Molecular cloning and characterization of the copper/zinc and manganese superoxide dismutase genes from the human parasite *Clonorchis sinensis*

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SUMMARY

A copper/zinc superoxide dismutase (Cu/ZnSOD) gene and a manganese superoxide dismutase (MnSOD) gene of the human parasite *Clonorchis sinensis* have been cloned and their gene products functionally characterized. Genes *Cu/ZnSOD* and *MnSOD* encode proteins of 16 kDa and 25·4 kDa, respectively. The deduced amino acid sequences of the two genes contained highly conserved residues required for activity and secondary structure formation of Cu/ZnSOD and MnSOD, respectively, and show up to 73·7% and 75·4% identities with their counterparts in other animals. The genomic DNA sequence analysis of Cu/ZnSOD gene revealed this as an intronless gene. Inhibitor studies with purified recombinant Cu/ZnSOD and MnSOD, both of which were functionally expressed in *Escherichia coli*, confirmed that they are copper/zinc and manganese-containing SOD, respectively. Immunoblots showed that both *C. sinensis* Cu/ZnSOD and MnSOD should be antigenic for humans, and both, especially the *C. sinensis* MnSOD, exhibit extensive cross-reactions with sera of patients infected by other trematodes or cestodes. RT-PCR and SOD activity staining of parasite lysates indicate that there are no significant differences in mRNA level or SOD activity for both species of SOD, indicating cytosolic Cu/ZnSOD and MnSOD might play a comparatively important role in the *C. sinensis* antioxidant system.

Key words: *Clonorchis sinensis*, copper/zinc superoxide dismutase, manganese superoxide dismutase, cDNA, genomic DNA, recombinant enzyme, helminths.

INTRODUCTION

Clonorchis sinensis, an oriental liver fluke, is the causative agent of clonorchiasis, which is an important endemic disease in Asian countries in which approximately 7 million people are infected (Crompton, 1999). This parasite causes typical consumption disease in the hepatobiliary system (Rim, 1986).

Highly reactive free oxygen radicals, including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot -}$) and singlet oxygen ($^1O_2$), are generated by numerous pathways in aerobic organisms (Fridovich, 1978). These reactive oxygen species might cause peroxidation of membrane lipids, DNA strand breakage, and inactivation of enzymes (Imlay & Linn, 1988). Such an oxidative stress burden is much higher against parasites because host immune effector cells that produced these reactive oxygen derivatives that were thought to contribute the killing of parasites by the host (Murray, 1981; Butterworth, 1984; Maizels, Bundy & Selkirk, 1993). Defence systems derived from enzymatic and non-enzymatic antioxidants in parasites might minimize the deleterious effects of the reactive oxygen radicals. Such a system is critical to enable parasites to survive. Several species of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, glutathione *S*-transferase (GST), glutathione reductase, cytochrome *c* oxidase and catalase, have been identified in various parasites (Chung *et al.* 1991; Choi *et al.* 2000). SODs are a group of ubiquitous metalloenzymes that catalyse the dismutation of $O_2^{\cdot -}$ radicals to O_2 and H_2O_2. In concert with catalase, SODs have strong anti-oxidant properties. Three genetically distinct forms of SOD occur that differ in their active site prosthetic metal ion and/or cellular localization in eukaryotic cells: cytosolic copper/zinc SOD (Cu/ZnSOD), a glycosylated extracellular copper/zinc SOD, and a mitochondrial manganese SOD (MnSOD). They are nuclear-encoded but are localized in different subcellular compartments. Cu/ZnSODs have been characterized in various helminth parasites, such as *Schistosoma mansoni* (Hong *et al.* 1992, 1993), *Onchocerca volvulus* (Henkle *et al.* 1991; James, McLean & Perler, 1994), *Dirofilaria immitis* (Callahan, Crouch & James, 1991), *Brugia pahangi* (Tang *et al.* 1994), *Brugia malayi*

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(Ou *et al*. 1995) and *Fasciola hepatica* (Kim *et al*. 2000), but no trematode MnSOD has been cloned or characterized to date. Under normal physiological conditions, mitochondria are the major source of $O_2^{\cdot-}$ production. Studies on nematodes have indicated that MnSOD also plays an important role in protecting cells from oxidative stress (Henkle-Duhrsen *et al*. 1995; Hunter, Bannister & Hunter, 1997). Manganese superoxide dismutase is uniformly distributed throughout the cytoplasm in prokaryotic cells, but for the highly partitioned eukaryotic cells, MnSOD is exclusively associated with mitochondria. The mitochondrial enzyme is encoded by a nuclear gene and must be transported across two mitochondrial membranes to the matrix. This process involves translation of a proenzyme, which in the case of the human MnSOD includes a 24-residue N-terminal leader targeting sequence, to the mitochondrion, where 90% of the cell's oxygen is consumed. The delivery of MnSOD to the mitochondrial matrix is essential for organelle function.

In an attempt to understand the structure and the functional mechanism of SODs in the process of self-protection of *C. sinensis* from attacks by the host defence system, we have isolated cytosolic Cu/ZnSOD cDNA and MnSOD cDNA, and characterized partially the biochemical properties of the bacterially expressed recombinant enzymes.

MATERIALS AND METHODS

Adult Clonorchis sinensis

The metacercariae of *C. sinensis* were obtained from naturally infected freshwater fish, *Pseu dorasbora parva*, collected in an endemic area in Korea. Dogs were each orally fed with 500 metacercariae and adult worms were harvested from the livers of experimental dogs 4 months later. The worms were washed 10 times with physiological saline at 4 °C and stored at −70 °C until required. For the preparation of parasite extracts, frozen worms were thawed at room temperature and resuspended in 2 volumes of phosphate-buffered saline solution (PBS; pH7·2), the parasites were then sonicated on ice. The insoluble cellular debris was spun down at 20 000 ***g*** at 4 °C for 20 min, and the supernatant was subjected to native PAGE for in-gel activity staining. Protein concentration was measured according to the method of Bradford (1976).

Isolation of RNA and cDNA synthesis

The adult worms (approximately 20 mg) were homogenized with a pellet pestle (Sigma) in the lysis buffer provided by a kit (Invitrogen, Carlsbad, CA). Isolation of total RNA was performed following the manufacturer's instructions. The first-strand cDNA synthesis and cDNA amplification by long-distance PCR were performed on total RNA using a cDNA Synthesis Kit (CLONTECH, Palo Alto, CA). The resulting double-strand cDNA library, approximately 0·5–3 kb in size, was divided into aliquots and stored at −70 °C until used.

Polymerase chain reaction (*PCR*)

Degenerate oligonucleotide primers (CZ-Df/CZ-Dr for Cu/ZnSOD and Mn-Df/Mn-Dr for MnSOD, Table 1) were designed based on two highly-conserved regions of Cu/ZnSOD or MnSOD cDNA sequences of other species (Hong *et al*. 1992; Henkle-Duhrsen *et al*. 1995; Kim *et al*. 2000). PCR reactions were performed on *C. sinensis* cDNA library. Thermal cycler profile included 35 cycles at 94 °C for 30 sec, a gradient annealing temperature from 40 to 60 °C for 30 sec and extension at 72 °C for 1 min. The amplifed products were analysed on 1% agarose gels. PCR bands with expected sizes were gel purified, directly subcloned into a pCR2.1 vector (Invitrogen), and sequenced. The nuclotide sequences of the cloned DNA fragments were determined from both directions by the dideoxynucleotide chain termination method using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Beaconsfield, UK), following the manufacturer's instructions, on an automated DNA sequencer (ABI, model 377). Sequence analysis was performed with BLAST and ENTREZ WEB services of the National Center for Biotechnology Information and the Prosite motif search facility of EXPASY (www.expay.ch/cgi-bin).

Rapid Amplification of cDNA Ends (*RACE*)

RACE reactions were performed using a RACE Kit (CLONTECH). The *C. sinensis* cDNA library produced above was used as a template. On the basis of a 260 bp fragment sequence for Cu/ZnSOD and a 147 bp fragment sequence for MnSOD, both obtained by PCR using corresponding degenerate primers, 2 pairs of gene-specific primers (CZ-GSP3/CZ-GSP5 for Cu/ZnSOD and Mn-GSP3/Mn-GSP5 for MnSOD, Table 1) were designed to match both 3′- and 5′-ends of Cu/ZnSOD cDNA or MnSOD cDNA. PCR amplifications of 3′- and 5′-end fragments were carried out according to the protocols provided by the manufacturer. The PCR products were cloned and sequenced as above.

Determination of genomic DNA sequence of C. sinensis *Cu/ZnSOD gene*

Genomic DNA was isolated from *C. sinensis* as previously described (Ismail *et al*. 1997). To determine the genomic DNA sequence of *C. sinensis* Cu/ZnSOD gene, pairs of specific primer with high annealing temperature were designed based on the neighbourhood sequences of start codon and stop codon

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Table 1. Primers used in this study

Primer	Sequence§ (5′->3′)	Description
Primers for Cu/ZnSOD study		
CZ-Df	GCSGGACCYCAYTTCAASCC	Degenerate primers
CZ-Dr	ACCACAAGCCARACGKCCWC	
CZ-GSP3	TCGTCATGTCGGCGATCTTGGCAATG	Gene specific primers used for RACE reactions
CZ-GSP5	TCGTGTACAACCATCGCCCGACCAAC	
CZ-EPf	GTATGTCGACTATGAAGGCAGTTTGTG-	For construction of expression plasmid
CZ-EPr	TAATGCGGCCGCCTACTGCGCTAATCC-	
gCZSODf	GCAGCTAACTATGAAGGCAGTTTGTG	For cloning of genomic DNA
gCZSODr	GAAGACTACTGCGCTAATCCAATGAC	
CZf	GCAGCTAACTATGAAGGCAGTTTGTG	For analysis of relative mRNA levels by RT-PCR
CZr	TCGTGTACAACCATCGCCCGACCAAC	
Primers for MnSOD study		
Mn-Df	GTNCARGGHTCNGGHTGG	Degenerate primers
Mn-Dr	RTARTAWGCATGTTCCCA	
Mn-GSP3	GGACTGAACCCGATAACCAAGCGTC	Gene specific primers used for RACE reactions
Mn-GSP5	GACGCTTGGTTATCGGGTTCAGTCC	
Mn-EPf	TTCATATGATGCAGAATTTGTTGGC-	For construction of expression plasmid
Mn-EPr	GACACTCGAGTTAAGACCTGGCGGA-	
Mnf	ATGCGCTTGAACCCACTATATCC	For analysis of relative mRNA levels by RT-PCR
Mnr	GACGCTTGGTTATCGGGTTCAGTCC	

§ S = C or G; W = A or T; Y = C or T; R = A or G; H = A, C or T; K = G or T; N = A, C, G, or T.
-Regions underlined represent restriction endonuclease sites.

respectively. One pair of primers, gCZSODf and gCZSODr (Table 1), produced a significant amplicon, which was then cloned, sequenced and analysed.

Phylogenetic analysis

The obtained sequences of *C. sinensis Cu/Zn*SOD or *MnSOD* were compared with corresponding sequences of several helminths deposited in GenBank. Multiple alignment analysis was performed with the DNAstar. Phylogenetic analyses on the aligned sequences were done using distance methods performed by the MEGA program version 2.1. A distance tree was constructed by use of the neighbour-joining (NJ) method. The stability of the trees obtained was estimated by bootstrap analysis for 1000 replications using the same program. For prediction of mitochondrial targeting sequence and its cleavage site, ExPASy system (http://psort.nibb.ac.jp/cgi-bin/runpsort.pl) was employed.

RT-PCR for analysis of cytosolic Cu/ZnSOD and MnSOD relative mRNA levels

RT-PCR was carried out using an Invitrogen RT-PCR kit according to the manufacturer's instructions except for the following details: 50 ng of Cu/ZnSOD or MnSOD specific primers (CZf/CZr and Mnf/Mnr, respectively, Table 1) were annealed to 250 ng of total RNA isolated from adult *C. sinensis* and extended at 55 °C for 30 min. The reactions were shifted to 95 °C and held for 2 min and then cycled at 95 °C for 10 s, 65 °C for Cu/ZnSOD and 55 °C for MnSOD for 1 min, and 68 °C for 1 min for 35 cycles in a Perkin-Elmer 9600 Thermal Cycler.

Construction of recombinant plasmid, expression and purification of recombinant protein

The whole coding region of Cu/ZnSOD was amplified by PCR, employing primers CZ-EPf and CZ-Epr (Table 1). The PCR product and vector pGEX-4T-2 were digested with the same enzymes, *Sal* I and *Not* I, and then ligated. The resulting plasmid (designated pGEX-Cu/ZnSOD) or parental pGEX-4T-2 was transformed into competent TOP10 *E. coli* cells (Pharmacia). The expression of fusion protein or the GST wild-type protein was performed by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and purified with a glutathione-Sepharose 4B column (Pharmacia). To cleave the fusion protein from the GST carrier, the protein was incubated with thrombin in PBS (50U/ml) for 16 h at room temperature with gentle shaking. For the expression of recombinant MnSOD, a similar procedure was used as that for the expression of Cu/ZnSOD, except that the expression vector was pET-28a instead of pGEX 4T-2. Primers Mn-EPf and Mn-EPr (Table 1) were used to construct the expression plasmid pET-MnSOD. The protein was expressed in *E. coli* BL21(DE3) with a 6 × His tag at its N-terminus. The recombinant MnSOD was purified using Ni-NTA resin.

SDS-PAGE and Western blotting

Proteins were separated by a standard SDS-PAGE protocol on 12% separating gels under reducing conditions by the method described previously (Laemmli, 1970). For Western blotting, the proteins resolved on SDS-PAGE were electrotransferred to

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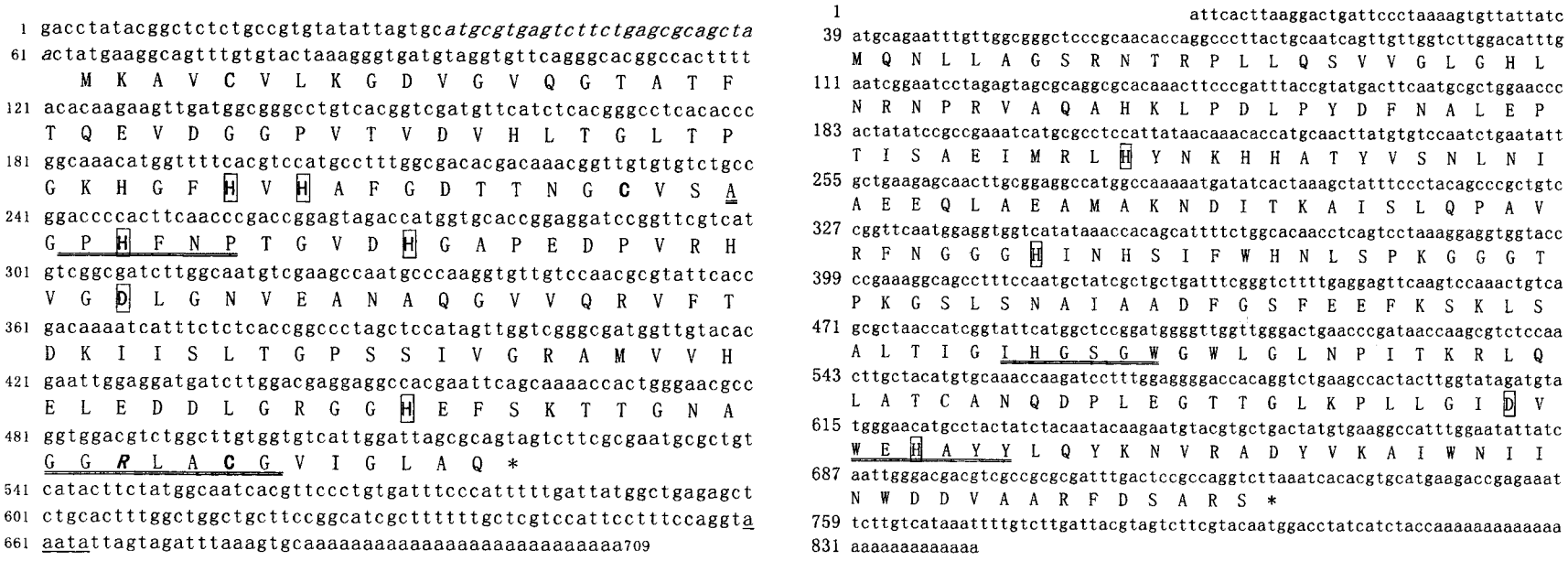

Fig. 1. Nucleotide and translated amino acid sequences of *Clonorchis sinensis* Cu/ZnSOD and MnSOD cDNAs. (A) Cu/ZnSOD: the consensus poly(A) signal in the 3′ terminus is underlined; the degenerate primers employed to produce the first fragment of 260 base pairs are double underlined; 6 His and 1 Asp residues (boxed) form the copper/zinc binding sites; 3 Cys residues (bold) are believed to form intra-chain and/or inter-chain disulfide bonds; the arginine residue (italic) is necessary to guide the superoxide anion to the active site. The contagious nucleotide acid sequence from 54 to 527 was also found in the genomic DNA, indicting this is a one-exon gene. (B) MnSOD: the degenerate primers employed to produce the first fragment of 147 base pairs are double underlined; 3 His and 1 Asp residues (boxed) form a Mn-binding site.

nitrocellulose membrane. Free binding sites in the membrane were blocked by incubation in blocking buffer (5% non-fat goat milk in PBS, pH 7·4) at room temperature for 1 h. The membrane was incubated with human sera (1 : 100) overnight. After washing, the membrane was further incubated with horseradish peroxidase-conjugated goat anti-human IgG for 2 h at a dilution of 1 : 1000. The colour reaction was developed with 3, 3′-diaminobenzidine tetrahydrochloride chromogen in the presence of hydrogen peroxide.

In-gel staining of SOD activity

To confirm SOD activity of the recombinant protein, samples were subjected to electrophoresis on 10% SDS-PAGE. After 3 extensive washes employing PBS, a photochemical method modified from Beauchamp & Fridovich (1971) and Chen *et al*. (2000) was used to visualize SOD activity. To distinguish between Cu/Zn and Mn or Fe dependent SODs, activity was measured on gel assay in the presence of 10 mM potassium cyanide (KCN), 10 mM hydrogen peroxide (H_2O_2), or 10 mM sodium azide (NaN_3).

Nucleotide sequence Accession number

The *C. sinensis* Cu/ZnSOD cDNA sequence, genomic sequence and MnSOD cDNA sequence are available in the GenBank database under the Accession numbers AY396147, AY675506, and AY563102, respectively.

RESULTS

Identification of cDNAs and genomic sequences encoding C. sinensis *Cu/ZnSOD and MnSOD*

PCR with the degenerate primers for Cu/ZnSOD produced a band with an expected size of 260 bp showing high similarity with cytosolic Cu/ZnSOD of other organisms. Based on this sequence, RACE-PCR amplifications resulted in a 709 bp full-length cDNA encoding *C. sinensis* Cu/ZnSOD (Fig. 1). PCR using the degenerate primers for MnSOD produced 1 band with an expected size of 147 bp showing high similarity with MnSOD of other organisms, and then an 843 bp full-length cDNA encoding *C. sinensis* MnSOD was obtained by the same procedure as that for the production of Cu/ZnSOD (Fig.1).

Genomic DNA sequence analysis revealed that no introns exist in the coding region of *C. sinensis* Cu/ZnSOD gene. This is very interesting and is the first identified intronless gene from helminth parasites to date (Fig.1).

Analysis of amino acid sequences of C. sinensis *Cu/ZnSOD and MnSOD*

The open reading frame (ORF) of *C. sinensis* Cu/ZnSOD cDNA encodes a polypeptide of 152 residues with a predictive monomeric *M*r of 15·5 kDa. When the sequence was aligned with those of Cu/ZnSOD from other organisms, high similarities were found (Fig. 2). It has been well characterized structurally that the active site of the oxidized Cu/ZnSOD enzyme consists of 1 copper and 1 zinc ion bridged by the imidazole ring of His61. The Cu, which is the active metal, is coordinated by a further 3 histidine ligands and a water molecule, while Zn is ligated by 2 additional histidines and an aspartic acid (Ciriolo *et al*. 2001). In *C. sinensis* Cu/ZnSOD, all of these amino acid residues crucial for activity are conserved. No signal peptide sequence was identified by the SignalP (Nielsen *et al*. 1997), excluding the possibility that this is a secretary protein.

The ORF of *C. sinensis* MnSOD cDNA encodes a polypeptide of 230 residues with a predictive monomeric Mr of 25·4 kDa. There are a number of residues that appear to be strictly conserved in MnSOD families, which are expected to represent groups essential for catalytic function. Four of these (His58, His106, Asp191, and His195) are metal ligands, two more (His62 and Tyr66) form the gateway to the active site, and another (Glu194) lies in the outer sphere of the metal binding site (Fig. 2). After removal of mitochondrial targeting sequences, alignment of MnSOD amino acid sequences of various organisms revealed that *C. sinensis* MnSOD has very high identities with those of other species (56·3%–75·4%) (Fig. 2) higher than the corresponding identities of Cu/ZnSOD (47·9%–73·7%). The striking amino acid similarity between phylogenetically distant organisms suggests that MnSOD is highly conserved.

Expression and characterization of C. sinensis *Cu/ZnSOD and MnSOD in* E. coli

After induction with IPTG, a high level of GST-Cu/ZnSOD fusion protein with a molecular weight of approximately 42 kDa, an expected size of fusion protein, was expressed in *E. coli* TOP10 (lane 5 in Fig. 3A). When the fusion protein was cleaved by thrombin, 2 bands of protein, one with an *M*r of 26 kDa being GST and another one with an *M*r of 16 kDa being *C. sinensis* Cu/ZnSOD, were observed (lane 7 in Fig. 3A). Both purified SOD and fusion protein demonstrated SOD activity in the 'in-gel' activity assay. To confirm the isoenzyme type of cloned SOD, a type-specific inhibitor study was performed using KCN, H_2O_2 and NaN_3. Fig. 4 revealed that enzymatic activity was not influenced by 10 mM NaN_3 but completely by 10 mM KCN and significantly by 10 mM H_2O_2. This inhibition pattern strongly suggested the *C. sinensis* SOD cloned in this study with an *M*r of 16 kDa was a Cu/Zn-containing SOD (Geller & Winge, 1983). Next, we investigated the formation of disulphide bonds in the protein structure. Fig. 5A shows the effects of reducing

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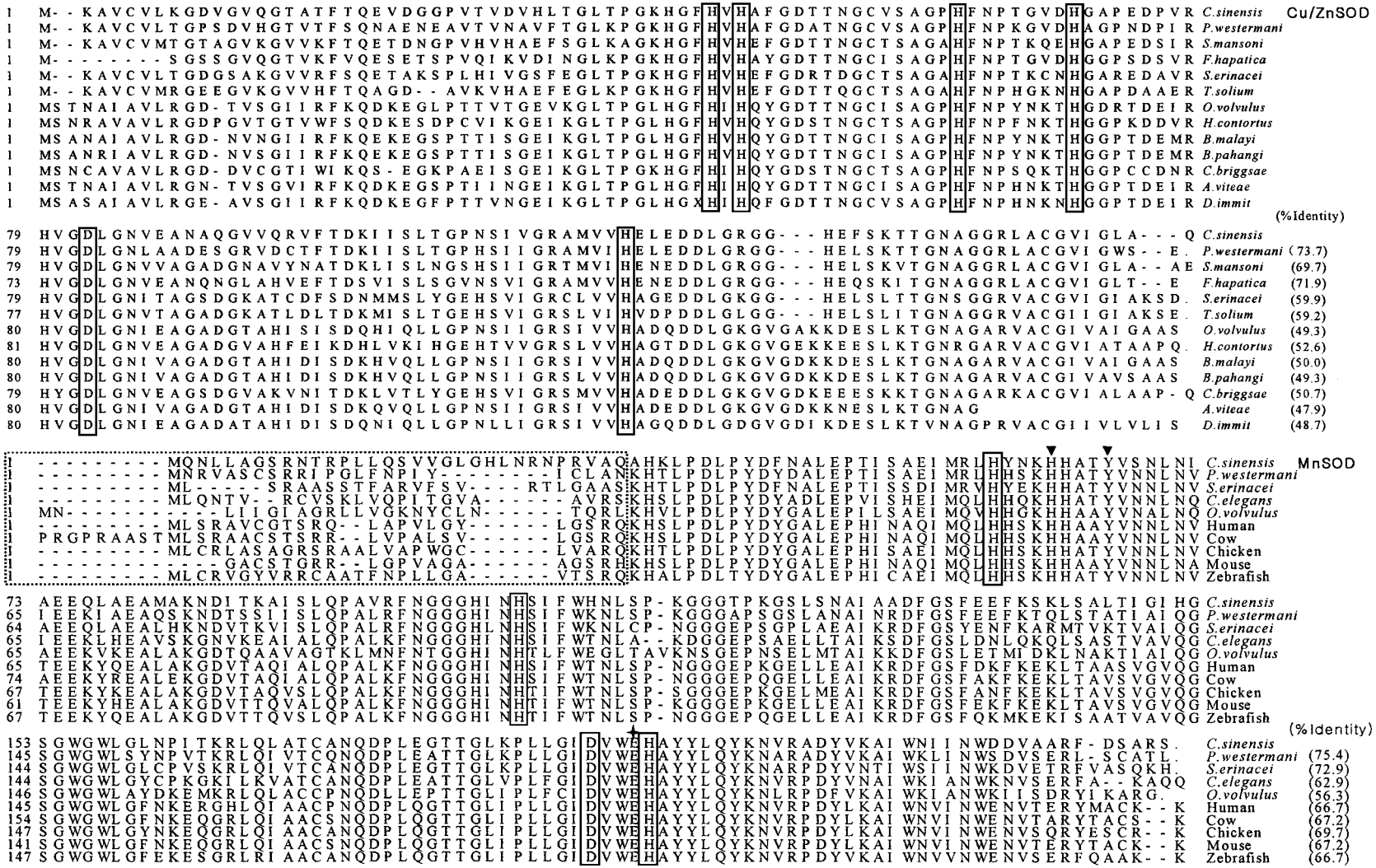

Fig. 2. Alignment of the deduced amino acid sequences of *Clonorchis sinensis* Cu/Zn-SOD and MnSOD with those of other organisms. Gaps are introduced to maximize alignment, (-) indicates a gap. The percentage identity of deduced amino acid sequences of *C. sinensis* SODs with those of other animals are presented at the end of each sequence alignments. (A) Cytosolic Cu/ZnSOD sequences, including *C. sinensis* (AY396147) in this study; *P. westermani* (AY396148); *F. hepatica* (AF071229); *S. erinacei* (AY396149); 5, *T. solium* (AF439353); *S. mansoni* (M97298); *B. pahangi* (X76284); *B. malayi* (AY428604); *O. volvulus* (X57150); *A. viteae* (*Acanthocheilonema viteae*, AJ240082); *H. contortus* (*Haemonchus contortus*, Z69621); *D. immit* (AF004949). The putative metal binding residues (boxed) are highly conserved. (B) Mitochondrial MnSOD sequences, their database Accession numbers are as follows: *P. westermani* (AY675508), *S. erinacei* (AY675509), *C. elegans* (*Caenorhabditis elegans*, NM_059889), *O. voluulus* (X82171), *E. coli* (NP_418344), Human (AAH12423), Cow (AAA30655), Chicken (AAG46055), Mouse (AAH18173), Zebrafish (AAH60895). The region boxed with a broken line includes putative mitochondrial targeting sequences. Amino acid residues in boxes are manganese-binding sites.

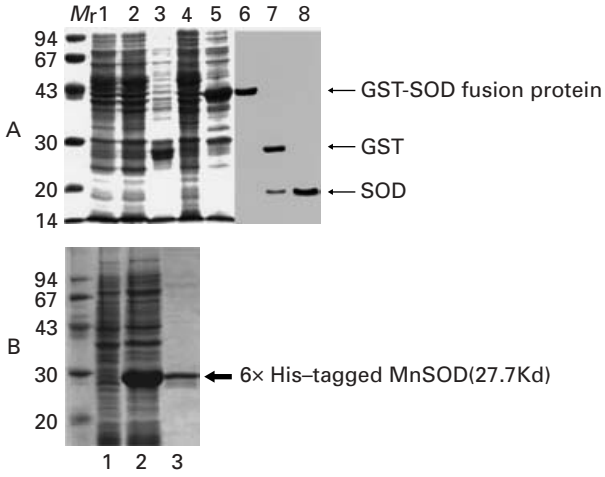

Fig. 3. Expression of *Clonorchis sinensis* Cu/ZnSOD and MnSOD in *E. coli*. (A) Cu/ZnSOD, showing Coomassie blue-stained SDS-polyacrylamide gel of proteins from lysates of *E. coli* without plasmid (lane1), uninduced pGEX-4T-2 (lane 2), pGEX-4T-2 induced with IPTG (lane 3), uninduced pGEX-Cu/ZnSOD (lane 4), pGEX-Cu/ZnSOD induced with IPTG (lane 5), purified GST-Cu/ZnSOD fusion protein (lane 6), purified GST-Cu/ZnSOD fusion protein cleaved with thrombin (lane 7), purified GST-free Cu/ZnSOD (lane 8). (B) MnSOD, showing Coomassie Blue-stained SDS-polyacrylamide gel of proteins from lysates of uninduced *E. coli* BL21 (DE3) harbouring plasmid pET-MnSOD (lane1), pET-MnSOD induced with IPTG (lane 2), Purified 6 × His-tagged MnSOD protein (lane 3).

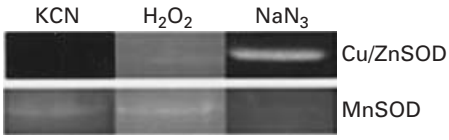

Fig. 4. SOD activity assay on a 12% SDS-polyacrylamide gel with NBT-riboflavin system. Cu/ZnSOD activity was significantly inhibited by KCN and H_2O_2, but not by NaN_3; MnSOD activity was significantly inhibited by NaN_3, but not by KCN or H_2O_2. NaN_3, 10 mM NaN_3; H_2O_2, 10 mM H_2O_2; KCN, 10 mM KCN.

reagent (β-mercaptoethanol) level on the behaviour of the purified *C. sinensis* Cu/ZnSOD in SDS-PAGE gels. When the protein was pre-treated before loading with a low concentration (5 mM) or no β-mercaptoethanol, 2 or 3 additional bands (lanes a and b in Fig. 5A) can be visualized when compared with that pre-treated with a high concentration (50 mM) of β-mercaptoethanol (lane c in Fig. 5A). All of these bands demonstrated SOD activity (Fig. 5B). This experiment provided enough evidence for the existence of disulphide bond(s) in *C. sinensis* Cu/ZnSOD molecules.

A 693-bp fragment coding for MnSOD, including the mitochondrial targeting sequence, was cloned into the *Nde*I and *Sal* I sites of the expression vector pET-28a and used to transform *E. coli* BL21(DE3).

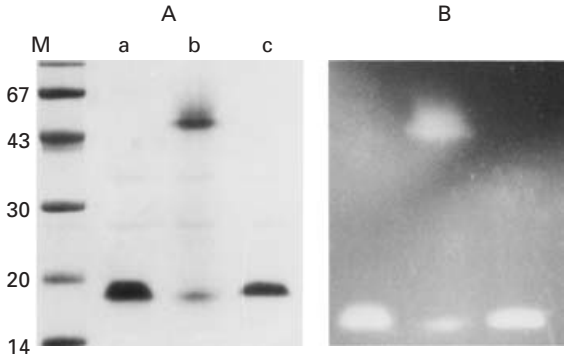

Fig. 5. Detection of disulfide bonds in the secondary structure of Cu/ZnSOD employing β-mercaptoethanol as reducing regent. Samples were separated on 12% SDS-PAGE and then were subjected to Coomassie Blue staining (A) or SOD activity staining (B). Purified SOD was treated with 5 mM (lane a), 50 mM (lane c) and or no (lane b) β-mercaptoethanol, and then heated for 5 min at 100 °C before loading the gels. The gel and sample treatment were identical for both panel A and panel B except the staining method. Two extra minor bands appearing on lanes a and b in panel A also showed SOD activity in panel B. M: molecular weight marker in kDa.

Analysis by SDS-PAGE of the cell extracts showed the overexpression of a protein of approximately 28 kDa after induction of the cells with IPTG for a period of 4 h (Fig. 3B). This was in good agreement with the molecular mass predicted from the derived amino acid sequence. The recombinant 6 × His-tagged MnSOD separated by SDS-PAGE and stained with Coomassie Blue was, apparently, the major induced protein. The proteins were banded to a Ni-NTA column and eluted with 0·3 M imidazole. The purification was monitored by removing aliquots for SDS-PAGE analysis. The purification yielded over 90% pure proteins as estimated by SDS-PAGE analysis (lane 3 in Fig. 3B).

Phylogenetic analysis

Phylogenetic analysis was performed in order to clarify the relationship of *C. sinensis* Cu/ZnSOD and those from other organisms. The topology of the resulting phylogenetic tree constructed using neighbour-joining (NJ) (Fig. 8) shows that the clade of *C. sinensis*, *P. westermani*, *F. hepatica*, *Taenia solium* and *Spirometra mansoni* is well separated from the other two clades on the tree. This result is inconsistent with that of the morphological taxonomy, where these species belong to a same phylum, Platyhelminthes. The MnSOD tree shows a similar topology as that of Cu/ZnSOD. The same and unique genomic organization feature of *C. sinensis* and *P. westermani* Cu/ZnSOD genes (both are one-exon genes), together with the topology of Cu/ZnSOD and MnSOD trees strongly indicate that these two species have very close phylogenetic relationship.

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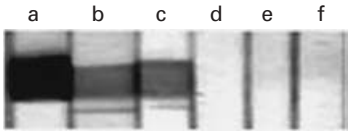

Fig. 6. Western blot analysis of pooled sera of clonorchiasis (a), paragonimiasis (b), fascioliasis (c), sparganosis (d), cysticercosis (e) patients and healthy human donors (f) versus recombinant *Clonorchis sinensis* Cu/Zn-SOD protein.

Immunological reactivity of C. sinensis *Cu/ZnSOD*

To test whether Cu/ZnSOD of *C. sinensis* is a natural target for the human immune system, and to clarify if there are cross-reactions among SOD proteins from helminth parasites, Western blots were performed. The results demonstrated that *C. sinensis* Cu/ZnSOD produced pronounced reactions to *C. sinensis*-infected patients' sera, suggesting the SOD enzyme is antigenic to humans. The immunoblots also showed that *C. sinensis* Cu/ZnSOD gave significant cross-reactions to sera of patients infected by *P. westermani* and *F. hepatica* with comparable strength as the homologous responses (Fig. 6), but did not cross-react with sera of patients with cysticercosis or with sparganosis, reflecting *C. sinensis* has closer phylogenetic relationships with *P. westermani* and *F. hepatica* than with *T. solium* and *S. erinacei*. With regards to MnSOD, there are strong and wide cross-reactions between *C. sinensis* MnSOD and the sera of patients infected by *P. westermani*, *F. hepatica*, *S. erinacei* and *T. solium*, even with some healthy sera (data not shown).

Relative expression of Cu/ZnSOD and MnSOD genes

The relative transcription level of *Cu/ZnSOD* and *MnSOD* during the adult stage was studied by RT-PCR analysis using two pairs of specific primers and using total RNA as template. The same amount of RNA and the same reaction conditions were used for both genes. No significant difference in the amount of the amplified SOD fragments (369 bp) was observed between the two genes. The occurrence of both species of SOD in adult *C. sinensis* was studied *in vitro* (Fig. 7, upper panel). A roughly equivalent abundance of KCN-sensitive SOD (putative Cu/ZnSOD) and KCN-resistant SOD (putative MnSOD) was observed in the supernatant of the parasite lysates. The transcriptional and post-transcriptional analyses indicate that cytosolic Cu/ZnSOD and MnSOD might play a comparative role in the *C. sinensis* antioxidant system.

DISCUSSION

Cu/ZnSOD is a dimer of molecular weight approximately 32 kDa. Each monomer folds as an 8-stranded

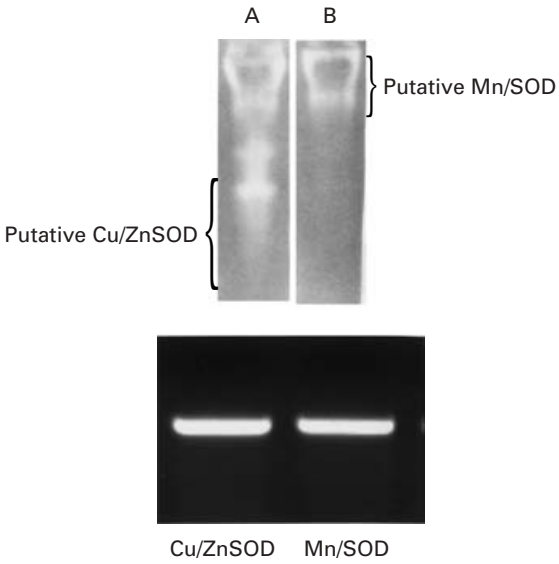

Fig. 7. The upper panel: activity detection and isoenzyme type identification of SOD in *Clonorchis sinensis* crude extract. (A) The soluble extract of adult *C. sinensis* (100 μg total protein) was separated on 12% non-denaturing polyacrylamide gels and stained immediately for SOD activity. (B) The sample and gel were identical to panel A except that the gel was treated with 10 mM KCN in the process of staining. The putative MnSOD and Cu/ZnSOD are indicted. The lower panel: reverse-transcriptase polymerase chain reaction (RT-PCR) of *Cu/ZnSOD and MnSOD*. PCR products were run on 1% agarose gels and stained with ethidium bromide.

Greek-key beta-barrel connected by 3 external loops. This enzyme has been reported in diverse organisms. We report here the cloning, expression and characterization of *C. sinensis* cytosolic Cu/ZnSOD. This is the first report of SOD cDNA sequences from this parasite. The nature of the protein cloned in this study was determined on the basis of its high similarity of deduced amino acid sequence with that of Cu/ZnSODs from other organisms, the absolute conservation of residues required for activity, the lack of signal peptide on the primary sequence, the SOD activity and its KCN-specific activity inhibition pattern.

The determination of the genomic DNA sequence of *C. sinensis* Cu/ZnSOD coding region showed it is a single-exon gene, an unusual structural feature. No intronless genes have been reported to date from helminth parasites, and very few SOD genes characterized to date in other eukaryotes were found to be intronless except *P. westermani* Cu/ZnSOD gene and yeast MnSOD gene (Marres *et al.* 1985). The significance of the lack of an intron in *C. sinensis* and *P. westermani* Cu/ZnSOD genes is not known. It would be interesting to determine whether this unique feature would be possessed by other trematodes except *S. mansoni*.

The residues known to be necessary for enzymatic activity were conserved in the deduced *C. sinensis*

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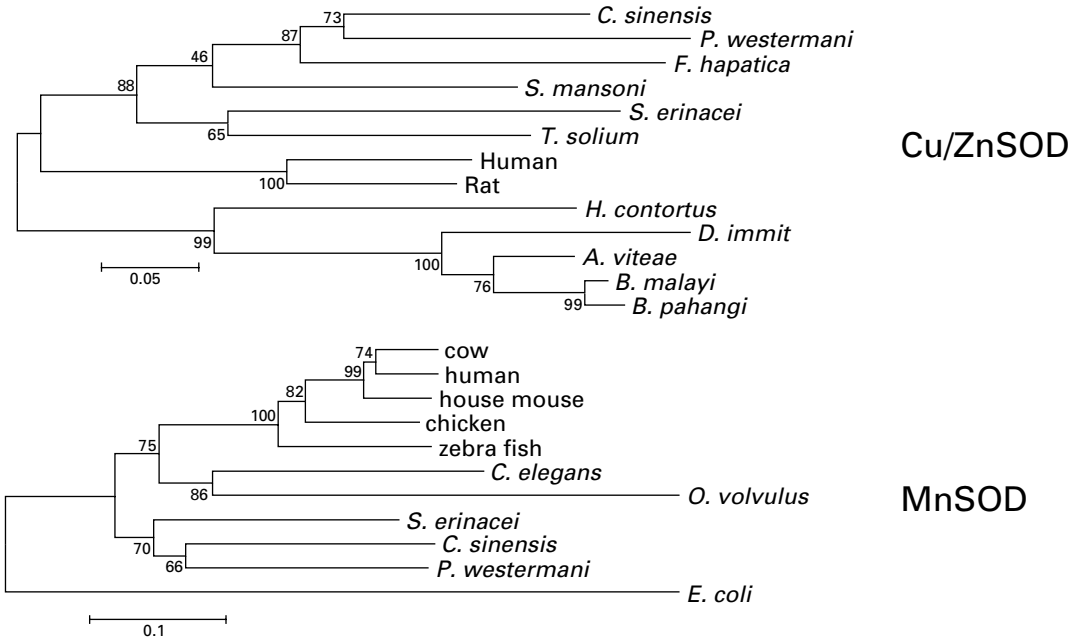

Fig. 8. Phylogenetic analysis of *Clonorchis sinensis* Cu/ZnSOD and MnSOD based on the amino acid sequences. The unrooted neighbour-joining method was used to construct the phylogenetic tree using the MEGA 2.1 program. The numbers at the nodes are the proportions of 1000 bootstrap re-samplings that support the topology shown. The scale bars indicate a distance of 0·05 or 0·1.

MnSOD. Furthermore, sequences of mature MnSODs from different organisms have higher similarity than Cu/ZnSODs do. But the N-terminal mitochondrial leader sequence region displayed the lowest level of primary sequence identity. The mature *C. sinensis* MnSOD subunit molecular mass of 21·9 kDa is typical of that found in other helminths studied so far (22·0 kDa in *C. elegans*; 23·4 kDa in *O. volvulus*). The potential N-glycosylation site (N-H-S/T at His-106 in the *C. sinensis* MnSOD sequence) is also highly conserved in most eukaryotic species. Interestingly, there are invariable penultimate arginine residues in the MnSOD putative mitochondrial leader sequences of other eukaryotic species, but this arginine residue is substituted by an alanine residue in *C. sinensis*, *P. westermani* and *S. erinacei* MnSODs.

Anti-Cu/ZnSOD and anti-MnSOD antibodies were detected from sera of *C. sinensis*-infected patients by recombinant *C. sinensis* Cu/ZnSOD and MnSOD in Western blots. Furthermore, extensive cross-reactions were observed at a significant degree between *C. sinensis* Cu/ZnSOD or MnSOD with their counterparts from other helminths, especially between MnSODs. The findings coincide with the high sequence similarities among each species of SODs, especially among MnSODs. However, considering the two facts that extracellular SODs are excreted actively into the extracellular compartment, and both forms of Cu/ZnSOD of a certain organism might share high sequence similarity (Henkle *et al*. 1991; James *et al*. 1994; Tang *et al*. 1994), it is a reasonable speculation that the immunological reactions probed by Western blotting in the present study not only resulted from cytosolic SODs, but also from extracellular SODs at a significant degree.

In our current study, both RT-PCR and activity staining in native PAGE of parasite extracts revealed no significant differences in the transcription level and activity between cytosolic Cu/ZnSOD and MnSOD. This finding is not in agreement with the previous conclusions that the more than 80–90% SOD activity in *B. malayi* and *S. mansoni* were due to Cu/ZnSOD (Hong *et al*. 1992; Ou *et al*. 1995).

Very few reports investigated anti-SOD antibody levels in naturally helminth-infected patients' sera. The stimulation of the host immune system against these antigens might be of use for an anti-*C. sinensis* vaccine. SODs have been recently evaluated as vaccine candidates for different diseases (Britton, Knox & Kennedy, 1994; Imlay & Imlay, 1996; Hess *et al*. 1997; Paramchuk *et al*. 1997; Golenser *et al*. 1998). Considering the importance of SOD for parasites' pathogenicity, and the extensive cross-reactions existing amongst SOD proteins of helminth parasites, we should take SOD as a valuable candidate for developing a broad-spectrum vaccine to combat these parasites, as preliminarily demonstrated by *Schistosoma* cytosolic Cu/ZnSOD (Carvalho-Queiroz *et al*. 2004).

Through the isolation, characterization and over-expression of *C. sinensis* SOD, we have taken the initial step in understanding the structure and the function SOD may play in parasite survival. A

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clearer and probably final determination will be attained through the gene inactivation. In conclusion, we isolated a gene encoding a cytosolic Cu/ZnSOD and a MnSOD of *C. sinensis* and characterized the biochemical properties of the recombinant enzyme. Overexpression of enzymatically active recombinant *C. sinensis* Cu/ZnSOD and MnSOD would allow us to investigate detailed enzymatic characteristics and molecular structure of the enzyme. The pathophysiological and/or biological roles of the enzyme in host-parasite interactions need further elucidation.

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