eluted from an UltroGel AcA54 gel filtration column (2.6 × 86 cm, bed volume 442 ml) equilibrated with Na-acetate buffer (0.1 M, pH 5.0). Elution was carried out with the same buffer. Peak fractions were pooled, concentrated, dialysed exhaustively against deionised water and then freeze-dried.

Dried material from gel filtration of glutaraldehyde treated SOD was assayed for enzyme activity using the cytochrome c reduction assay[14] and the dilution method of Ysabaert-Vanneste and Vanneste.[15]

Metal (Cu and Zn) analyses, using atomic absorption spectroscopy, were carried out on the freeze-dried material. A Perkin Elmer 2380 atomic absorption spectrometer was used for the analyses. Following metal analysis, the polymer was reconstituted with copper and zinc by the published method.[16] Metal reconstituted polymer was freeze-dried and assayed for enzyme activity as described above.

### *Clearance and Tissue Distribution of Polymeric SOD in Rats*

Tetrameric and native SOD were iodinated with $^{125}$I using the lactoperoxidase method.[17]

Male Wistar rats were injected with either $^{125}$I-tetrameric or native SOD (2.5 mg in 0.4 ml isotonic phosphate buffer solution (PBS), pH 7.4). Blood samples were taken, at time intervals after injection, in capillary tubes (10 $\mu$l) from the ends of the tail. The samples were counted for $^{125}$I.

On completion of the clearance studies described above, approximately 60 minutes after injection, the animals were sacrificed. Spleen, muscle, kidneys, liver and bladder

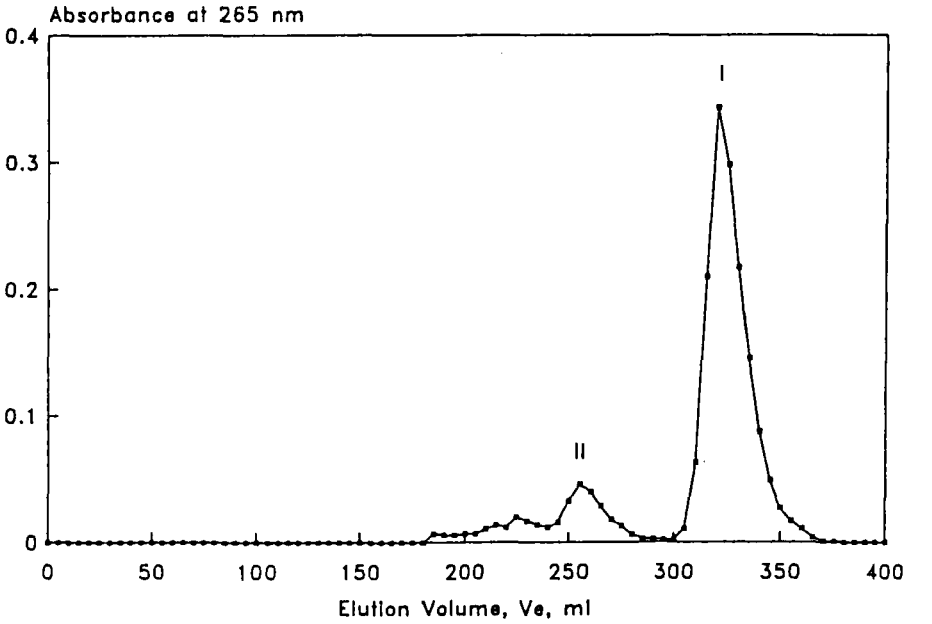


FIGURE 1 A typical UltroGel AcA54 elution profile for glutaraldehyde treated Cu/Zn SOD. Elution was carried out with Na-acetate buffer (0.1 M, pH 5.0), at a flow rate of 12 ml hour$^{-1}$. Bed volume was 442 ml.

contents were removed from each animal. Tissues were homogenised in HEPES buffer (0.1 M, pH 7.4) containing NaCl (0.15 M). Homogenates and bladder contents were sampled for $^{125}$I counting.

## RESULTS

A typical UltroGel AcA54 gel filtration elution profile for elution of glutaraldehyde treated SOD is shown in Figure 1. Peak II fractions were pooled and treated as described in the materials and methods section.

Calibration of the UltroGel AcA54 gel filtration column revealed the molecular

TABLE I
The activity of tetrameric SOD before and after reconstitution with copper and zinc.

| SOD Type | Activity Units mg$^{-1}$ | % Activity* |
|---|---|---|
| Native | 3333 | 100 |
| Tetramer before metal reconstitution | 1555 | 46.7 |
| Tetramer after metal reconstitution | 2887 | 86.6 |

* Percentage activity values shown are expressed as a percentage of the activity of native SOD. $n = 3$

TABLE II

The results of copper and zinc analyses carried out on tetrameric SOD before and after metal reconstitution.

| SOD type | Metal Content, moles/mole SOD* | |
|---|---|---|
| | Cu | Zn |
| Native | 2 | 2 |
| Tetramer before metal reconstitution | 2 | 2 |
| Tetramer after metal reconstitution | 4 | 4 |

* Values given are approximate values per mole of native or tetrameric SOD

weight of peak II material to be 64,000. This is therefore taken to represent a tetramer of the native SOD subunit of molecular weight 16,000.

The specific activity of the tetrameric form of SOD before and after reconstitution with copper and zinc is shown in Table I. Before reconstitution with the metals, specific activity was found to be 46.7% that of the native enzyme, while after reconstitution specific activity recovered was 86.6% that of the native enzyme. Metal analysis of the tetramer before reconstitution revealed that almost 50% of the total metal content of the enzyme (compared with native enzyme) had been lost during glutaraldehyde treatment. Metal analysis of the copper/zinc reconstituted tetramer

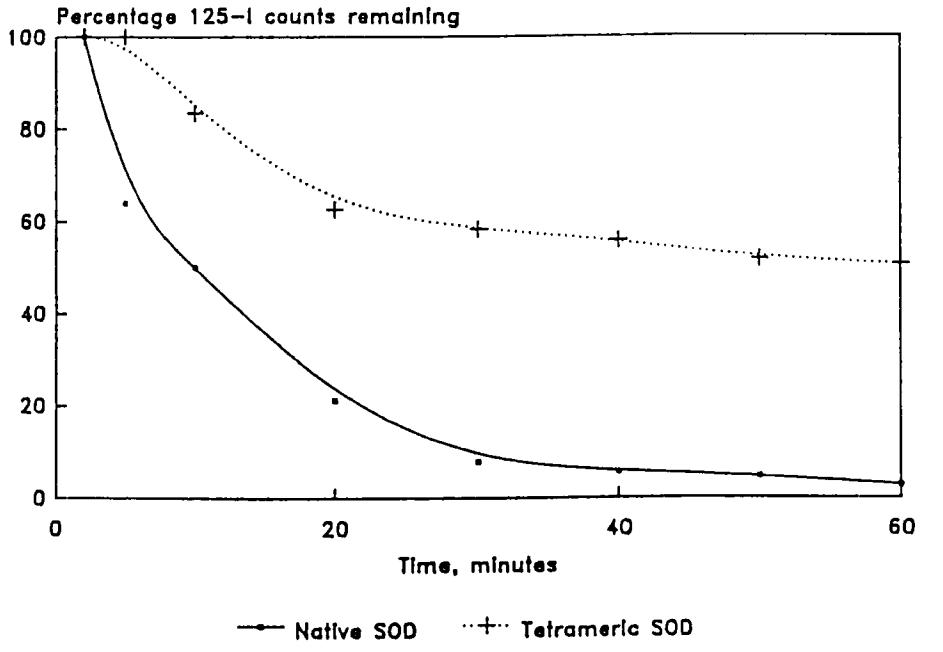


FIGURE 2 Clearance of native and tetrameric SOD from rats. 0.4 ml (containing 2.5 mg) of each of $^{125}$I-labelled native and tetrameric SOD were injected into rats via the penile vein. Blood samples were taken at time intervals after injection in capillary tubes (10 $\mu$l) from the ends of the tails. Samples were counted for $^{125}$I.

TABLE III

The serum half-lives of native and tetrameric SOD when injected into rats. 0.4 ml (containing 2.5 mg) of each of $^{125}I$ labelled native and tetrameric SOD were injected into rats. Blood samples were taken at time intervals after injection in capillary tubes (10 $\mu$l) from the ends of the tails. Samples were counted for $^{125}I$.

| SOD Type | Half-Life, $t_{1/2}$ minutes |
|---|---|
| Native | 11 |
| Tetrameric | 65 |

showed the presence of approximately 100% of each of the metals compared with the native enzyme. These results are shown in Table II. The loss of metal during glutaraldehyde treatment probably accounts for the low specific activity of the tetramer before metal reconstitution.

Figure 2 shows blood decay curves after intravenous injections of $^{125}I$-labelled tetrameric and native bovine SOD. Serum half-lives were determined from these curves and are shown in Table III.

Native bovine SOD has been reported to have a half-life of approximately 6 minutes in rats,[18] the value obtained here for native SOD was 11 minutes. Tetrameric SOD was found to have a half-life of 65 minutes, a factor of approximately six greater than that of native SOD.

Tissue distributions of $^{125}I$-tetrameric and native SOD, approximately 60 minutes after intravenous injection, are shown in Table IV. It can be seen that the tetramer has been preferentially accumulated in the liver and posssibly in the spleen, when compared to the native form of the enzyme. It is apparent that native SOD is rapidly removed by the kidneys, quickly appearing in the bladder. $^{125}I$ counts from tetrameric SOD are present in the kidneys, but do not appear to any great extent in the bladder contents.

TABLE IV

Tissue distribution of $^{125}I$-labelled native and tetrameric SOD, 60 minutes after injection into rats. Animals were sacrficed after clearance studies. Tissues were homogenised in HEPES buffer (0.1 M, pH 7.4) containing NaCl (0.15 M), and sampled for $^{125}I$ counting.

| Sample | $^{125}I$ counts* cpm | |
|---|---|---|
| | Native SOD | Tetrameric SOD |
| Spleen (whole organ) | 387,421 | 668,612 |
| Muscle (per g) | 33,006 | 27,679 |
| Kidneys (2 organs) | 18,006,879 | 9,122,094 |
| Liver (whole organ) | 247,180 | 7,652,931 |
| Bladder contents (per ml) | 2,636,644 | 961,740 |
| Total Counts | 21,311,130 | 18,433,056 |

*$n = 3$

## DISCUSSION

A chemically cross-linked polymer of Cu/Zn SOD has been prepared using glutaraldehyde. A polymer, of molecular weight 64,000, was isolated. This polymer is presumed to be equivalent to a tetramer of the SOD 16,000 molecular weight subunit. The tetramer was shown to have a six-fold increased circulation half-life when injected into rats. This demonstration of an increased circulating time provides another possible means of obtaining high molecular weight active SOD for use as a therapeutic agent.

The tissue distribution of the tetramer was found to be different to that of the native enzyme, with the tetramer apparently being removed from the circulation by the liver.

It has previously been demonstrated that all organs other than the kidneys play an insignificant role, at least quantitatively, in the elimination of native bovine SOD from rats.[18] The results reported here, indicate that little elimination of tetrameric SOD is carried out by the kidneys. The liver appears to be the important organ for removal of tetrameric SOD from the circulation.

Bovine SOD does not invoke an immune response when injected into humans; the antigenicity of the tetramer isolated needs to be examined and compared with that of native SOD.

Glutaraldehyde reacts predominantly with the amino acid side chains of lysine residues in proteins (this was confirmed for tetrameric SOD by amino acid analysis). However, there is evidence to suggest that some glutaraldehyde molecules may have reacted at only one functional site with a lysine residue side chain, since some precipitation is observed when the pH of a solution of tetramer is raised above pH 7.0. This may be significant in that it may leave one reactive site free for reaction with other molecules on intravenous injection of the tetramer. This has not been investigated further.

The major problem with the technique outlined here for production of high molecular weight SOD is the low recovery of the enzyme after treatment with glutaraldehyde (approximately 3–5%). This is due to the complicated nature of the glutaraldehyde cross-linking reaction which is extremely difficult to control. For the technique to prove worthwhile, an extensive study of the interaction of glutaraldehyde with SOD needs to be undertaken to determine the conditions and stopping procedure which will give optimum recovery of the tetramer.

If recovery after treatment with glutaraldehyde could be improved, and the tetramer was shown to be non-antigenic, then the procedure outlined would be attractive for production of high molecular weight SOD.

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*References*

1. W.F. Petrone, D. K. English, L. Wong and J. M. McCord (1980) Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma. *Proceedings of the National Academy of Science U.S.A.*, 77, 1159–1163.

2. J.M. McCord (1985), Oxygen-derived free radicals in postischemic tissue injury. *The New England Journal of Medicine*, **312**, 159–163.
3. F. Edsmyr, W. Huber and K.B. Menander (1976) *Current Therapeutic Research*, **19**, 198–211.
4. K. Lund-Olesen and K.B. Menander (1974) *Current Therapeutic Research*, **16**, 706–717.
5. H. Merberger, G. Bartsch, W. Huber, K.B. Menarberger and T. L. Sculte (1973) *Current Therapeutic Research*, **18**, 466–475.
6. M. Rister, K. Banermester, U. Gravert- and E. Gladtke (1978) *The Lancet*, **1**, 1094.
7. J.M. McCord (1980) Free radicals and myocardial ischemia: overview and outlook. *Journal of Free Radicals in Biological Medicine*, **4**, 9–14.
8. W. Huber and M.G.P. Saifer (1977) in *Superoxide and dismutases*, eds. A. M. Michelson, J. M. McCord and I. Fridovich, Academic Press, pp. 517–536.
9. K. Wong, L.G. Cleland and M.J. Poznansky (1980) Enhanced anti-inflammatory effect and reduced immunogenicity of bovine liver superoxide dismutase by conjugation with homologous albumin. *Agents Actions*, **10**, 231–239.
10. E. Boccu, G.P. Velo and F.M. Veronese (1982) Pharmacokinetic properties of polyethylene glycol derivatized superoxide dismutase. *Pharmacological Research Communications*, **14**, 113–120.
11. P.S. Pyatak, A. Abuchowski and F.F. Davies (1980) Preparation of a polyethylene glycol: superoxide dismutase adduct, and an examination of its blood circulation life and anti-inflammatory activity. *Research Communications in Chemical and Pathological Pharmacology*, **29**, 113–127.
12. *Chem. Mark. Rep.*, **234**(19), p.4 (1988).
13. R.A. Hallewell, I. Laria, A. Tabrizi, G. Carlin, E.D. Gatzoff, J.A. Tainer, L.S. Cousens and G.T. Mullenbach (1989) Genetically engineered polymers of human CuZn superoxide dismutase. *The Journal of Biological Chemistry*, **264**(9), 5260–5268.
14. J.M. McCord and I. Fridovich (1969) Superoxide dismutase: an enzyme function for erythrocuprein (homocuprein). *The Journal of Biological Chemistry*, **244**, 6049–6055.
15. M. Ysabaert-Vanneste and W.H. Vanneste (1980) Quantitative resolution of Cu, Zn- and Mn-superoxide dismutase activities. *Analytical Biochemistry*, **107**, 86–95.
16. J. Bannister, W. Bannister and E. Wood (1971) Bovine erythrocyte cupro-zinc protein: Isolation and general characterisation. *European Journal of Biochemistry*, **18**, 178–186.
17. G.S. David and R.A. Reisfeld (1975) Protein iodination with solid state lactoperoxidase. *Biochemistry*, **13**(5), 1015–1021.
18. B. Odlind, L.-E. Appelgren, A. Bayati and M. Wolgast (1988) Tissue distribution of $^{125}$I-labelled bovine superoxide dismutase (SOD) in the rat. *Pharmacology and Toxicology*, **62**, 95–100.

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