Molecular Cloning and Expression of Cu/Zn-Containing Superoxide Dismutase from *Fasciola hepatica*

TONG-SOO KIM,¹ YOUNGHUN JUNG,² BYOUNG-KUK NA,³ KI-SUN KIM,² AND PYUNG-RIM CHUNG^{2*}

Department of Parasitology,¹ and Division of Respiratory Viruses,³ National Institute of Health, Seoul 122-701, and Department of Parasitology, Inha University College of Medicine, Incheon 400-103,² Korea

Received 4 January 2000/Returned for modification 9 March 2000/Accepted 23 April 2000

The cytosolic superoxide dismutase (SOD) of *Fasciola hepatica*, a causative agent of fascioliasis, was purified and characterized. The enzyme consists of two identical subunits, each with an apparent molecular mass of 17.5 kDa. An analysis of the enzyme's primary structure and inhibition studies revealed that the enzyme is a copper/zinc-containing SOD (Cu/Zn-SOD). The enzyme activity was relatively stable in a broad pH range, from pH 7.0 to 10.0, and the enzyme showed maximum activity at pH 7.5. This enzyme also displayed strong antigenicity against sera of bovine and human subjects with fascioliasis. The SOD gene fragment was amplified by PCR with degenerate oligonucleotide primers derived from amino acid sequences conserved in the Cu/Zn-SODs of other organisms. An *F. hepatica* cDNA library was screened with the SOD gene fragment as a probe. As a result, a complete gene encoding the Cu/Zn-SOD was identified, and its nucleotide sequence was determined. The gene had an open reading frame of 438 bp and 146 deduced amino acids. Comparison of the deduced amino acid sequence of the enzyme with previously reported Cu/Zn-SOD amino acid sequences revealed considerably high homologies. The coding region of the *F. hepatica* Cu/Zn-SOD was cloned and expressed in *Escherichia coli*. Staining of native polyacrylamide gel for SOD activity of the expressed protein revealed SOD activity that was inactivated by potassium cyanide and hydrogen peroxide but not by sodium azide. This means that the presence of the recombinant fusion protein is indicative of Cu/Zn-SOD. The expressed protein also reacted with sera of bovine and human subjects with fascioliasis, but it did not react with sera of uninfected bovine and human subjects.

Worldwide, *Fasciola hepatica*, a liver fluke, is a causative agent of fascioliasis in mammals (11, 34, 49), including humans (2, 15, 38). Following ingestion of metacercariae by the hosts, the juvenile worms burrow through the host gut walls and migrate to the liver, where they cause extensive damage before moving into the bile ducts. Finally, the parasites pass the bile duct walls and develop into mature forms that live in the microenvironment of the bile ducts. Although the adult worms have a predominantly anaerobic metabolism and inhabit in the bile duct, where the oxygen tension is relatively low (58), oxygen is still required for other functions such as egg generation, which generate reactive oxygen species (59). In addition to this normal confrontation with oxidative stress, the parasite is exposed to reactive oxygen species generated by host effector cells such as macrophages, eosinophils, neutrophils, and platelets (3, 37).

Free oxygen radicals generated by these effector cells via the oxidative burst are thought to contribute to the killing of parasites by hosts (37, 42, 44). To defend themselves against oxygen-mediated killing mechanisms of hosts, parasites have developed antioxidant enzyme systems. It has been suggested that antioxidant suppression of host oxidative killing may play a protective role in the parasite life cycle (9). Therefore, antioxidant enzymes have been considered important virulence factors in a number of parasites (16, 33, 40, 43).

Prominent among antioxidants are superoxide dismutases (SODs), which catalyze the decomposition of superoxide, the first reactive species in the reduction of molecular oxygen into hydrogen peroxide and molecular oxygen (4, 7, 18, 19). SODs

are postulated to play a role in the protection of parasites against the cellular, oxygen-mediated killing mechanisms of the hosts (10, 21, 23, 26, 27, 29).

Up to now, SODs have been characterized and cloned from various helminth parasites of different species such as *Schistosoma mansoni*, *Onchocerca volvulus*, *Dirofilaria immitis*, and *Brugia pahangi* (10, 21, 22, 25, 27, 29, 56). In these parasites, SODs have been found to be surface-located or secreted (23, 25, 26, 29, 56). This has led to the hypothesis that the enzymes play a special defensive role for the parasite at the interface of the host-parasite interaction. Like the above-mentioned parasites, *F. hepatica* has developed SODs to defend itself from oxidative killing mechanisms of the host (48). However, at present there is little information indicating that *F. hepatica* SOD is associated with the pathogenicity of the parasite. Therefore, in order to elucidate a possible role of the SOD of *F. hepatica* in the defense against the oxygen-dependent killing mechanisms of the host, we carried out the purification and characterization of cytosolic SOD from *F. hepatica*, and we cloned the gene and functionally expressed it in *E. coli*.

MATERIALS AND METHODS

Collection of parasite. *F. hepatica* adult worms were obtained from the liver of an infected bovine. The collected worms were washed several times with phosphate-buffered saline (PBS [pH 7.4]) and then incubated in the same buffer at 37°C for 3 h to eliminate any residual host matter. After the parasites were washed with PBS several times, they were stored at –70°C until used.

Preparation of parasite extract. *F. hepatica* adult worms were homogenized with a Teflon homogenizer in PBS supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetic acid, and 10 mM leupeptin. The homogenates were centrifuged at 28,000 × g for 30 min at 4°C, and the supernatants were collected and used for further studies. The protein concentration was determined by the method of Lowry et al. (35), with bovine serum albumin as a standard.

Purification of SOD. *F. hepatica* extract was applied to a diethylaminoethyl (DEAE)-Sephacel column (1.6 by 12 cm; Pharmacia, Uppsala, Sweden) equilibrated with 50 mM sodium phosphate buffer (pH 7.5). The column was extensively washed with the same buffer, and the absorbed proteins were eluted with

* Corresponding author. Mailing address: Department of Parasitology, Inha University College of Medicine, Incheon 400-103, Korea. Phone: 82-32-890-0981. Fax: 82-32-884-2104. E-mail: chungpr@dragon.inha.ac.kr.

a linear gradient of 0.5 M NaCl. Fractions exhibiting SOD activity were pooled and purified further by successive carboxymethyl (CM) Sepharose Fast Flow chromatography (1.6- by 12-cm column; Pharmacia), equilibrated with 0.1 M sodium acetate buffer (pH 5.5), and eluted with a linear gradient of 0.5 M NaCl. Fractions exhibiting SOD activity were collected, concentrated, and applied to Superose 12 molecular-sieve chromatography columns (1.6 by 30 cm; Pharmacia) equilibrated with 50 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl. Active fractions were collected, concentrated, and applied to Mono-Q ion-exchange chromatography columns (0.5 by 5 cm; Pharmacia) equilibrated with 10 mM Tris-HCl buffer (pH 7.0). After extensive washing with the same buffer, adsorbed proteins were eluted with a linear gradient of 0.5 M NaCl. All the purification procedures were performed at 4°C.

Enzyme assay. SOD activity was determined by the neotetrazolium chloride (NTC) reduction assay based on the method of Noridaka et al. (45). The assay mixtures (0.5 ml) contained 50 µl of 0.5 M sodium phosphate (pH 7.5), 25 µl of 16% Triton X-100, 2.5 µl of 10 mM EDTA, 75 µl of 1.2 mM NTC, 2.5 µl of xanthine oxidase (1.0 U), 10 µl of sample, 25 µl of 4 mM hypoxanthine, and distilled water. The A_{540} was monitored with a spectrophotometer (model DU-600; Beckman, Palo Alto, Calif.) after the addition of 0.5 ml of a solution containing 1 M formate buffer (pH 3.5), 10% Triton X-100, and 40% formaldehyde. One unit of enzyme activity was defined as the amount of the enzyme required to cause 50% inhibition in the rate of reduction of NTC under the conditions of the assay.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (31). Native polyacrylamide gel electrophoresis was performed at 4°C in the absence of SDS. Gels were stained with Coomassie brilliant blue and destained.

Staining for SOD activity. To identify the SOD activity on the native gel, the gel was stained by the riboflavin-nitroblue tetrazolium method (6). In brief, the gels were soaked simultaneously in a solution of 0.2% nitroblue tetrazolium, 0.028 M N,N,N',N' -tetramethylethylenediamine (TEMED), and 2.8×10^{-5} M riboflavin in 50 mM potassium phosphate buffer (pH 7.8) for 30 min at room temperature. After that, the gels were illuminated until chromatic zones indicating SOD activity were visible in a uniformly blue background.

Determination of molecular weight of purified enzyme. Apparent molecular weight of the purified enzyme was determined by SDS-PAGE as described above. The molecular weight standard proteins were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α -lactalbumin (14,400) (Pharmacia). The native molecular weight of the enzyme was determined by molecular-sieve chromatography with a Superose 12 column.

Determination of the metallic cofactor of SOD. To determine the types of SOD on the native gel, the purified enzyme was mixed with potassium cyanide (KCN; 3 and 6 mM), sodium azide (NaN_3 ; 5 and 10 mM), or hydrogen peroxide (H_2O_2 ; 5 and 10 mM) and incubated at 37°C for 30 min. After incubation, the enzyme activity in each sample was assayed as described above.

Effect of pH on SOD activity. To determine the optimal pH value for the SOD activity, the enzyme activity was measured by standard assay method, with 50 mM sodium phosphate buffers (pH 6.0 to 7.0), 50 mM Tris-HCl buffers (pH 8.0 to 9.0), or 50 mM glycine-NaOH buffers (pH 10.0 to 11.0) instead of standard buffer. Following 30 min of incubation at 37°C, the SOD activity was measured and compared.

N-terminal amino acid sequencing. The purified enzymes (10 µg) were subjected to SDS-10% polyacrylamide gel electrophoresis and then electroblotted onto polyvinylidene difluoride membrane as described previously (39). Blots were briefly stained with Coomassie brilliant blue and destained, and the stained protein was excised and subjected to N-terminal amino acid sequencing with a MilliGen/Bioscience 6600 Prosequence system (Millipore, Bedford, Mass.).

Western blot analysis. After SDS-PAGE, the proteins were electrotransferred from the gel to nitrocellulose membrane (0.45 µm; Bio-Rad) by the method of Towbin et al. (60). After transfer, the membrane was blocked in PBST (0.05% Tween 20 in PBS) containing 3% skim milk for 1 h at room temperature and then incubated with diluted (1:1,000) sera of bovine or human subjects with fascioliasis, paragonimiasis, and clonorchiasis or sera from healthy sera controls for 2 h at room temperature. After being washed three times with PBST, the membrane was incubated with peroxidase-conjugated anti-human immunoglobulin G (IgG) or peroxidase-conjugated anti-bovine IgG (Sigma) for 2 h at room temperature. After an additional three washes with PBST, the membrane was incubated in a freshly prepared mixture of substrate solution (4 mg of 3,3'-diaminobenzidine per ml, 0.01% of hydrogen peroxide in 0.1 M PBS [pH 7.2]) for 10 min at room temperature. The reaction was stopped by washing the membrane with distilled water several times. Bovine serum samples were collected from *F. hepatica*-positive and parasite-free bovines in a slaughterhouse in order to get the positive and negative controls. Human *F. hepatica*-positive sera were collected from three fascioliasis patients.

mRNA purification. *F. hepatica* adult worms were homogenized in guanidium thiocyanate and layered on a CsCl step gradient, and total RNA was extracted by a method described previously (12). mRNA was selected by oligo(dT) chromatography (23).

Construction of cDNA library. cDNA library of *F. hepatica* was constructed using the Librarian Express cDNA library kit (Invitrogen, San Diego, Calif.). The pcDNA3.1+ vector was linearized with *Bst*XI and *Not*I enzymes. The first strand

of cDNA was synthesized with avian myeloblastosis virus reverse transcriptase from mRNA using an oligo(dT) primer that contained a *Not*I restriction site. After the second strand of cDNA was synthesized, *Bst*XI/*Eco*RI adapters were ligated to the double-stranded cDNA. This cDNA was then trimmed with *Not*I enzyme to produce cDNA with *Bst*XI-*Not*I ends. All of this cDNA was then run out on a low-melt agarose gel with molecular weight markers. The cDNA with a size of >500 bp was cut out from the gel and recovered from the agarose. This size-enriched cDNA was then ligated into the linearized pcDNA3.1+ plasmid. The ligated plasmid-cDNA was then transformed into *E. coli* TOP10F' (Invitrogen) and amplified by overnight growth on ampicillin plates. These plates were scraped and pooled into a glycerol stock that was aliquoted, and stored at -70°C. The number of primary recombinants in the library was determined to be 1.38×10^6 by serial dilution of the unamplified library. Restriction analysis of 10 clones showed that 10 out of 10 contained inserts. The inserts had the sizes listed above, and the average size of the 10 clones was 0.74 kb.

PCR. Two degenerate oligonucleotide primers were designed based on conserved amino acids of copper/zinc-containing (Cu/Zn-SODs) from various eukaryotic organisms. The sequence of the forward primer (primer 1) was 5'-GC(T/G)GG(A/T)(G/C)C(T/G)CATTT(T/C)AATCC-3', and the sequence of the reverse primer (primer 2) was 5'-CC(A/G)CA(A/T)GC(A/T)A(A/C)ACGA(G/C)(G/C)ACCAGCATT-3'. Using the two primers, PCR analysis was performed on an *F. hepatica* cDNA library to check the presence of SOD cDNA sequence. Two microliters of *F. hepatica* library was heated at 90°C for 5 min to denature the cell and was used as the DNA template in the PCR. A 50-µl volume of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl_2 , a 10 µM concentration of each deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany), and 20 pmol of each primer was subjected to 45 cycles of amplification with a Thermal Cycler (model 480; Perkin Elmer, Foster City, Calif.). Each step was done at 94°C for 1 min in denaturation, 50°C for 2 min in annealing, and 72°C for 2 min in extension. The amplified product was purified from the gel, ligated into pCR2.1 vector, and transformed into competent *E. coli* INV α F' cells, using an Original TA cloning kit (Invitrogen).

Screening of *F. hepatica* cDNA library. The cDNA library was screened by a standard colony hybridization assay using the PCR product amplified with degenerate oligonucleotide primers as a probe. The probe was labeled to a specific activity of $>10^9$ cpm/µg with [α - ^{32}P]dCTP (Amersham, Arlington Heights, Ill.) by random priming method (16). A total of 10^6 CFU of *F. hepatica* cDNA library was plated, and the cells were transferred onto a nylon membrane (0.2-µm pore size; Amersham). The transferred cells were hybridized with probe in a standard buffer solution at 50°C for 16 h. Membranes were washed two times for 15 min each time with a solution containing $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% (wt/vol) SDS at 68°C and washed twice for 15 min each time with maleic acid buffer solution after being subject to reaction with $1 \times$ blocking solution for 30 min. After that, additional washings were carried out for 20 min each in $2 \times$, $1 \times$, and $0.5 \times \text{SSC}$ solution. Ten positive clones were identified, and two of them that yielded larger sizes were subjected to a second round of screening to isolate larger cDNAs. Finally, one *F. hepatica* Cu/Zn-SOD cDNA was identified and sequenced. The cDNA contained the complete coding region and 5' and 3' untranslated regions.

DNA sequencing and sequence analysis. The nucleotide sequence of the cloned gene was determined by the dideoxynucleotide chain termination method (53) using the Sequenase version 2.0 DNA sequencing kit (Amersham) and also by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Beaconsfield, United Kingdom), following the manufacturer's instruction.

Northern blot analysis. Total RNA was purified from adult worms of *F. hepatica* by the method described earlier. Northern blot analysis was performed by standard procedure (51). The purified RNA (10 µg) was separated on a 1% agarose gel containing 0.67 M formaldehyde and transferred onto a Hybond-N nylon membrane (Amersham). The membrane was prehybridized and hybridized using an *F. hepatica* SOD cDNA labeled with ^{32}P by random-priming method as a probe (17).

Expression and purification of the fusion protein. *F. hepatica* SOD protein was expressed in *E. coli* with prokaryotic expression vector pGEX-4T-2 (Pharmacia), which contains an isopropyl- β -D-thiogalactoside (IPTG)-inducible *tac* promoter, the T5 promoter transcription-translation system and a glutathione *S*-transferase (GST) coding sequence. The whole coding region of *F. hepatica* Cu/Zn-SOD cDNA was amplified by PCR, using forward primer CP1 (5'-GGATCCATGTCGGGTTCAGTGGC-3'), which contains a *Bam*HI site upstream of the start codon, and reverse primer CP2, (5'-GAATTCCTATTCCGTCAGACCAATTA-3'), which contains an *Eco*RI site downstream of the stop codon. The CP1 and CP2 primers were designed such that the ATG and TAA of the amplified product would be in frame with the GST. The PCR product was purified, ligated into pCR2.1 vector, and transformed into competent *E. coli* INV α F' (Invitrogen) again. After purification of plasmid DNA, the nucleotide sequence of insert was confirmed by sequencing. The plasmid DNA was digested with *Bam*HI and *Eco*RI and ligated to pGEX-4T-2 vector predigested with the same enzymes using standard techniques (51). The resulting plasmid (named pGEX/FhSOD) was transformed into competent *E. coli* BL-21 cells (Pharmacia) and spread on Luria-Bertani agar plates containing 100 µg of ampicillin per ml. The expression of fusion protein was performed by adding IPTG to a final concentration of 1

FIG. 1. Purification profiles of SOD from *F. hepatica*. (A) DEAE-Sephacel ion-exchange chromatography; (B) CM Sepharose Fast Flow ion-exchange chromatography; (C) Superose 12 molecular sieve chromatography; (D) Mono-Q ion-exchange chromatography. $\blacktriangle$, SOD activity; $\circ$, protein concentration. The straight line represents a 0 to 0.5 M NaCl gradient. For detailed explanations, see Materials and Methods. ABS, absorbance.

mM, and the fusion protein was purified with a glutathione-Sepharose 4B column (Pharmacia). To cleave the fusion protein from the GST carrier, the protein was incubated with thrombin (Sigma), 1:500 (wt/wt), in cleavage buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2.5 mM CaCl_2 , 0.1% β -mercaptoethanol) for 4 h at room temperature.

Nucleotide sequence accession number. *F. hepatica* Cu/Zn-SOD cDNA has been assigned EMBL/GenBank accession number AF071229.

RESULTS

Purification of SOD. When extract of *F. hepatica* was subjected to DEAE-Sephacel ion-exchange chromatography, one SOD-active peak was detected (Fig. 1A). These fractions were collected, desalted, and concentrated. The enzyme was purified further by successive CM Sepharose Fast Flow ion-exchange chromatography, Superose 12 molecular-sieve chromatography, and Mono-Q ion-exchange chromatography (Fig. 1B, C, and D). The purified enzyme was found to have a molecular mass of approximately 17.5 kDa on SDS-PAGE (Fig. 2). The native molecular mass of the enzyme was 34 kDa when estimated by Superose 12 molecular-sieve chromatography (data not shown). This indicates that the enzyme has dimeric structure consisted of two identical subunits. Table 1 summarizes the purification of the SOD. SOD was purified to a specific activity of about 727.1 U/mg protein, with a yield of 14.6%.

Characterization of the *F. hepatica* SOD. To determine the metal cofactor of the *F. hepatica* SOD, studies on the inhibitors of SODs containing various cofactors (Cu/Zn, Mn, or Fe) were performed. It was inhibited by KCN and H_2O_2 , which are both known to inhibit Cu/Zn-SOD, but not by NaN_3 (Table 2). This suggests that *F. hepatica* SOD was Cu/Zn-SOD. The pH profile of the SOD was determined. It showed activity over a broad pH range of 7.0 to 11.0, and the maximum level of activity was

FIG. 2. SDS-PAGE analysis of purified *F. hepatica* Cu/Zn-SOD. Lane M, molecular weight marker proteins; lane 1, purified *F. hepatica* Cu/Zn-SOD.

TABLE 1. Purification scheme of *F. hepatica* SOD

Step	Total protein (mg)	Total activity (U) ^a	Specific activity (U/mg) ^a	Purification (fold)	Yield (%)
Crude extract	920.0	5,980.0	6.5	1.0	100.0
DEAE-Sephacel ^b	349.8	3,818.2	10.9	1.7	63.8
CM Sepharose ^c	100.8	2,666.4	26.5	4.1	44.6
Superose 12 ^d	13.5	1,790.0	132.6	20.4	29.9
Mono-Q ^e	1.2	872.5	727.1	111.9	14.6

^a One unit is defined as the amount of SOD needed to cause a 50% inhibition in the rate of oxidation of neotetrazolium chloride at 37°C.

^b DEAE-Sephacel ion-exchange chromatography.

^c CM Sepharose Fast Flow ion-exchange chromatography.

^d Superose 12 molecular-sieve chromatography.

^e Mono-Q ion-exchange chromatography.

at pH 7.5 (data not shown). The activities below pH 7.0 were not assayed, because the xanthine oxidase is inactive below a pH of 6.5. The N-terminal amino acid sequence of the first 12 residues of the enzyme was Met-Ser-Gly-Ser-Ser-Gly-Val-Gln-Gly-Thr-Val-Lys. The purified enzyme reacted with sera from bovine and human subjects with fascioliasis but not with sera from healthy controls (Fig. 3).

Cloning and characterization of *F. hepatica* Cu/Zn-SOD cDNA. A comparison of the amino acid sequences of known Cu/Zn-SODs from various organisms revealed certain regions of the protein that are strongly conserved (4, 21, 24, 25, 29). By using these regions, we designed two degenerate oligonucleotide primers. PCR amplification of an aliquot of the *F. hepatica* cDNA library using these primers yielded a product with the expected size of 264 bp. Sequence analysis confirmed that the 264-bp fragment contained nucleotide and deduced amino acid sequence homologies to the Cu/Zn-SODs of the other organisms. To obtain full-length cDNA clones, we used the 264-bp product as a probe for screening of *F. hepatica* cDNA library. The primary screening of the cDNA library yielded 10 hybridizing colonies that consisted of mixed populations of full-size and truncated colonies. Two of the 10 colonies that yielded larger sizes were used as a probe in a second round of screening to isolate larger cDNAs. Finally, one *F. hepatica* cDNA was selected and sequenced. It was 600 bp and contained the complete open reading frame of 438 bp with 5' and 3' nontranslated regions (Fig. 4). The 3' nontranslated region included a poly(A) tail and possessed the modified 5-TACTGAAA-3 conserved octamer-like sequence present in the 3' nontranslated regions of the *O. volvulus* and *S. mansoni* Cu/Zn-SOD cDNAs at positions 490 to 497 (5'-TACTGTAA-3'). In Northern blot hybridization analysis, a strong hybridized signal of around 600 bp was identified (Fig. 5). This size is consistent with that expected based upon the size of the *F. hepatica* Cu/Zn-SOD gene.

TABLE 2. Sensitivity of the *F. hepatica* SOD to various inhibitors

Inhibitor and concn	Inhibition rate (%)
KCN	
3 mM.....	53.3
6 mM.....	98.2
NaN ₃	
5 mM.....	0
10 mM.....	2.2
H ₂ O ₂	
3 mM.....	34.7
6 mM.....	51.4

FIG. 3. Western blot analysis of the purified enzyme with sera collected from bovine and human subjects with fascioliasis. Lanes 1 to 3, sera from bovines with fascioliasis; lanes 4 to 6, sera from healthy bovine; lanes 7 to 10, sera from humans with fascioliasis; lanes 11 to 14, sera from healthy humans.

Analysis of the deduced amino acid sequence of *F. hepatica* Cu/Zn-SOD cDNA. The complete *F. hepatica* Cu/Zn-SOD cDNA encodes a 146-amino-acid protein with a predicted molecular mass of 17,592 Da. As shown in Fig. 4, the amino acid residues known to be responsible for binding to copper/zinc (His-39, His-41, His-56, His-64, His-73, His-113, and Asp-76) were present in the sequence (4, 19, 55). The arginine residue (Arg-136) which is believed to be necessary to guide the superoxide anion to the active site (4, 19) was also present. Therefore, all of the Cu/Zn binding sites and active sites were conserved. The two cysteine residues (Cys-50 and Cys-139) which are believed to form a disulfide bond were present. Residues involved in Cu/Zn-SOD dimer formation (Gly-30, Leu-31, Gly-34, His-36, Arg-72, Gly-78, Ile-106, Leu-137, and Val-141) were also conserved (14). It had a putative N-glycosylation site at Phe-59. When the deduced amino acid sequence of the *F. hepatica* Cu/Zn-SOD was aligned with Cu/Zn-SODs of the other parasites for sequence homology comparison, a significant amount of sequence identity was found (Fig. 6). When gaps were used in all of the sequences to improve the alignments, the homologies were 64.8% for the *F. hepatica* and *S. mansoni* Cu/Zn-SODs, 51.6% for the *F. hepatica* and *B. pahangi* Cu/Zn-SODs, and 48.4% for the *F. hepatica* and *O. volvulus* Cu/Zn-SODs.

Expression and purification of *F. hepatica* Cu/Zn-SOD. The expression of the cloned *F. hepatica* Cu/Zn-SOD gene was induced by adding IPTG and analyzed on SDS-PAGE followed by Coomassie blue staining. The size of the expressed protein was approximately 43 kDa, which corresponded with the predicted molecular weight of the protein, with 26 kDa being from the GST and 17.6 kDa being from *F. hepatica* Cu/Zn-SOD, as predicted from the cDNA (Fig. 7). *F. hepatica* Cu/Zn-SOD obtained from cleavage of the fusion protein with thrombin did run as 17.5 kDa. A nondenaturing PAGE analysis of the protein showed a SOD activity. The SOD activity was sensitive to KCN and H₂O₂ but resistant to NaN₃, which is indicative of Cu/Zn-SOD (Fig. 8). The expressed protein reacted strongly to sera from bovine and human subjects with fascioliasis, but not to sera from uninfected subjects (Fig. 9). These suggested that the expressed protein might be a useful antigen for the diagnosis of fascioliasis. However, the expressed *F. hepatica* Cu/Zn-SOD showed cross reactivities with sera from human paragonimiasis and clonorchiasis patients. These may be due to high homologies observed in that Cu/Zn-SODs. Therefore, before *F. hepatica* Cu/Zn-SOD is used for diagnostic purposes in connection with fascioliasis, more specific monoclonal antibodies against the *F. hepatica* Cu/Zn-SOD should be made.

						M	S	G	S	S	G	V	Q	G	T	V	K	
1																		12
1	A	GCT	ATT	TGC	GTA	<u>ATG</u>	<u>TCG</u>	<u>GGT</u>	<u>TCC</u>	<u>AGT</u>	<u>GGC</u>	GTG	CAA	GGA	ACT	GTC	AAG	49
						CP1												
13	F	V	Q	E	S	E	T	S	P	V	Q	I	K	V	D	I	N	29
50	TTC	GTT	CAA	GAG	TCT	GAG	ACG	TCC	CCA	GTG	CAA	ATA	AAG	GTT	GAC	ATC	AAT	100
30	G	L	K	P	G	K	H	G	F	H	V	H	A	Y	G	D	T	46
101	GGC	CTT	AAG	CCG	GGT	AAG	CAC	GGC	TTC	CAT	GTT	CAT	GCT	TAT	GGA	GAT	ACA	151
47	T	N	G	C	I	S	A	G	P	H	F	N	P	T	G	V	D	63
152	ACG	AAT	GGT	TGT	ATA	TCC	<u>GCG</u>	<u>GGA</u>	<u>CCT</u>	<u>CAT</u>	<u>TTC</u>	<u>AAC</u>	<u>CCA</u>	ACC	GGT	GTG	GAT	202
						Primer 1												
64	H	G	G	P	S	D	S	V	R	H	V	G	D	L	G	N	V	80
203	CAT	GGA	GGT	CCA	AGC	GAT	AGT	GTG	CGT	CAT	GTT	GGC	GAT	CTG	GGA	AAT	GTT	253
81	E	A	N	Q	N	G	L	A	H	V	E	F	T	D	S	V	I	97
254	GAG	GCA	AAT	CAA	AAC	GGA	CTC	GCC	CAC	GTC	GAA	TTT	ACA	GAT	AGT	GTC	ATT	304
98	S	L	S	G	V	N	S	V	I	G	R	A	M	V	V	H	E	114
305	TCT	CTC	TCT	GGA	GTG	AAC	TCA	GTT	ATT	GGC	AGA	GCC	ATG	GTT	GTA	CAT	GAA	355
115	N	E	D	D	L	G	R	G	G	H	E	Q	S	K	I	T	G	131
356	AAT	GAG	GAC	GAC	CTC	GGT	CGC	GGA	GGA	CAC	GAG	CAG	AGT	AAA	ATA	ACC	GGC	406
132	N	A	G	G	R	L	A	C	G	V	I	G	L	T	E	*		147
407	<u>AAC</u>	<u>GCT</u>	<u>GGA</u>	<u>GGC</u>	<u>CGT</u>	<u>TTG</u>	<u>GCT</u>	<u>TGT</u>	<u>GGT</u>	<u>GTA</u>	<u>ATT</u>	<u>GGT</u>	<u>CTG</u>	<u>ACG</u>	<u>GAA</u>	<u>TAA</u>	TTA	457
	Primer 2												CP2					
458	GTC	GGT	CAA	CTT	TTC	ACG	TGT	TTA	GTT	TAA	TTT	ACT	GTA	AGA	CAA	ATA	TCG	508
509	TTG	ACT	CAT	TAA	ATT	TTG	ACT	CTC	TTC	CCA	AAT	GCT	CAT	TGG	GTA	CAA	TAC	559
560	ATA	TTT	TCT	CTA	CTT	GCT	CAA	AAA	AAA	AAA	AAA	AAA	AAA	AA				600

FIG. 4. Nucleotide and deduced amino acid sequences of *F. hepatica* Cu/Zn-SOD cDNA. Conserved amino acid residues for Cu and Zn binding and active site formation are shown in boldface. The positions of the primers used in this study are highlighted by underlining. Primers 1 and 2 were used to amplify the 264-bp PCR product employed for screening the cDNA library. Primers CP1 and CP2 were used for construction of the expression plasmid. The boxed amino acid residues indicate the N-terminal amino acid sequence of the purified enzyme, as determined by protein sequencing.

DISCUSSION

Parasites have been known to develop several possible mechanisms to circumvent the immune responses of the hosts

FIG. 5. Northern blot analysis of *F. hepatica* Cu/Zn-SOD mRNA transcripts. A 10- μ g quantity of poly(A)⁺ mRNA was electrophoresed on a 1.2% formaldehyde-agarose gel, transferred to nylon membrane and hybridized with a ³²P-labeled probe. The size of the hybridizing mRNA was about 600 kb. The positions of RNA size markers are indicated on the left.

during invasion and maintenance. One possible mechanism of the immune evasion could be the expression of antioxidant enzymes that would suppress oxidative killing by the host effector cells (9). Prominent among the antioxidant enzymes are SODs that are believed to play a major role in the antioxidant system of the parasites. Studies which demonstrate the presence of SOD activity in many parasites support the hypothesis that parasites use SOD not only in its usual function but also as a way of protecting themselves against the cellular, oxygen-mediated killing mechanisms of the hosts (10, 21, 28, 41, 52, 57).

In this study, we have characterized biochemical properties of a purified SOD from *F. hepatica* adult worms and cloned and expressed its gene as a prerequisite for further investigations of its possible pathophysiological role in *F. hepatica* pathogenesis. The enzymatic and sequence analysis of the purified *F. hepatica* SOD revealed characteristics and a structure similar to that of cytosolic Cu/Zn-SODs from the other species (10, 21, 25, 26, 27, 32, 41, 50, 56). It reacted with sera from bovine and human subjects with fascioliasis but not with sera from healthy controls. Recently, it has been reported that two forms of Cu/Zn-SODs were detected in *F. hepatica* (48). One

FIG. 6. Alignment of the deduced amino acid sequence of Cu/Zn-SOD of *F. hepatica* with those of other known Cu/Zn-SODs. Gaps are introduced to maximize alignment. FhSOD, *F. hepatica* Cu/Zn-SOD (in this study); SMSOD, *S. mansoni* Cu/Zn-SOD (25); BPSOD, *B. pahangi* Cu/Zn-SOD (56); OVSOD, *O. volvulus* Cu/Zn-SOD (20); HUMANSOD, human Cu/Zn-SOD (54).

is a 60-kDa extracellular SOD found in in vitro culture medium of *F. hepatica* adult worms and the other is a 32-kDa cytosolic SOD detected in a detergent-extractable fraction of the parasite. Although the enzyme that we describe here showed enzymatic characteristics similar to those of the 32-kDa cytosolic SOD, it seems unlikely that the enzyme is homologous to the 32-kDa SOD, since their molecular weights and N-terminal amino acid sequences were not matched. Therefore, it is possible to postulate that the two enzymes are isoenzymes of *F. hepatica*.

The N-terminal amino acid sequence and molecular weight of the purified native *F. hepatica* Cu/Zn-SOD correspond exactly to those of the deduced amino acid sequence, demonstrating the homogeneity of the purified enzyme and indicating that no maturation or modification of the N-terminal region is functionally necessary.

It is known that there are two forms of Cu/Zn-SODs, extracellular and cytosolic SOD, in helminth parasites which are analogous to the human SODs (21, 25, 26, 29, 48). Since superoxide anions are not able to penetrate biological membranes (36), it seems likely that extracellular SOD is more effective as a defense mechanism of parasites against oxidative killing of hosts than cytosolic SOD. Therefore, the hypothesis

that extracellular SOD plays a more important protection role against oxidative killing of hosts than cytosolic SOD is acceptable. However, if the cytosolic SOD is located with surface-associated form, it also provides sufficient protection against extracellular superoxide toxicity. The purified and cloned *F. hepatica* Cu/Zn-SOD in this study was thought to be a cytosolic enzyme when it was determined that its N-terminal amino acid sequence did not include a signal peptide sequence. However, the fact that the cloned gene contained a potential site for N-linked glycosylation enhances the possibility that the enzyme may locate in surface-associated form. However, to more precisely define the physiological role of the enzyme, more studies on localization of the enzyme should be performed.

The whole open reading frame region of *F. hepatica* Cu/Zn-SOD was cloned and expressed in *E. coli*. As a result, we were able to produce a large quantity of a highly purified, enzymatically active protein that behaves essentially the same as the native enzyme purified from *F. hepatica*. It also showed strong antigenicity against the sera from bovine and human subjects with fascioliasis but not against sera from healthy controls. On the basis of these results, the enzyme will be useful for various further studies. First, it will facilitate functional and structural studies of the protein, including its crystallization. Second, it will be used for the design of the drugs that can specifically inhibit the parasite enzyme. SODs of parasites have been identified as attractive targets for chemotherapy (1, 61). Since the

FIG. 7. Expression and purification of the GST-*F. hepatica* SOD (FhSOD) fusion protein. Lane M, molecular weight marker proteins; lane 1, uninduced *E. coli* lysate; lane 2, IPTG-induced *E. coli* lysate; lane 3, purified GST-FhSOD fusion protein; lane 4, GST-FhSOD fusion protein after treatment of thrombin; lane 5, purified FhSOD.

FIG. 8. Activity staining of expressed *F. hepatica* SOD. Lane 1, purified SOD only; lane 2, with 3 mM KCN; lane 3, with 6 mM KCN; lane 4, with 5 mM NaN_3 ; lane 5, with 10 mM NaN_3 ; lane 6, with 5 mM H_2O_2 ; lane 7, with 10 mM H_2O_2 .

FIG. 9. Western blot analysis of the expressed protein with sera of bovine and human subjects with fascioliasis. Lanes 1 to 3, sera from bovines with fascioliasis; lanes 4 to 6, sera of healthy bovines; lanes 7 to 10, sera from humans with fascioliasis; lanes 11 to 13, sera from humans with paragonimiasis; lanes 14 to 16, sera from humans with clonorchiasis; lanes 17 to 20, sera of healthy humans.

effective dismutation of the superoxide anion is important to the survival of the parasites, inhibition of parasite SODs would very likely lead to the parasites being more susceptible to oxidative killing by hosts. Indeed, many chemotherapeutic drugs for various parasites are based on their effects through free-radical-mediated mechanisms (13). Therefore, comparison of the inhibition characteristics of *F. hepatica* Cu/Zn-SOD and mammalian SODs may ultimately lead to the design of drugs for treatment of fascioliasis. Finally, that parasite SODs are recognized as antigens by infected hosts and, more notably, SODs in administered vaccines that have an immunoprotective role are ideas that have been described earlier (8, 30, 47). Furthermore, vaccination of mice with *Brucella abortus* SOD induced a significant level of protection against virulent *B. abortus* infection and *Norcardia asteroides* with an antibody to the extracellular SOD increased its susceptibility to killing by leukocytes (5, 46). Such observations indicate that *F. hepatica* Cu/Zn-SOD may also be an attractive vaccine candidate and target for immunodiagnosis of fascioliasis.

ACKNOWLEDGMENTS

This work was supported by the 2-year Basic Medical Research grant 1997-021-F0020 (1997 to 1999) from the Ministry of Education of the Republic of Korea.

We are deeply grateful to Myung-Kee Hwang for his technical assistance and to Young-Hwan Chung for collecting experimental materials in the public butcheries of Kangwon province.

REFERENCES

- Asada, K., S. Kanematsu, S. Okaka, and T. Hayakawa. 1980. Phylogenetic distribution of three types of superoxide dismutase in organisms and in cell organelles, p. 136–153. In J. V. Bannister and H. Hill (ed.), *Chemical and biochemical aspects of superoxide and superoxide dismutases*. Elsevier/North-Holland, New York, N.Y.
- Ashton, W. L. G., P. L. Boardman, C. J. D'Sa, P. H. Everall, and A. W. J. Houghton. 1970. Human fascioliasis in Shropshire. *Br. Med. J.* **3**:500–502.
- Badwey, J. A., and M. L. Karnovsky. 1980. Active oxygen species and the functions of phagocytic leukocytes. *Annu. Rev. Biochem.* **49**:695–726.
- Bannister, J. V., W. H. Bannister, and G. Rottilio. 1987. Aspects of the structure, function and application of superoxide dismutases. *Crit. Rev. Biochem.* **22**:111–180.
- Beaman, L. V., and B. I. Beaman. 1990. Monoclonal antibodies demonstrate that superoxide dismutase contributes to protection of *Norcardia asteroides* within the intact host. *Infect. Immun.* **58**:3122–3128.
- Beauchamp, C. O., and I. Fridovich. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**:276–287.
- Beyer, W., J. Imlay, and I. Fridovich. 1991. Superoxide dismutases. *Prog. Nucleic Acids Res. Mol. Biol.* **40**:221–253.
- Bruchhaus, I., N. W. Brattig, and E. Tannich. 1992. Recombinant expression, purification and biochemical characterization of a superoxide dismutase from *Entamoeba histolytica*. *Arch. Med. Res.* **23**:27–29.
- Callahan, H. L., R. K. Crouch, and E. R. James. 1988. Helminth anti-oxidant enzymes: a protective mechanism against host oxidants? *Parasitol. Today* **4**:218–225.
- Callahan, H. L., R. K. Crouch, and E. R. James. 1991. *Dirofilaria immitis* superoxide dismutase: purification and characterization. *Mol. Biochem. Parasitol.* **49**:245–252.
- Chauvin, A., E. Moreau, and C. Boulard. 1997. Diagnosis of bovine fascioliasis using serology of pools of sera. *Vet. Res.* **28**:37–43.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299.
- Decampo, R., and S. N. J. Moreno. 1986. Free radical metabolism of antiparasitic agents. *Fed. Proc.* **45**:2471–2476.
- Deng, H.-X., A. Hentati, J. A. Tainer, Z. Iqbal, A. Cayabyab, W.-Y. Hung, E. D. Getzoff, P. Hu, B. Herzfeldt, R. P. Ross, C. Warner, G. Deng, E. Soriano, C. Smyth, H. E. Parge, A. Ahmed, A. D. Roses, R. A. Hallowell, M. A. Pericak-Vance, and T. Siddique. 1993. Amyotrophic lateral sclerosis and structural defects in Cu, Zn superoxide dismutase. *Science* **261**:1047–1051.
- el-Shabrawi, M., H. el-Karaksy, S. Okasha, A. el-Hennacoy. 1997. Human fascioliasis: clinical features and diagnostic difficulties in Egyptian children. *J. Trop. Pediatr.* **43**:162–166.
- Fairfield, A. S., A. Abosch, A. Ranz, J. W. Eaton, and S. R. Meshnick. 1988. Oxidant defense enzymes of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **30**:77–82.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6–13.
- Fridovich, I. 1983. Superoxide radical: an endogenous toxicant. *Annu. Rev. Toxicol.* **23**:239–257.
- Fridovich, I. 1986. Superoxide dismutases. *Adv. Enzymol.* **58**:61–97.
- Getzoff, E. D., J. A. Tainer, P. K. Weiner, P. A. Kollman, J. S. Richardson, and D. C. Richardson. 1983. Electrostatic recognition between superoxide and copper, zinc superoxide dismutase. *Nature (London)* **306**:287–290.
- Henkle, K. J., E. Liebau, S. Müller, B. Bergmann, and R. D. Walter. 1991. Characterization and molecular cloning of a Cu/Zn superoxide dismutase from the human parasite *Onchocerca volvulus*. *Infect. Immun.* **59**:2063–2069.
- Henkle-Duhrsen, K., W. Tawe, C. Warnecko, and R. D. Walter. 1995. Characterization of the manganese superoxide dismutase cDNA and gene from the human parasite *Onchocerca volvulus*. *Biochem. J.* **308**:441–446.
- Henkle-Duhrsen, K., R. S. Tuan, G. Wildenberg, M. L. Eschbach, W. Tawe, P. Zipfel, and R. D. Walter. 1997. Localization and functional analysis of the cytosolic and extracellular Cu/Zn superoxide dismutases in the human parasitic nematode *Onchocerca volvulus*. *Mol. Biochem. Parasitol.* **88**:187–202.
- Heussler, V. T., M. Eichhorn, and D. A. Dobbelaere. 1992. Cloning of a full-length cDNA encoding bovine interleukin 4 by the polymerase chain reaction. *Gene* **114**:273–278.
- Hong, Z., D. J. Kosman, A. Thakur, D. Rekosh, and P. T. Loverde. 1992. Identification and purification of a second form of Cu/Zn superoxide dismutase from *Schistosoma mansoni*. *Infect. Immun.* **60**:3641–3651.
- Hong, Z., P. T. Loverde, M. L. Hammarskjöld, and D. Rekosh. 1992. *Schistosoma mansoni*: cloning of a complementary DNA encoding a cytosolic Cu/Zn superoxide dismutase and high-yield expression of the enzymatically active gene product in *Escherichia coli*. *Exp. Parasitol.* **75**:308–322.
- Hong, Z., P. T. Loverde, A. Thakur, M. L. Hammarskjöld, and D. Rekosh. 1993. *Schistosoma mansoni*: a Cu/Zn superoxide dismutase is glycosylated when expressed in mammalian cells and localized to a subtegumental region in adult schistosomes. *Exp. Parasitol.* **76**:101–114.
- Ismail, S. O., Y. A. W. Skeiky, A. Bhatia, L. A. Omara-Opyene, and L. Gedamu. 1994. Molecular cloning, characterization, and expression in *Escherichia coli* of iron superoxide dismutase cDNA from *Leishmania donovani*. *Infect. Immun.* **62**:657–664.
- James, E. R., D. C. McLean, Jr., F. Perler. 1994. Molecular cloning of an

- Onchocerca volvulus* extracellular Cu-Zn superoxide dismutase. Infect. Immun. **62**:713–716.
30. Lattemann, C. T., Z. X. Yan, A. Matzen, T. F. Meyer, and H. Apfel. 1999. Immunogenicity of the extracellular copper/zinc superoxide dismutase of the filarial parasite *Acanthocheilichonema viteae* delivered by a two-phase vaccine strain of *Salmonella typhimurium*. Parasite Immunol. **21**:219–224.
 31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
 32. Leid, R. W., and C. M. Suquet. 1986. A superoxide dismutase of metacystodes of *Taenia taeniaeformis*. Mol. Biochem. Parasitol. **18**:301–311.
 33. Leid, R. W., C. M. Suquet, and L. Tanigoshi. 1989. Oxygen detoxifying enzymes in parasites: a review. Acta Leiden **57**:107–114.
 34. Lenton, L. M., F. L. Bygrave, C. A. Behm, and J. C. Boray. 1996. *Fasciola hepatica* infection in sheep: changes in liver metabolism. Res. Vet. Sci. **61**:152–156.
 35. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Foline phenol reagent. J. Biol. Chem. **193**:265–275.
 36. Lynch, R. E., and I. Fridovich. 1978. Permeation of the erythrocyte stroma by superoxide radical. J. Biol. Chem. **253**:4696–4699.
 37. Maizels, R. A., D. A. P. Bundy, M. E. Selkirk, D. F. Smith, and R. M. Anderson. 1993. Immunological modulation and evasion by helminth parasites in human populations. Nature (London) **365**:797–805.
 38. Mas-Coma, M. S., J. G. Esteban, and M. D. Bargues. 1999. Epidemiology of human fascioliasis: a review and proposed new classification. Bull. W. H. O. **77**:340–346.
 39. Mastudaira, P. 1987. Sequence from picomole quantities of proteins electrophoretically onto polyvinylidene difluoride membranes. J. Biol. Chem. **193**: 265–275.
 40. Mei, H., and P. T. LoVerde. 1997. *Schistosoma mansoni*: the developmental regulation and immunolocalization of antioxidant enzymes. Exp. Parasitol. **86**:69–78.
 41. Michalski, W. P., and S. J. Prowse. 1991. Superoxide dismutases in *Eimeria tenella*. Mol. Biochem. Parasitol. **47**:189–195.
 42. Murray, H. W. 1981. Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. J. Exp. Med. **153**:1302–1315.
 43. Nare, B., J. M. Smith, and R. K. Prichard. 1990. *Schistosoma mansoni*: levels of antioxidants and resistance to oxidants increase during development. Exp. Parasitol. **70**:389–397.
 44. Nathan, C., N. Nogueira, C. Juangbhanich, J. Ellis, and Z. A. Cohn. 1979. Activation of macrophages *in vivo* and *in vitro*. Correlation between hydrogen peroxide release and killing of *Trypanosoma cruzi*. J. Exp. Med. **149**: 1056–1068.
 45. Noridaka, O., M. Sinbu, T. Mastro, and O. Akila. 1982. Hyohininukem superoxide dismutase. Nipponkai **92**:583–590. (In Japanese.)
 46. Onate, A. A., R. Vemulapalli, E. Andrews, G. G. Schurig, S. Boyle, and H. Folch. 1999. Vaccination with live *Escherichia coli* expressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. Infect. Immun. **67**:986–988.
 47. Owhashi, M., F. Tomiyoshi, and H. Hayashi. 1994. Isolation and characterization of a neutrophil chemotactic factor from *Trichomonas foetus* organisms. Immunol. Cell Biol. **72**:249–255.
 48. Piacenza, L., R. Raid, F. Goni, and C. Carmona. 1998. CuZn superoxide dismutase activities from *Fasciola hepatica*. Parasitology **117**:555–562.
 49. Rangel-Ruiz, L. J., R. Marquez-Izquierdo, and G. Bravo-Nogueira. 1999. Bovine fascioliasis in Tabasco, Mexico. Vet. Parasitol. **81**:119–127.
 50. Rhoads, M. L. 1983. *Trichinella spiralis*: identification and purification of superoxide dismutase. Exp. Parasitol. **56**:41–54.
 51. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 52. Sanchez, M. M., M. Monteoliva, A. Fatou, and R. M. A. Garcia. 1988. Superoxide dismutase from *Ascaris suum*. Parasitology **97**:345–353.
 53. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
 54. Sherman, L., N. Dafni, J. Lieman-Hurwitz, and Y. Groner. 1983. Nucleotide sequence and expression of human chromosome 21-encoded superoxide dismutase mRNA. Proc. Natl. Acad. Sci. USA **80**:5465–5469.
 55. Tainer, J. A., E. D. Getzoff, J. S. Richardson, and D. C. Richardson. 1983. Structure and mechanism of copper, zinc superoxide dismutase. Nature (London) **306**:284–287.
 56. Tang, L., X. Ou, K. Henkle-Duhrsen, and M. E. Selkirk. 1994. Extracellular and cytoplasmic CuZn superoxide dismutases from *Brugia lymphatic* filarial nematode parasites. Infect. Immun. **62**:961–967.
 57. Tannich, E., I. Bruchhaus, R. D. Walter, and R. D. Horstman. 1991. Pathogenic and nonpathogenic *Entamoeba histolytica*: identification and molecular cloning of an iron-containing superoxide dismutase. Mol. Biochem. Parasitol. **49**:61–72.
 58. Tielens, A. G. M., J. M. van Den Heuvel, and S. G. van Den Bergh. 1982. Changes in energy metabolism of the juvenile *Fasciola hepatica* during its development in the liver parenchyma. Mol. Biochem. Parasitol. **6**:277–286.
 59. Tielens, A. G. M. 1994. Energy generation in parasitic helminthes. Parasitol. Today **10**:346–352.
 60. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
 61. Wilson, C. B., V. Tsai, and J. S. Remington. 1980. Failure to trigger the oxidative metabolic burst by normal macrophages: possible mechanism for survival of intracellular pathogens. J. Exp. Med. **151**:328–346.

Editor: W. A. Petri, Jr.