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# Overexpression, purification, crystallization and preliminary X-ray diffraction analysis of Cu,Zn superoxide dismutase from Peking duck

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The cDNA encoding Peking duck Cu,Zn superoxide dismutase (dSOD) was cloned and sequenced. The recombinant enzyme was overexpressed in *Escherichia coli*, purified to homogeneity and crystallized using the sitting-drop vapour-diffusion technique. Trigonal crystals of dSOD were obtained at 278 K at low ionic strength and around neutral pH. These crystals belong to space group $P3_221$, with unit-cell parameters $a = 124.4$, $c = 163.5$ Å, $\gamma = 120^\circ$. The asymmetric unit contains four dimers (eight monomers of Cu,Zn dSOD) and has a 56% solvent content, with a $V_M$ of 2.8 Å$^3$ Da$^{-1}$. On a Rigaku R-AXIS IIc image-plate area-detector system, the crystal diffracted to 2.9 Å. Unusual supermolecular double-helix packing with $9_22$ non-crystallographic symmetry in crystals has been observed in the initial structural analysis.

## 1. Introduction

Usually, a series of protein structures of distinct biological origins and functional status can provide a wealth of information from which to gain insight into the structure–function relationship or evolutionary understanding. Such concepts apply particularly well to the enzyme Cu,Zn superoxide dismutase (Cu,Zn SOD). Superoxide dismutases (SOD; EC 1.15.1.1) are metalloenzymes which play an important role in protecting organisms from the damage caused by the superoxide anion ($O_2^{\cdot-}$). The superoxide radical, the first intermediate produced by aerobic metabolism, can be converted by a metal ion of SOD into molecular oxygen and hydrogen peroxide (Fridovich, 1975). Although a series of three-dimensional structures of Cu,Zn SODs have been reported (*e.g.* Tainer *et al.*, 1982; Kitagawa *et al.*, 1991; Parge *et al.*, 1992; Djinovic, Coda *et al.*, 1992; Djinovic, Gatti *et al.*, 1992; Djinovic Carugo *et al.*, 1994, 1996), novel structures of this enzyme from different organisms providing fresh information have been appearing in recent literature (*e.g.* Ferraroni *et al.*, 1999; Hough & Hasnain, 1999; Hough *et al.*, 2000; Forest *et al.*, 2000). To date, however, there is still no structural model of an avian SOD available (data from the PDB). Here, we report the overexpression, purification, crystallization and preliminary X-ray analysis of a Cu,Zn SOD from a particular bird, the Peking duck, which will lead to an avian crystal structure model.

Roast Peking duck is a Chinese dish famous for its distinctive flavour and extraordinary tenderness, both of which are a consequence of the particular physiological habits and characteristics of the Peking duck. The Peking duck (*Anas platyrhynchos domestica*) is a domestic species of *Anas platyrhynchos*. Peking ducks compulsively consume much more than they physiologically require. Because of their high-lipid and high-protein diets, the ducks become incredibly fat (above 3 kg on average) in a short time (45 d or so) after they are born (data from the duckery). Despite their excessive obesity compared with other duck species, Peking ducks rarely suffer from cardiovascular and cerebrovascular diseases such as atherosclerosis and hypercholesterolaemia (Wang *et al.*, 1986). Because of this characteristic, the Peking duck has been used as a model to explore effective factors against cardiocerebrovascular diseases such as atherosclerosis (Ye *et al.*, 1995). Investigations showed that Peking ducks did not exhibit apparent atherosclerotic plaques in the aorta after 18 weeks being fed on high-cholesterol and high-fatty-acid diets (Lu *et al.*, 1998), contrary to the case of Japanese quail (Yuan *et al.*, 1997). Efforts have been made to determine the effective factors in this phenomenon (*e.g.* Lu *et al.*, 1998), which may lead to identification of functional molecules and provide a model for treatment of relevant diseases. In view of the close relationship between lipid peroxidation and atherosclerosis as well as aging (Rikans & Hornbrook, 1997), thorough investigation of antioxidant enzymes including superoxide dismutase becomes of particular interest. The results reported in this paper establish a sound basis for these investigations.

Firstly, we extracted Cu,Zn SOD from Peking duck erythrocytes (dSOD). The enzyme, however, was difficult to purify to homogeneity because there were at least five isoelectric variants, whose pI values as determined by isoelectrofocusing electrophoresis were 5.0, 5.3, 5.9, 6.1 and 6.3. Consequently, single crystals suitable for data collection were not obtained. Isoelectric variants of Cu,Zn SOD have also been discovered in rat (Crost & Sausa, 1980), cow (Malinowski & Fridovich, 1979; Civalleri *et al.*, 1982), human (Crost, 1978; Arai *et al.*, 1986) and chicken (Lonnerdal *et al.*, 1979). Although some investigators have attempted to explore the nature of the isoenzymes (*e.g.* Sumio, 1990; Schinina *et al.*, 1996), the exact mechanism is not yet clear. Nevertheless, recombinant SODs are expected to be single entities easy to purify to homogeneity, as genetic variation is ruled out. Therefore, we cloned the cDNA of Peking duck Cu,Zn SOD and expressed the enzyme in *E. coli* at a high level (>100 mg $l^{-1}$). The overexpression and successful crystallization of dSOD provides a sound basis for the investigation of its structure–function relationship.

## 2. Experiments and results

### 2.1. Overexpression of dSOD

By using the 3′ RACE method, the partial cDNA including the encoding region and 3′-UTR was cloned from the total RNA extracted from the liver of Peking duck. The open reading frame (ORF) of the cDNA encodes a mature peptide of 153 residues, whose sequence was aligned with those of human, bovine and yeast counterparts as shown in Fig. 1.

```
                          20                  40
duck  : MATLKAVCVMKGDGPVEGVIHFQQQGN-GPVKVTGRISG-LSDGD
human : ...-....L....Q.I.N.E.KESN....W.S.K.-.TE.L
bovine: ...-....L....Q.T..EAK.--DT.V...S.T.-.TE..
yeast : .-V-Q..A.L...AG.S..VK.E.ASESE.TT.SYE.A.NSPNAE

                  60                  80
duck  : HGFHVHEFGDNTNGCTSAGAHFNPEGKKHGGPKDAERHVGDLGNV
human : ...........A.....P....LSR......E.........
bovine: .....Q.....Q.....P....LS.......D.........
yeast : R...I.....A....V...P....FK.T..A.T.EV.....M...

              100                 120
duck  : TA-KGGVADVEIEDSVISLTGPHCIIGRTMVVHAKSDDLGKGGDN
human : ..D.D....S..G....S.D.....L...E.A.......NE
bovine: ..D.N...I.D.V.PL...S.EYS.........E.P....R..NE
yeast : KTDEN...KGSFK..L.K.I..TSVV..SV.I..GQ.....DTE

          140
duck  : ESTLTGNAGPRLACGVIGIAKC      - 154
human : ...K.....S..........Q       - 154
bovine: ...S.....S..........        - 152
yeast : ..LK.......P......LTN       - 154
```

**Figure 1**
Amino-acid sequence deduced from the open reading frame of the partial cDNA encoding Peking duck SOD in comparison with those of the human, bovine and yeast counterparts. Gaps (dashes) are introduced to facilitate alignment. Amino acids identical to the duck enzyme are indicated as dots in the human, bovine and yeast sequence; residues conserved in all the four SODs are highlighted. The first residue, the initial methionine, is removed in both the native and the recombinant enzyme. The only difference between them is that the native SOD presents a blocked N-terminus, whereas the recombinant one does not.

Two oligonucleotide primers were synthesized and PCR was performed to clone the ORF of dSOD into the expression vector. Primer 1, 5′-CCCATATGGCGAC-GCTGAAGGCC-3′, was designed to provide an *Nde*I restriction site (underlined) juxtaposed with the ATG start codon. Primer 2, 5′-GCCGAATCC**ATTA**GCACT-TGGCTATTCCGAT-3′, was designed to change the stop codon from TGA, as originally existed in the cDNA of dSOD, to TAAT (bold) preferred by *E. coli* in order to improve the termination efficiency during translation and also to generate a *Bam*HI restriction site (underlined) adjacent to the 3′ end of the encoding region. The partial cDNA of dSOD was used as a template to perform PCR. The PCR product was inserted into the entry vector pBlueSript-KS(+) (Stratagene) using the T/A cloning technique. The plasmid pBlueScript-dSOD was subsequently digested by the restriction endonucleases *Nde*I and *Bam*HI before being cloned into the corresponding restriction sites in pET-3a (Novagen) to create the expression vector pET-3a-dSOD.

The final constructed plasmid pET-3a-dSOD was transformed into the host strain BL21(DE3)pLysS, where dSOD could be produced with high yield in the cytoplasm of *E. coli.* The recombinant *E. coli* cells were grown at 310 K in 5 ml Luria broth medium containing 50 µg $ml^{-1}$ carbenicillin and 34 µg $ml^{-1}$ chloramphenicol until the $OD_{600}$ was approximately 0.5. The entire 5 ml starter culture was inoculated with 500 ml Luria broth medium containing carbenicillin and chloramphenicol at the same concentrations as above. The cultures were shifted to 298 K when the $OD_{600}$ was close to 0.5 and were incubated for 30 min prior to induction. Afterwards, $CuSO_4$, $ZnSO_4$ and isopropyl-$\beta$-thiogalactopyranoside (IPTG) were added to final concentrations of 1.2, 0.25 and 0.4 m*M*, respectively. After 5 h of induction, the induced cultures were harvested by centrifugation at 4000*g* for 20 min at 278 K. The results from SDS–PAGE showed that the recombinant dSOD (16 kDa) was expressed at a very high level in the cytoplasm of *E. coli* (Fig. 2) and comprised more than 50% of the total cell protein after induction. Moreover, the bulk of the target product was in the soluble fraction.

### 2.2. Purification of recombinant enzyme

The cell pellets were treated with BugBuster Protein Extraction Reagent (Novagen). According to the instructions provided by the manufacturer, 5 ml BugBuster per gram of wet cell paste was added to completely resuspend the cell pellets at room temperature. After incubation on a shaking platform for 10–20 min, soluble proteins and nucleotide acids were released during cell lysis. The insoluble cell debris were removed by centrifugation at 16 000*g* for 20 min at 278 K. The supernatant containing active recombinant dSOD was loaded directly onto a HiLoad column (16/30) packed with Q Sepharose Fast Flow (Pharmacia Biotech) which was pre-equilibrated with 20 m*M* Tris–HCl pH 7.8. Two peaks appeared in the flowthrough fraction when the column was eluted using the same buffer. The eluent in the second peak that displayed high superoxide dismutase activity was pooled and then concentrated to the desired degree by ultrafiltration using centrifugation concentration tubes (Pall) with a nominal 10 kDa cutoff. The concentrated sample, which showed a visible blue colour, was immediately applied to a Mono Q column (5/5, Pharmacia Biotech) pre-equilibrated with 20 m*M* Tris–HCl pH 8.0 and eluted with a linear gradient of 0–500 m*M* sodium chloride. The peak

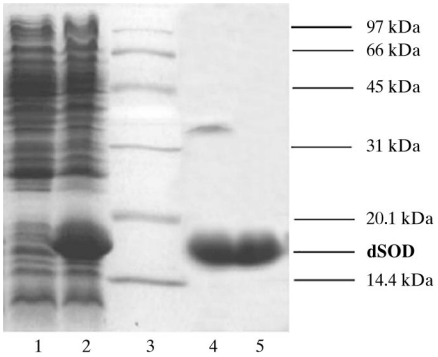


**Figure 2**
Overexpression and purification of dSOD analyzed by SDS–PAGE (15% T) and stained by Coomassie brilliant blue. Lane 1, total cell protein of BL21(DE3)pLysS before induction; lane 2, total cell protein of BL21(DE3)pLysS after induction; lane 3, standard protein size marker (Bio-Rad); lane 4, the elution peak containing dSOD from the Sepharose Q fast flow column; lane 5, the final purified dSOD from the Mono Q column.

containing duck SOD was pooled, concentrated as above and desalted with HiTrap desalting columns (Pharmacia Biotech), after which the sample was lyophilized.

The purity and rough size of the subunit of the purified recombinant dSOD were analyzed by SDS–PAGE, with the results (Fig. 2) displaying a single band with a molecular mass of 16 kDa. The exact molecular mass measured by electrospray mass spectrometry is 15 541.0 Da, which agrees with the estimated value of 15 541.8 Da from the amino-acid sequence deduced from cDNA. Nearly all Cu,Zn SODs are homodimers, except some prokaryotic enzymes such as the monomeric Cu,Zn SOD extracted from *E. coli*. Size-exclusion chromatography on Superdex 75 (Pharmacia) was used to determine the oligomeric state of dSOD: the enzyme shows a molecular weight of approximately 32 kDa, confirming the dimeric form of dSOD.

### 2.3. Test of SOD activity

Superoxide dismutase activity was measured by auto-oxidation of pyrogallol as described in Zhang *et al.* (1997). The enzymatic activity of recombinant dSOD is greater than 2000 units $mg^{-1}$, approaching that of the native enzyme extracted from Peking duck erythrocytes (2600 units $mg^{-1}$).

### 2.4. Crystallization

Diffraction-quality crystals at 278 K were grown by the sitting-drop vapour-diffusion method (Ducruix & Giegé, 1992). Purified protein was dissolved in 0.1 mol $l^{-1}$ cacodylate buffer pH 6.8 to a final concentration of 60 mg $ml^{-1}$; the precipitant and the reservoir were composed of 52 and 48% of a mixture of 2-methyl-2,4-pentanediol (MPD) and hexane-1,6-diol (HD) in a 3:2 ratio, respectively. During crystallization, 30 µl of protein solution mixed with 30 µl of precipitant was placed in sitting drops, while 1.5 ml of reservoir was used as equilibrating agent. Large single crystals with average dimensions 0.2 × 0.2 × 0.8 mm could be obtained in two months when the final reservoir concentration approached 65%. These crystals were light-blue elongated hexagonal rods. The crystals were stabilized by adding 20–50 µl of pure MPD after nucleation at 6 d intervals for about one month. The crystals stabilized by the reservoir can diffract to 2.9 Å resolution.

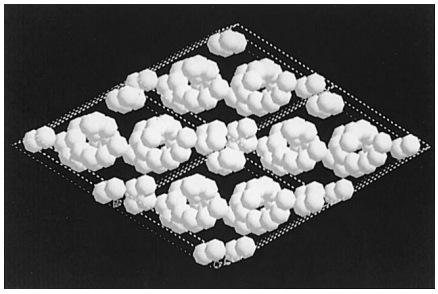

**Figure 3**
Molecular packing of the dSOD in the trigonal crystals. The two interwinding helices with a $9_2$2 non-crystallographic symmetry form a supermolecular double helix. The six adjacent double helices generate a hexagonal channel along the *c* axis into which 12 dSOD dimers are packed within a period of the helix. Spheres represent dSOD dimers.

### 2.5. X-ray diffraction analysis

All X-ray characterization and data collection were carried out using a Rigaku R-AXIS IIc image-plate area-detector system. Graphite-monochromated Cu $K\alpha$ radiation ($\lambda$ = 1.5418 Å) was provided by a Rigaku RU-300 rotating-anode generator operated at 40 kV, 100 mA and equipped with a 0.5 mm diameter collimator. The intensity data were collected at 288 K using one crystal of dSOD with dimensions of approximately 0.25 × 0.25 × 0.8 mm, mounted with the *c** axis along the rotation axis. The crystal-to-detector distance was set at 120 mm and 66 × 1.5° oscillation images were recorded at 45 min exposure time per image. Indexing, integration and scaling of intensity data were carried out using the R-AXIS IIc accompanying processing program *PROCESS* (Sato *et al.*, 1992; Rigaku, 1993). Processing of the data showed that the crystals belonged to the trigonal form, with space group $P3_221$ or $P3_121$ and unit-cell parameters $a$ = 124.4, $c$ = 163.5 Å, $\gamma$ = 120°. The results of the translation searches for each of the possible enantiomorphic space groups show that the $P3_221$ space group is more reasonable for the correlation coefficient and $R$ factor. For four dimeric molecules in the asymmetric unit, the estimated $V_M$ value of 2.79 $Å^3$ $Da^{-1}$ is well within the normal range of 1.6–3.6 $Å^3$ $Da^{-1}$ typical for globular proteins (Matthews, 1968). The four dSOD dimers were identified by a molecular-replacement calculation in the subsequent analysis. This corresponds to a solvent content of approximately 56%. From a total of 67 322 measurements, there were 20 323 unique reflections with $I > 2\sigma(I)$ in the resolution range 50–3.0 Å. The final $R_{merge}$ on all data was 0.111 and the completeness of the data set was 79.9%.

Since there are eight dSOD molecules (four dimers) with a total molecular weight of 124 kDa in the asymmetric unit, the present resolution obtained using the in-house facility is reasonable. We are trying to improve the data to high resolution using synchrotron radiation.

Preliminary structural analysis with the present data by means of molecular replacement has shown unusual molecular packing. 24 dSOD molecules in a unit cell are packed as two interwinding helices with a $9_2$2 non-crystallographic symmetry and form a supermolecular double helix in these trigonal crystals. The six adjacent double helices generate a hexagonal channel along the *c* axis into which 12 dSOD dimers are packed within a period of each helix. A schematic view of this unique packing is depicted in Fig. 3. Such distinct packing is rarely observed in protein crystals, which may produce crystallographic interest in the structure being solved.

## References


Arai, K., Lizuka, S., Makita, A., Oikawa, K. & Taniguchi, N. (1986). *J. Immunol. Methods*, **91**, 139–143.

Civalleri, L., Pini, C., Rigo, A., Federico, R., Calabrese, L. & Rotilio, G. (1982). *Mol. Cell Biochem.* **47**, 3–9.

Crost, N. (1978). *Biochem. Genet.* **16**, 739–742.

Crost, N. & Sausa, P. (1980). *Biochem. Genet.* **18**, 693–696.

Djinovic, K., Coda, A., Antolini, L., Pelosi, G., Desideri, A., Falconi, M., Rotilio, G. & Bolognesi, M. (1992). *J. Mol. Biol.* **226**, 227–238.

Djinovic, K., Gatti, G., Coda, A., Antolini, L., Pelosi, G., Desideri, A., Falconi, M., Marmocchi, F., Rotilio, G. & Bolognesi, M. (1992). *J. Mol. Biol.* **225**, 791–809.

Djinovic Carugo, K., Battistoni, A., Carri, M. T., Poticelli, F., Desideri, A., Rotilio, G., Coda, A., Wilson, K. S. & Bolognesi, M. (1996). *Acta Cryst.* D**52**, 176–188.

Djinovic Carugo, K., Polticelli, F., Desideri, A., Rotilio, G., Wilson, K. S. & Bolognesi, M. (1994). *J. Mol. Biol.* **240**, 179–183.

Ducruix, A. & Giegé, R. (1992). *Crystallization of Nucleic Acids and Proteins: A Practical Approach*, pp. 73–98. Oxford University Press.

Ferraroni, M., Rypniewski, W., Wilson, K. S., Viezzoli, M. S., Banci, L., Bertini, I. & Mangani, S. (1999). *J. Mol. Biol.* **288**, 413–426.

Forest, K. T., Langford, P. R., Kroll, J. S. & Getzoff, E. D. (2000). *J. Mol. Biol.* **296**, 145–153.

Fridovich, I. (1975). *Annu. Rev. Biochem.* **44**, 147–159.

Hough, M. A. & Hasnain, S. S. (1999). *J. Mol. Biol.* **287**, 579–592.

Hough, M. A., Strange, R. W. & Hasnain, S. S. (2000). *J. Mol. Biol.* **304**, 231–241.

Kitagawa, Y., Tanaka, N., Hata, Y., Kusunoki, M., Lee, G. P., Katsube, Y., Asada, K., Aibara, S. & Morita, Y. (1991). *J. Biochem. (Tokyo)*, **109**, 477–485.

Lonnerdal, B., Keen, C. L. & Hurley, L. S. (1979). *FEBS Lett.* **108**, 51–55.

Lu, X. Y., Chen, B. S. & Wang, K. Q. (1998). *Chin. J. Biochem. Mol. Biol.* **14**, 471–474.

Malinowski, D. P. & Fridovich, I. (1979). *Biochemistry*, **18**, 237–244.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Parge, H. E., Hallewell, R. A. & Tainer, J. A. (1992). *Proc. Natl Acad. Sci. USA*, **89**, 6109–6113.
Rigaku (1993). *Instruction Manual for R-AXIS IIC and Software (Version 2.0)*. Manual No. ME201LR2.
Rikans, L. E. & Hornbrook, K. R. (1997). *Biochim. Biophys. Acta*, **1362**, 116–127.
Sato, M., Yamamoto, M., Imada, K., Katsube, Y., Tanaka, N. & Higashi, T. (1992). *J. Appl. Cryst.* **25**, 348–357.
Schinina, M. E., Carlini, P., Polticelli, F., Zappacosta, F., Bossa, F. & Calabrese, L. (1996). *Eur. J. Biochem.* **237**, 433–439.
Sumio Y. (1990). *Arch. Biochem. Biophys.* **279**, 60–69.
Tainer, J. A., Getzoff, E. D., Richardson, J. S. & Richardson, D. C. (1982). *J. Mol. Biol.* **160**, 181–217.
Wang, K. Q., Li, Z. G., Hao, Q. L., He, J. L., Wu, G., Chen, B. S., Xue, H., Wang, J. M., Li, X. Z. & Hu, Y. M. (1986). *Acta Acad. Med. Sin.* **8**, 88–94.
Ye, P., Chen, B. & Wang, S. (1995). *Atherosclerosis*, **117**, 43–50.
Yuan, Y. V., Kitts, D. D. & Godin, D. V. (1997). *Br. J. Nutr.* **78**, 993–1014.
Zhang, B. R., Diao, A. P. & Ouyang, J. (1997). *Microbiol. Bull.* **24**, 178–180.