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Identification and Purification of a Second Form of Cu/Zn Superoxide Dismutase from *Schistosoma mansoni*

ZHI HONG,[1]† DANIEL J. KOSMAN,[1] ARVIND THAKUR,[2] DAVID REKOSH,[1,2,3] AND PHILIP T. LoVERDE[2]*

Departments of Biochemistry,[1] Microbiology,[2] and Oral Biology,[3] State University of New York at Buffalo, Buffalo, New York 14214

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Our laboratories previously isolated a putative extracellular or membrane-associated Cu/Zn superoxide dismutase (Cu/Zn-SOD) gene, designated a signal peptide-containing (SP) Cu/Zn-SOD, from *Schistosoma mansoni*. SOD activity was thus investigated throughout the life cycle of *S. mansoni* and found in all stages: eggs, miracidia, cercariae, schistosomula, lung-stage worms, and adult worms. The adult worms had the highest SOD activity (53 ± 9 nitrite units), which was five times higher than that of eggs or miracidia and twice as high as that of 3-h-old mechanically transformed schistosomula. Cu/Zn-SOD constituted over 95% of the total SOD activity found in *S. mansoni*, compared with that of Mn-SOD. Most of Cu/Zn-SOD specific activity was associated with a detergent-extractable fraction of the parasite. Isoelectric focusing gel electrophoresis analysis revealed that there were four major pI variants of Cu/Zn-SOD present in the adult worms. Only two of these Cu/Zn-SOD pI variants were present in the 3-h-old mechanically transformed schistosomula. Fast protein liquid chromatography gel filtration fractionation of adult parasite extract was carried out to correlate the SP Cu/Zn-SOD with the SOD activity by using anti-SP Cu/Zn-SOD monoclonal antibodies, which separated the immunoreactive gene product and the SOD activity into different fractions. Quantitative tissue fractionation also revealed a discordant distribution of the gene product compared with that of Cu/Zn-SOD activity. These results indicated the existence of another Cu/Zn-SOD(s) in the parasite. Purification of the Cu/Zn-SOD activity from the adult worms showed that it represented the two lower-pI variants found in both adult worms and 3-h-old schistosomula. Peptide sequence analysis of the purified Cu/Zn-SOD confirmed that there is a second form of Cu/Zn-SOD in the parasite.

Schistosoma mansoni, an intravascular human parasite, lives in a hostile environment in close contact with host humoral and cellular cytotoxic factors. To establish itself in the host, the parasite has evolved a number of immune evasion mechanisms (13, 53). Once established, the *S. mansoni* parasite develops a partial immunity, termed concomitant immunity, which consists of various immune components or mechanisms that allow extended survival of both host and parasite (10, 54).

The major effector mechanisms involve various types of leukocytes and macrophages potentiated by antibodies, cytokines, and/or complement (7, 48, 57). One of the killing mechanisms that might be used by the effector cells is the release of toxic oxygen species by the sensitized phagocytes and the subsequent oxidative damage to the parasites (1). One of the toxic oxygen species, $O_2 \cdot^-$, is produced by activated eosinophils, neutrophils, and macrophages through a respiratory burst involving a membrane-associated NADPH oxidase system (45). The toxicity of $O_2 \cdot^-$ radicals and its derivatives, such as H_2O_2, $\cdot OH$, and $^1O_2$ (singlet O_2), on schistosome parasites has been evaluated by in vitro studies (36–38). Mkoji et al. (37) found that the adult worms have much greater resistance (2% killed) to oxidative killing, while under the same conditions, most of the schistosomula (95%) are killed. The differences in the susceptibility to in vitro oxidative killing correlates with the differences in the levels of endogenous antioxidant enzymes. However, in vivo studies on oxidative killing of schistosomes are not available.

Superoxide dismutase (SOD), first characterized by McCord and Fridovich (32), is a ubiquitous metalloenzyme (EC 1.15.1.1) in aerobes. SOD enzymes have Cu and Zn (Cu/Zn-SOD), Mn (Mn-SOD), or Fe (Fe-SOD) at the active site. They protect the organism against the toxic effects of superoxide radicals by catalyzing its dismutation to molecular oxygen and hydrogen peroxide (19). Cu/Zn-SOD occurs primarily in the cytosol of eukaryotic cells and the chloroplasts of plants. This cytosolic enzyme has a dimer structure and is responsible for removing toxic superoxide radicals generated by a number of oxidative metabolisms. An extracellular form of Cu/Zn-SOD has been recently found in mammalian body fluids and also in some tissues (29, 30). This secretory enzyme is a tetrameric glycoprotein with a subunit molecular mass of 30 kDa (55). Both forms of Cu/Zn-SODs are related and are believed to derive from a common ancestor which is different from that of Mn-SOD and Fe-SOD (19).

Numerous Cu/Zn-SODs have been characterized and/or purified from various parasites of different species (9, 22, 26, 28, 35, 41, 44, 46, 49). For example, Cu/Zn-SOD is believed to be excreted by *Trichinella spiralis*, since its activity has been detected in the culture medium (44). A putative extracellular or membrane-associated Cu/Zn-SOD (signal peptide-containing [SP] Cu/Zn-SOD) gene has also been isolated from *S. mansoni*. Its gene product has a hydrophobic signal peptide and an N-linked glycosylation site (49). A malaria parasite, *Plasmodium berghei*, seems to have acquired host Cu/Zn-SOD as part of its own defense enzyme (16). The presence of various forms of Cu/Zn-SODs in helminth and

* Corresponding author.
† Present address: Molecular Genetics Department, Schering-Plough Research, Bloomfield, NJ 07003.

protozoan parasites suggests the need to study the importance of these enzymes in the detoxifying pathways against the toxic oxidants released by host phagocytes.

Schistosome SOD activity reported herein is shown to be developmentally regulated throughout the life cycle of schistosomes, from the egg stage to the adult worm stage. SOD activity in adult worms is found to be five times as high as that in eggs and miracidia and twice as high as that from young larvae such as cercariae and schistosomula. More interestingly, the SOD activity is in a detergent-extractable fraction that contains the outer membrane (tegument) components of the parasite. Over 95% of the activity is sensitive to cyanide, which is a potent inhibitor of Cu/Zn-SODs, indicating that Cu/Zn-SOD is the major species of the SOD enzymes to defend the parasite against toxic oxidants released by activated phagocytes.

The SP Cu/Zn-SOD gene isolated in our laboratory from *S. mansoni* exhibited properties such as its 20-kDa molecular size, hydrophobic amino terminus, and N-linked glycosylation site, suggesting its similarity to a human extracellular Cu/Zn-SOD (23, 49). Our attempt to link the SP Cu/Zn-SOD with the major SOD activity found in the Triton X-100 extract of adult parasites and 3-h-old schistosomula and further characterization of SP Cu/Zn-SOD gene product by different fractionation lead to the identification of another Cu/Zn-SOD.

MATERIALS AND METHODS

Parasites. *S. mansoni* NMRI (17) was maintained in the laboratory with *Biomphalaria glabrata* snails and golden hamsters. Schistosome eggs were isolated from the infected hamster liver by homogenization and filtration (21). Miracidia were collected from hatched eggs. Infectious cercariae released into spring water by infected snails after light activation were collected and mechanically transformed into schistosomula by passing the cercariae through an emulsifying needle connected to two syringes (4), then separating the cercarial bodies from their tails (27), and finally incubating the bodies in RPMI 1640 medium at 37°C for 3 h. Adult schistosomes were obtained by hepatic portal perfusion of hamsters 40 to 45 days postinfection (15). Immature parasites (15 to 20 days old) were collected by perfusion from either lung or liver tissue. All parasites were either used fresh or stored at −70°C.

Parasite extracts. Frozen parasites were thawed at room temperature and resuspended in 5 volumes of phosphate-buffered saline solution (PBS; pH 7.2). The parasites were then disrupted by sonication (Branson Sonifier 450) (three times, 30 s each time) on ice. For adult worm large-scale preparation, a French pressure cell (AMINCO) was used at 1,200 lb/in^2 to maximize tissue disruption. The insoluble cellular debris was spun down at 5,000 × *g* at 4°C for 20 min. A fraction containing surface tegument components was prepared by suspending intact parasites in ice-cold PBS with 0.2% Triton X-100 or 0.2% Nonidet P-40 and vortexing the mix at full speed three times for 30 s each time. The mixture was then centrifuged at 3,000 × *g* for 15 min to separate the detergent-extractable fraction from the carcass. The detergent-extracted bodies were then disrupted by sonication as described above to generate the interior fraction.

Chemicals and enzymes. Most enzymes and chemicals, including bovine Cu/Zn-SOD, xanthine oxidase, bicinchoninic acid assay solution, riboflavin, nitroblue tetrazolium, hypoxanthine, and iodoacetamide, were purchased from Sigma Chemical Co. Trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone treated) and cyanogen bromide were from Pierce Immunotechnology.

Protein concentration assay. The protein content of different parasite extracts was determined by bicinchoninic acid assay (52). Bovine serum albumin (1 mg/ml) was used as the standard.

SOD nitrite assay. SOD activity was determined by inhibition of nitrite formation. One unit of SOD activity is defined as the amount of enzyme that inhibits 50% nitrite formation under the assay conditions specified by Oyanagui (39). The diazo dye formed as a function of nitrite concentration was detected spectrometrically at a 550-nm wavelength. Total SOD activity was measured in the absence of potassium cyanide, and Mn-SOD activity was determined in the presence of 1 mM potassium cyanide, which specifically inhibits Cu/Zn-SOD activity (3). The Cu/Zn-SOD activity is the difference of total SOD activity and Mn-SOD activity.

SOD IEF gel assay. Isoelectric focusing (IEF) analysis was carried out as described previously (25). The SOD activity on the gel was stained by riboflavin photooxidation and $O_2 \cdot^-$ reduction of nitroblue tetrazolium (6). The Mn-SOD can be distinguished from Cu/Zn-SOD by its presence on the gel when stained in the presence of 2 mM KCN.

Anti-SP Cu/Zn-SOD mouse monoclonal antibodies. Specific mouse monoclonal antibodies were raised against the schistosome SP Cu/Zn-SOD recombinant gene product by a modified protocol by Claflin and Williams (12). Briefly, two mice were immunized with a recombinant fusion protein containing 90% of the coding sequence of the SP Cu/Zn-SOD gene without the putative signal peptide (21 amino acids). The primary injection in complete Freund's adjuvant and two subsequent booster injections in incomplete Freund's adjuvant were given intraperitoneally, 50 μg of antigen for each injection per mouse. The final booster dose (50 μg) in PBS was given intraperitoneally 4 days before fusion. Fusion was carried out by mixing mouse spleen cells (1×10^8) with Sp2/O myeloma cells (2.5×10^7) in a Universal centrifuge tube. The fusion was induced by 35% polyethylene glycol. The hybrids were cloned out in 96-well cluster dishes and screened by enzyme-linked immunosorbent assay (ELISA) and Western blot (immunoblot) analysis. Specificity was determined in ELISA by using only monoclonal antibodies that recognized the glutathione-*S*-transferase (GST)–SOD fusion protein but not purified GST alone.

Western blot analysis. Western blots were made with Immobilon P membranes from Millipore as described elsewhere (56). All reactions took place in PBS solution containing 2% evaporated milk and 0.05% Tween 20 at room temperature. The primary monoclonal antibodies were used at 1:10,000 dilutions. Specific protein was visualized by staining with anti-immunoglobulin G (IgG) alkaline phosphate-conjugated second antibodies.

Purification and enrichment of SOD. Total adult parasite extract in 20 ml of PBS was made from 3 g of frozen adult parasites by sonication and French pressure cell disruption as described above. To the extract, solid $(NH_4)_2SO_4$ was added to 50% saturation, and the precipitate was removed by centrifugation (4,000 × *g*). Then saturated $(NH_4)_2SO_4$ was added slowly to the parasite extract to 65% saturation, and the precipitate was again removed. The SOD activity was precipitated in 65 to 90% $(NH_4)_2SO_4$ and resuspended into 2 ml of 10 mM Tris-HCl (pH 7.5). Ammonium sulfate was removed by dialysis overnight at 4°C against three changes of 1 liter each of 10 mM Tris-HCl. The SOD solution was concentrated to 0.5 ml by a Centricon (3,000-molecular-weight cutoff) and injected onto a fast protein liquid chro-

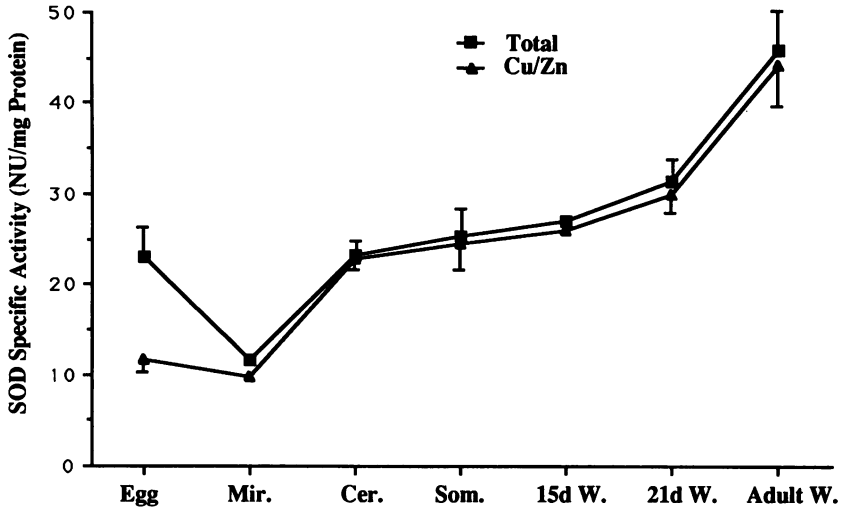

FIG. 1. SOD specific activity throughout the schistosome life cycle. Total parasite extracts were made as described in Materials and Methods. The various parasite stages are likewise described in the Materials and Methods. Protein content was determined by bicinchonic acid assay and normalized to 40 μg for each SOD nitrite assay. Total, total SOD activity, including cyanide-sensitive Cu/Zn-SOD and cyanide-insensitive Mn-SOD; Cu/Zn, Cu/Zn-SOD. Bars represent standard errors of the mean from three repeated independent experiments. Only one experiment was carried out employing miracidia (Mir.) and 15-day-old worms (15d W.) (no bar). Cer., cercariae; Som., schistosomula.

matograph (FPLC) with a Mono-Q column. The flow rate was set to 1 ml/min, and the fraction size was 1 ml per tube. A salt gradient was created between a low-salt buffer (10 mM Tris-HCl) and a high-salt buffer (10 mM Tris-HCl plus 1.0 M NaCl). Schistosome Cu/Zn-SOD eluted off the column right after the run-through which was in the second and third fraction collected after injection. This corresponded to a salt concentration of between 0 and 0.1 M NaCl. The gradient was held at 10% (0.1 M NaCl) for 2 min and then raised within 1 min to 100%. Fractions of the first 5-ml eluent including the run-through were collected, and an aliquot of 10 μl from each fraction was analyzed by a sodium dodecyl sulfate (SDS)-polyacrylamide gel stained with silver. Fractions which contained the purified SOD were pooled and stored at −20°C.

Mono-Q fractions were sometimes contaminated with a few proteins of high molecular weight, especially during purification from large amounts of material. An additional purification using a Superose 12 column was required. Mono-Q fractions were concentrated to 0.2 ml by using a Centricon as described earlier and then injected onto a Superose 12 column equilibrated with 10 mM Tris-HCl (pH 7.5). The flow rate was 0.5 ml/min, and the fraction size was 1 ml. The first 8 ml after sample injection was the void volume and was discarded. Eluent from 9 to 20 ml was collected. Schistosome Cu/Zn-SOD eluted off the column between 15 and 17 ml, and these fractions were combined and stored at −20°C. To enrich for hamster SOD, erythrocytes (RBC) were collected, washed, and sonicated in PBS. The supernatant was subjected to ammonium sulfate (65 to 90%) precipitation. The precipitate was dissolved in 1 ml of PBS and dialyzed overnight at 4°C against three changes of 1 liter each of PBS. This sample was then used in the SOD IEF assay described above.

Protein modification, digestion, and sequencing. The protein was reduced and carboxymethylated by iodoacetamide before Edman degradation sequencing (40). Trypsin digestion was carried out at 37°C for 8 h by using a ratio of 1/20 (the amount of trypsin/the amount of protein digested). Cyanogen bromide digestion was carried out overnight at room temperature (31). Products from the above treatments were isolated by high-pressure liquid chromatography on a reverse-phase column (μBondapak C_{18}; Waters) with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The samples were then evaporated to dryness. Amino acid sequences were obtained from the N termini of digested fragments and the intact protein by using an automated protein sequencer (Applied Biosystems model 470A).

RESULTS

SOD activity in the life cycle of *S. mansoni*. Specific SOD activity (in nitrite units per milligram of protein) was determined by SOD nitrite assay in total parasite extracts of different stages, from eggs, miracidia, cercariae, schistosomula, 15-day-old worms, and 21-day-old worms to adult worms. According to the evaluation by Oyanagui (39), 8.5 nitrite units (NU) are equivalent to 1 cytochrome *c* unit (standard SOD unit). It was found that SOD activity gradually increases during the parasite life cycle, reaching the maximum in the adult stage (Fig. 1). Greater than 95% of the SOD activity came from cyanide-sensitive Cu/Zn-SOD. The specific total SOD activity in adult schistosomes was 53 ± 9 NU/mg of protein, which is twice as high as that of cercariae and 3-h-old schistosomula (23 to 25 NU/mg of protein) and five times as high as that of eggs and miracidia (9 to 11 NU/mg of protein). The amount of SOD activity in the egg stage is difficult to evaluate because of the possible contamination with host tissues during the preparation of eggs.

Detergent-extractable SOD activity. As free-living cercariae penetrate the skin of the vertebrate host and become endoparasitic, a number of changes occur. Among them, the glycocalyx of the cercariae is lost and the exposed outer covering of the parasite (the tegument) is changed from an anucleated syncytium covered by a trilaminar membrane to one covered by a heptalaminar membrane (33). As the tegument is the interface between the parasite and the host, it was of interest to determine SOD activity in a fraction that

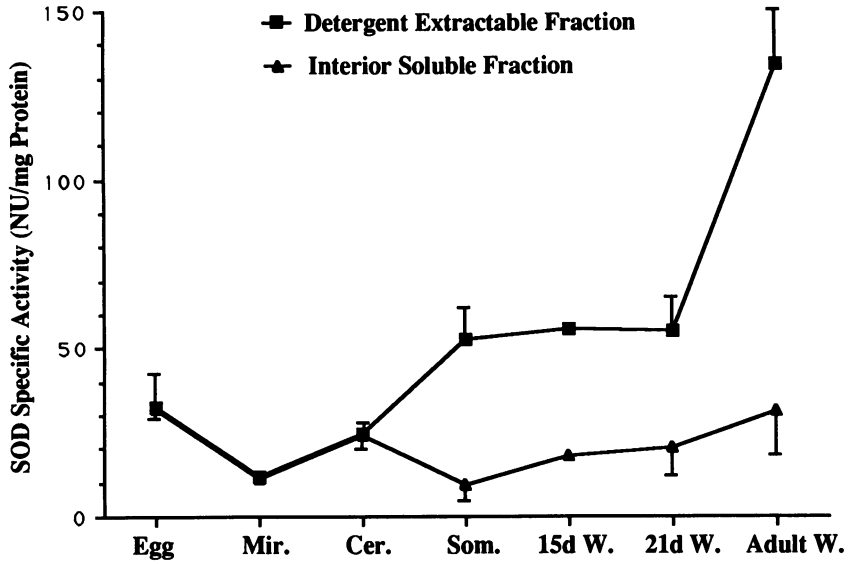

FIG. 2. Distribution of total SOD activity within different stages of the schistosome life cycle. The intact parasites were fractionated into a detergent-extractable fraction that contains tegument components and an interior (detergent-resistant carcass) soluble fraction (see Materials and Methods for procedures). Forty micrograms of protein was used for each SOD nitrite assay. Bars represent standard errors of the mean from three repeated independent experiments. Only one experiment was carried out employing miracidia (Mir.) and 15-day-old worms (15d W.) (no bar). Cer., cercariae; Som., schistosomula.

contained components of the tegument. The result shown in Fig. 2 demonstrates an increase of the total specific SOD activity in the nonionic detergent fractions (containing components from the tegument) once the parasite becomes an endoparasite, with the highest specific activity found in the fraction from adult parasites.

Characterization of SOD activity by IEF gel assay. The SOD activity from detergent-extractable fractions and from interior fractions of adult worms and 3-h-old schistosomula was characterized on IEF activity gels. The result is shown diagrammatically in Fig. 3. Both Mn-SOD and Cu/Zn-SOD activities were found in the parasite. The Mn-SOD had pI values close to neutral pH (6.9 to 7.2) and was cyanide insensitive. The Cu/Zn-SOD was slightly acidic (pH 5.3 to 6.3) and cyanide sensitive and could be visualized only in those gels stained in the absence of KCN (lanes 1, 3, 5, and 7). There were four Cu/Zn-SOD pI variants (pI = 5.3, 5.7, 6.0, and 6.3) in adult schistosomes (lanes 1 and 3). Only two of the four pI variants found in the adult (6.0 and 6.3) were present in the 3-h-old schistosomula (lanes 5 and 7). The reason for multiple pI variants and different SOD activity patterns between the adult parasites and the young schistosomula is not clear. It could be the result of the expression of different gene products or differential posttranslational modifications of the same gene product, including proteolytic degradation, different metal content, or host-associated SOD. In order to address this question, we attempted to correlate the SOD activity bands to the previously identified SP Cu/Zn-SOD gene product (49).

Production of anti-SP Cu/Zn-SOD monoclonal antibodies. The SP Cu/Zn-SOD cDNA was isolated by polymerase chain reaction-reverse transcription using adult parasite total RNA as a template. It was then subcloned into pGEM3Zf(−) vector (Promega) at the *Sma*I site. The coding region without the first 21 amino acids was removed by *Nde*I and *Bam*HI digestion, repaired with T4 DNA polymerase, and inserted into the *Sma*I site of pGEX-3X (50) in frame with the carrier protein, *Schistosoma japonicum* glutathione-*S*-transferase (Sj26), to create plasmid pGEX-SODΔSP. This plasmid was transformed into bacterial (DH5α) cells and induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to express large amounts of the fusion protein (Fig. 4). The fusion protein (Sj26-SODΔSP) was purified by electroelution and used to immunize mice in order to make monoclonal antibodies. Six monoclonal antibodies that specifically recognize the SP Cu/Zn-SOD gene product were produced, and four of them (4G3 [IgG2b], 8B2 [IgG1], 4A7 [IgG2a], and 1A2 [IgG1]) were grown as mouse ascitic fluid and purified with a Bio-Rad MAPSII protein A column. All of the monoclonal antibodies recognized the SP Cu/Zn-SOD gene product on a Western blot membrane. Attempts either to immunoprecipitate the SP Cu/Zn-SOD gene product from the parasite extract or to inhibit any of the SOD activity failed (data not shown).

Separation of immunoreactive SP Cu/Zn-SOD from the major parasite SOD activity by FPLC on a Superose 12 column. The failure of the monoclonal antibodies to immunoprecipitate the SP SOD gene product or to inhibit SOD activity prompted us to carry out further experiments to correlate SOD activity with immunoreactivity. Extracts of adult worms were prepared as described in Materials and Methods and subjected to FPLC Superose 12 fractionation. The result showed that the immunoreactive SP Cu/Zn-SOD gene product eluted off the column right after the void volume. This fraction contained molecules much larger than 200 kDa (Fig. 5A). In contrast, all of the Cu/Zn-SOD activity eluted off the column in fractions containing molecules 20 to 40 kDa in size (Fig. 5B). Other attempts to correlate SOD activity with immunoreactivity by IEF gel electrophoresis or FPLC on a Mono-Q column also failed (data not shown). These results suggested to us that the major Cu/Zn-SOD activity in schistosomes was not related to the SP Cu/Zn-SOD gene product.

Tissue-specific distribution of the SP Cu/Zn-SOD gene product and SOD activity. To further confirm the hypothesis that the SP Cu/Zn-SOD gene product was not related to the major

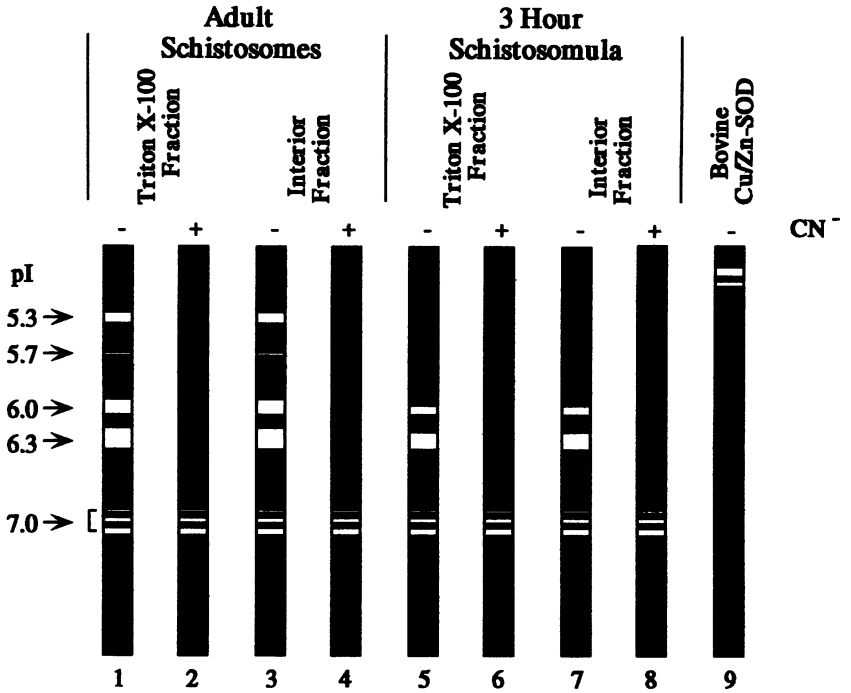

FIG. 3. IEF analysis of SOD activity in schistosome extracts. IEF tube gels (7%) were prepared with 2% Ampholine (pH 3.5 to 10). SOD samples normalized to 100 NU were loaded on the top of the gel (acidic end), electrophoresis was performed, and the gels were stained and developed for SOD activity as described in Materials and Methods. A diagrammatic representation of the IEF gels is shown. Clear bands represent the presence of SOD activity. Bovine Cu/Zn-SOD was from Sigma. The pI value was determined by cutting a blank gel after electrophoresis into 0.5-cm pieces and measuring the pH of each piece to plot a pH gradient curve. The gels in lanes 2, 4, 6, and 8 were stained in the presence of 2 mM KCN to show Mn-SOD activity.

SOD activity present in the adult schistosome extract, another experiment was performed. Adult schistosomes were fractionated into a detergent-extractable fraction, an interior fraction, and an insoluble fraction. Each fraction was then Western blotted with the anti-SP Cu/Zn-SOD monoclonal antibodies as the first antibody and ^{125}I-labelled anti-mouse IgG as the second antibody. The specific immunoreactivity was defined as the amount of ^{125}I-labelled anti-mouse IgG (in counts per minute) bound to the blot per milligram of protein. SOD specific activity was measured as nitrite units per milligram of protein. The results (Fig. 6) showed that the detergent-extractable fraction contained the highest SOD specific activity, which was normalized to 100%, and the lowest specific immunoreactivity (1%). On the other hand, the insoluble cellular debris contained the highest immunoreactivity, which was normalized to 100%, and the lowest SOD specific activity (5.4%). The levels in the soluble interior fraction (worm carcass after detergent extraction) were 17.6% for SOD enzyme activity and 4% for SP Cu/Zn-SOD gene product immunoreactivity. The different distribution patterns of the major SOD activity and immunoreactive SP Cu/Zn-SOD gene product were consistent with the results from the FPLC fractionation experiments (Fig. 5) and strongly bolstered the hypothesis that the two were not related.

Purification of Cu/Zn-SOD activity from adult schistosomes. The major Cu/Zn-SOD enzyme activity present in the adult worm extracts was purified to homogeneity by $(NH_4)_2SO_4$ precipitation and FPLC on Mono-Q and Superose 12 columns. Purity was checked by SDS-polyacrylamide gel electrophoresis coupled with silver staining (Fig. 7, lane 2). The apparent molecular size of the purified protein on SDS-

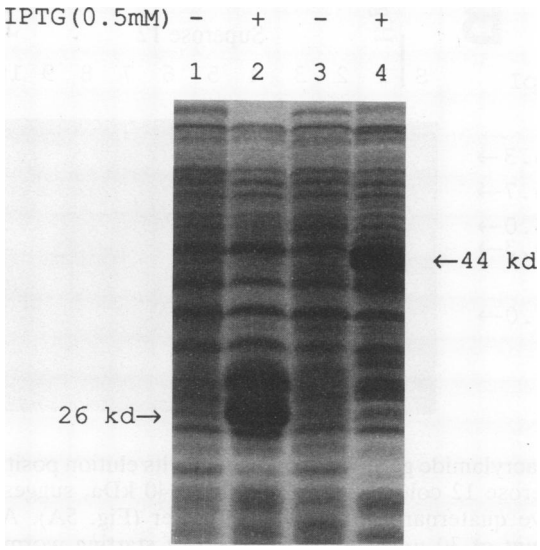

FIG. 4. Expression of SP Cu/Zn-SOD fusion protein in *Escherichia coli*. pGEX-SODΔSP expression plasmid was constructed as described in Results. Both the pGEX vector plasmid and the pGEX-SODΔSP expression plasmid were transformed into DH5α bacterial cells. The transformed cells were induced by 0.5 mM IPTG at 37°C for 3 h. Lanes 1 and 3, uninduced cells containing either the vector pGEX or the expression plasmid pGEX-SODΔSP, respectively; lanes 2 and 4, induced cells corresponding to lanes 1 and 3, respectively. Each lane contained a total cell extract equivalent to 0.5 ml of the bacterial cultures.

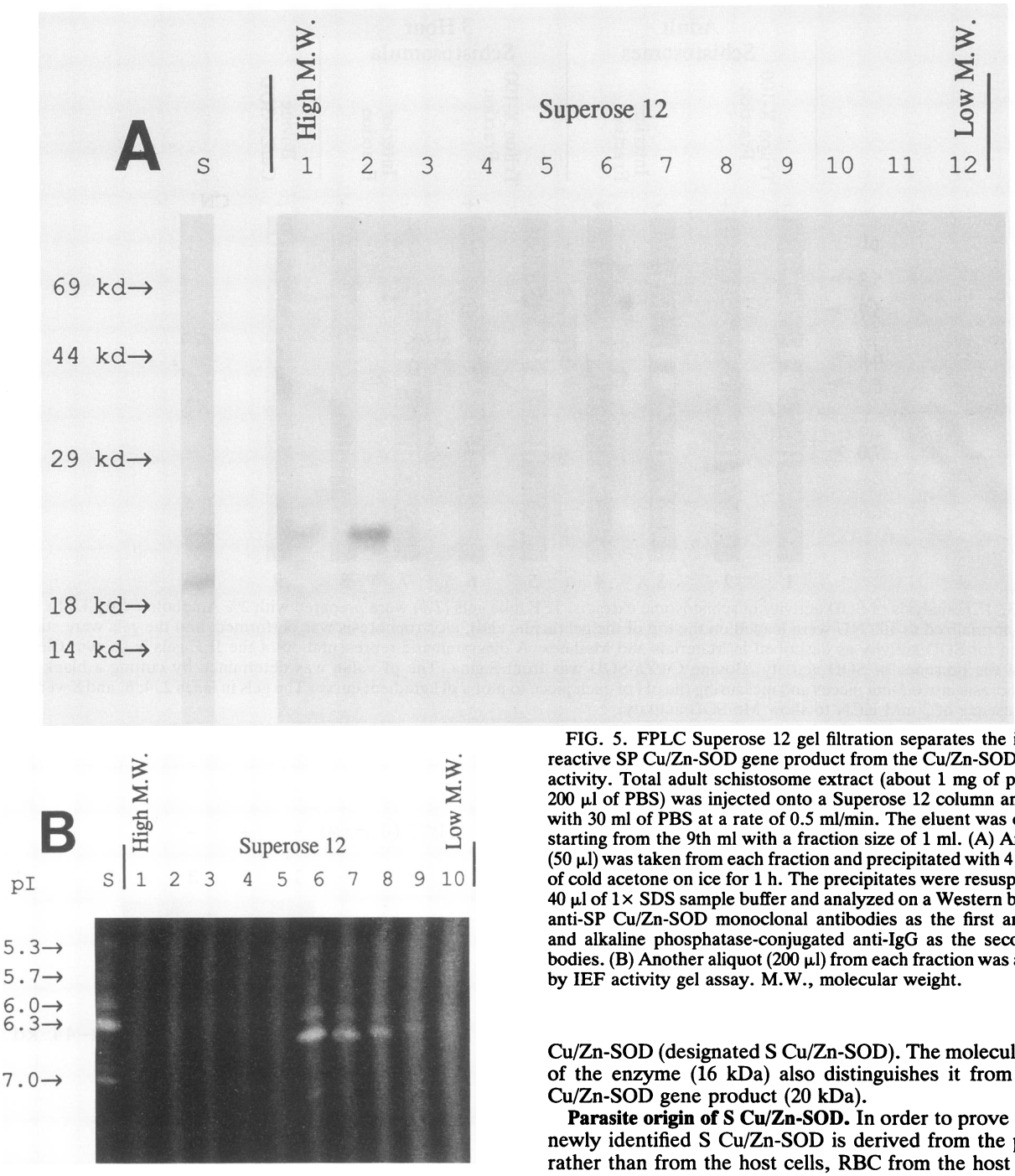

FIG. 5. FPLC Superose 12 gel filtration separates the immunoreactive SP Cu/Zn-SOD gene product from the Cu/Zn-SOD enzyme activity. Total adult schistosome extract (about 1 mg of protein in 200 μl of PBS) was injected onto a Superose 12 column and eluted with 30 ml of PBS at a rate of 0.5 ml/min. The eluent was collected starting from the 9th ml with a fraction size of 1 ml. (A) An aliquot (50 μl) was taken from each fraction and precipitated with 4 volumes of cold acetone on ice for 1 h. The precipitates were resuspended in 40 μl of 1× SDS sample buffer and analyzed on a Western blot using anti-SP Cu/Zn-SOD monoclonal antibodies as the first antibodies and alkaline phosphatase-conjugated anti-IgG as the second antibodies. (B) Another aliquot (200 μl) from each fraction was analyzed by IEF activity gel assay. M.W., molecular weight.

polyacrylamide gels was 16 kDa, while its elution position on Superose 12 column was about 20 to 40 kDa, suggesting a native quaternary structure of a dimer (Fig. 5A). A total amount of 30 μg of SOD from 3 g of starting worms was purified on the basis of an estimation from the silver staining using SP Cu/Zn-SOD purified from bacterial cells as a standard (Fig. 7, lanes 3, 4, and 5). The SOD activity of the purified enzyme was analyzed by IEF gel electrophoresis (Fig. 8, lane 1). The purified enzyme generates two of the Cu/Zn-SOD activity bands (pI = 6.0 and 6.3) that exist in both adult schistosomes and 3-h-old schistosomula (Fig. 8, lanes 4 and 5). The newly purified enzyme is soluble in a variety of buffers (pH 7 to 8) and is considered to be a soluble Cu/Zn-SOD (designated S Cu/Zn-SOD). The molecular mass of the enzyme (16 kDa) also distinguishes it from the SP Cu/Zn-SOD gene product (20 kDa).

Parasite origin of S Cu/Zn-SOD. In order to prove that the newly identified S Cu/Zn-SOD is derived from the parasite rather than from the host cells, RBC from the host animal, golden hamsters, were collected and the hamster Cu/Zn-SOD activity was partially purified (65 to 90% ammonium sulfate precipitate). IEF analysis showed that hamster RBC Cu/Zn-SOD focuses at a position very similar to the top SOD activity band (pI = 5.3) of adult parasite extract (Fig. 8, lane 2 and 4). When the partial purified hamster SOD sample was mixed with the purified parasite S Cu/Zn-SOD (Fig. 8, lane 3), it gave a pattern very similar to that of adult worm extract (Fig. 8, lane 4). The second SOD activity band from the top (pI = 5.7) was faintly present in both adult parasite extract and hamster RBC extract. The origin of the band (host or parasite) has not been determined.

Peptide sequences of S Cu/Zn-SOD. The N terminus of the purified S Cu/Zn-SOD was unblocked, and about 20 amino

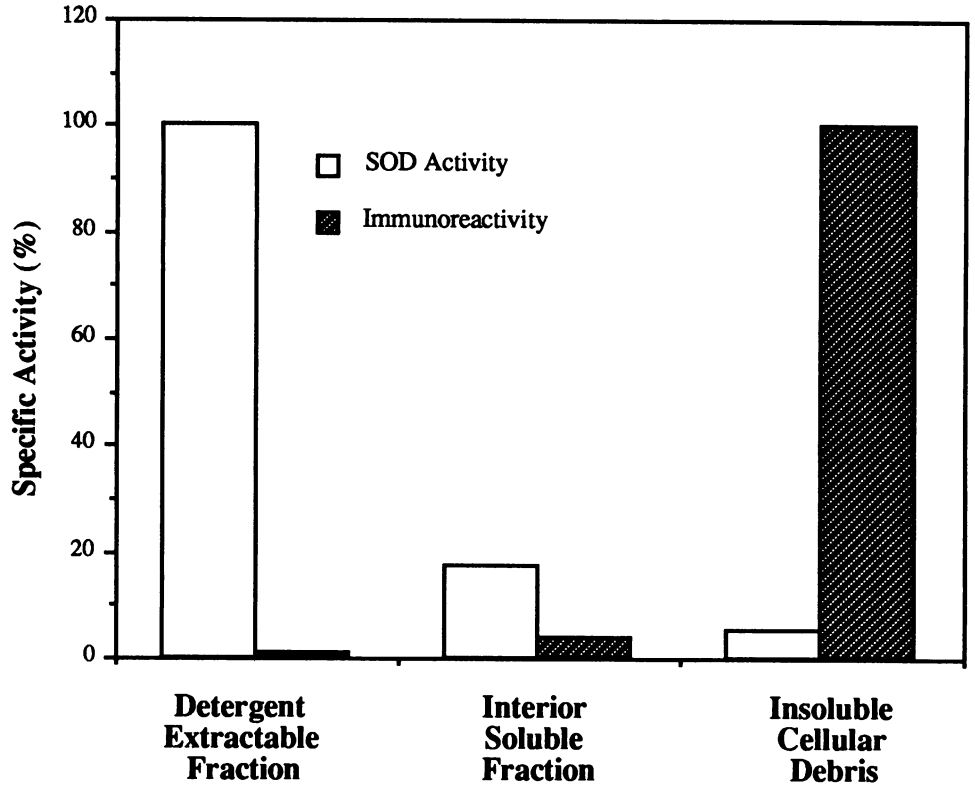

FIG. 6. The distribution of SP Cu/Zn-SOD gene product and SOD activity in adult schistosomes. Intact adult parasites were fractionated into a detergent-extractable fraction, interior (detergent-resistant carcass) soluble fraction, and insoluble cellular debris (see Materials and Methods). The protein content was normalized, and 40 μg of protein was used for both SOD nitrite assay and Western blot analysis. The specific immunoreactivity (in counts per minute per milligram of protein) on the Western blot was quantitated by using the anti-SP Cu/Zn-SOD monoclonal antibodies as the first antibodies and ^{125}I-labelled anti-IgG as the second antibodies. SOD specific activity (in nitrite units per milligram of protein) and the specific immunoreactivity were then compared in different fractions as indicated. One hundred percent SOD specific activity was 146.5 NU/mg, and 100% specific immunoreactivity was 250,000 cpm/mg of protein.

acids of sequence were obtained by Edman degradation. The protein was also digested with trypsin and cyanogen bromide (CNBr) to yield two internal peptides, which were also purified and partially sequenced. A comparison of these peptide sequences with the sequence of SP Cu/Zn-SOD showed that they were not derived from it, proving that this enzyme represents a distinctly different gene product (Fig. 9). A further comparison with sequences contained in the SwissProt data base by using the FASTA sequence comparison program (43) aligned the peptide sequences to corresponding positions of 19 cytosolic Cu/Zn-SODs from other species with a high degree of homology (Fig. 9). It is likely that the S Cu/Zn-SOD is a cytosolic form of schistosome Cu/Zn-SOD.

DISCUSSION

Superoxide radicals have been shown to cause damage to nearby cells by peroxidation of membranes, proteins, and DNA and also by inhibition of some critical enzyme activities (18). Thus, it is important to have SOD activity which can remove the toxic radicals. $O_2 \cdot^-$ radicals are released by immune effector cells as a host defense mechanism to kill invading parasites (2). Therefore, in these parasites in which SOD is at the host-parasite interface, it is believed that SOD plays an important role in the survival of the parasite from the host immune response. Other antioxidants, such as glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase, and cytochrome *c* peroxidase, may also play a role in parasite survival (8, 36–38).

It is well known that schistosomes have evolved mechanisms of immune evasion and thus are able to establish themselves in their vertebrate hosts during primary infection. Once established, the adult worms seem to be resistant to immune elimination. A number of schistosome immune evasion mechanisms have been identified (42). One of these is the antioxidant suppression of host oxidative killing in which SOD constitutes a part of the antioxidant activity (8). In schistosomes, Mkoji et al. (37) showed that the parasite resistance against in vitro oxidative killing is correlated with the levels of endogenous SOD activity.

We report here some of our recent investigations on the characterization of a previously isolated putative schistosome Cu/Zn-SOD (SP Cu/Zn-SOD) and the identification of a second form of schistosome Cu/Zn-SOD (S Cu/Zn-SOD). The enzyme activity of the S Cu/Zn-SOD was characterized at all the developmental stages and also in different tissue components of 3-h-old schistosomula and of adult worms. These results, which are consistent with previous reports (38), support the antioxidant role of the parasitic SOD against the host toxic oxidant, $O_2 \cdot^-$.

The increase of schistosome Cu/Zn-SOD specific activity during the intravascular migratory phase of development in the hosts (Fig. 1) correlates with the survival (evasive immunity) of the adult parasites, indicating that SOD might

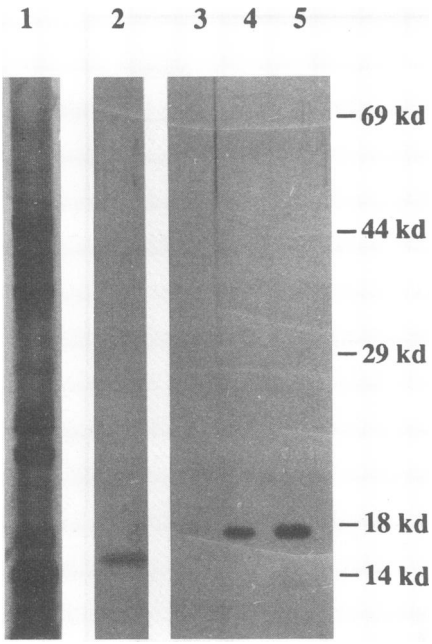

FIG. 7. Purification of schistosome S Cu/Zn-SOD from adult parasite total-worm extract. Ammonium sulfate precipitation of a total adult worm extract was subjected to FPLC using a Mono-Q column as described in Methods and Materials. Lane 1, 65 to 90% $(NH_4)_2SO_4$ precipitate, which is the starting material for the Mono-Q column; lane 2, purified S Cu/Zn-SOD (16 kDa) from Mono-Q fractions; lanes 3 to 5, 10, 50, and 100 ng of purified SP Cu/Zn-SOD gene product expressed in *E. coli.*

be one of mechanisms used by the parasite to circumvent damage due to the host immune response. Especially the enrichment of SOD enzyme activity in the fraction containing tegument components (Fig. 2) may allow the parasite to prevent oxidative damage caused by host effector cells more efficiently. Ultrastructural studies showed that eosinophils,

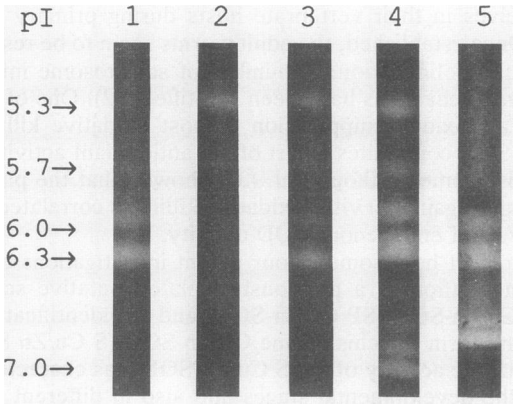

FIG. 8. IEF analysis of schistosome S Cu/Zn-SOD and host hamster RBC SOD. Purified schistosome S Cu/Zn-SOD (lane 1) and partially purified (65 to 90% ammonium sulfate precipitate) hamster RBC SOD (lane 2) were analyzed by IEF gel electrophoresis and compared with those from adult schistosome (lane 4) and 3-h-old schistosomula (lane 5). The mixture of purified schistosome S Cu/Zn-SOD and partially purified hamster SOD was also subjected to IEF gel electrophoresis, revealing an activity pattern similar to that of an adult schistosome extract (lane 3).

neutrophils, and macrophages have very close contact and are even fused with the outer surface of schistosomes during attack (11, 14, 34). The intimate contact by these phagocytes should be expected to potentiate the toxic effects of the oxidants, which in turn will require more scavenger enzymes from the parasite for detoxification. Comparative IEF analysis between adult parasites and schistosomula suggested an intriguing mechanism for developmental regulation rather than a simple overexpression of the SOD activity (Fig. 3). This may involve a host SOD acquisition mechanism as well as different gene expression or different posttranslational modifications. The top SOD activity band of the adult parasites on IEF gels (Fig. 8, lane 4) has the same pI value (5.3) as for hamster RBC Cu/Zn-SOD (Fig. 8, lane 2), while this top SOD activity band is missing in the 3-h-old mechanically transformed schistosomula (Fig. 8, lane 5). One explanation is that the adult parasite preparation is contaminated by the hamster RBC as the parasite feeds on host RBC. However, there exists the possibility that schistosomes acquire the host SOD during the intravascular life cycle stages, especially since it has been shown that there is an active material exchange between the host RBC and the parasite (20). A similar observation, that a malaria parasite may acquire the host SOD molecules, was also reported (16), although further studies are needed to address this question.

We failed in these studies to ascribe any SOD activity to the SP Cu/Zn-SOD gene product previously identified by Simurda et al. (49). Different lengths of the coding regions and different fusion proteins were expressed in bacterial cells in large quantities, but the solubility of the recombinant SP Cu/Zn-SOD seems to prevent us from recovering any SOD activity (unpublished data). In vitro denaturation and reoxidization experiments were not successful (unpublished data). Specific antibody reagents also failed to immunoprecipitate the SP Cu/Zn-SOD from parasite extracts; instead, the SP Cu/Zn-SOD was associated with high-molecular-weight aggregates (Fig. 5). In addition, the antibodies also failed to inhibit any SOD activity in parasite extract (unpublished data). In spite of our failure to demonstrate enzymatic activity, we still believe that schistosome SP Cu/Zn-SOD gene encodes a specialized Cu/Zn-SOD, since the predicted protein contains all of the copper-zinc-binding sites and activity sites. The insolubility of the SP Cu/Zn-SOD and its aggregate formation in extraction buffers suggest an association with membranes. The extra N-terminal hydrophobic sequence (20 to 30 amino acids) and the putative N-linked glycosylation site seem to support our hypothesis that SP Cu/Zn-SOD requires a special N terminus which may guide the enzyme to a special environment (such as a membrane phase) for its enzymatic function (24). Current efforts to demonstrate SOD activity are directed towards protein engineering such as making chimeric SOD molecules by polymerase chain reaction to replace the N terminus with a consensus N terminus from yeast Cu/Zn-SOD.

The newly purified schistosome S Cu/Zn-SOD is believed to be cytosolic in part because of its peptide alignment to other cytosolic SODs (Fig. 9). The purified S Cu/Zn-SOD focuses at the same positions as that from schistosomula which never ingest host RBC, further indicating its parasite origin.

Our interest is to understand why schistosomes have acquired two different forms of Cu/Zn-SODs and what their special functions might be. Interestingly, in *T. spiralis* (44), *Taenia taeniaeformis* (28), and *Onchocerca volvulus* (22), Cu/Zn-SOD activity has been demonstrated in the culture fluids excreted by these parasites. In each case the Cu/Zn-

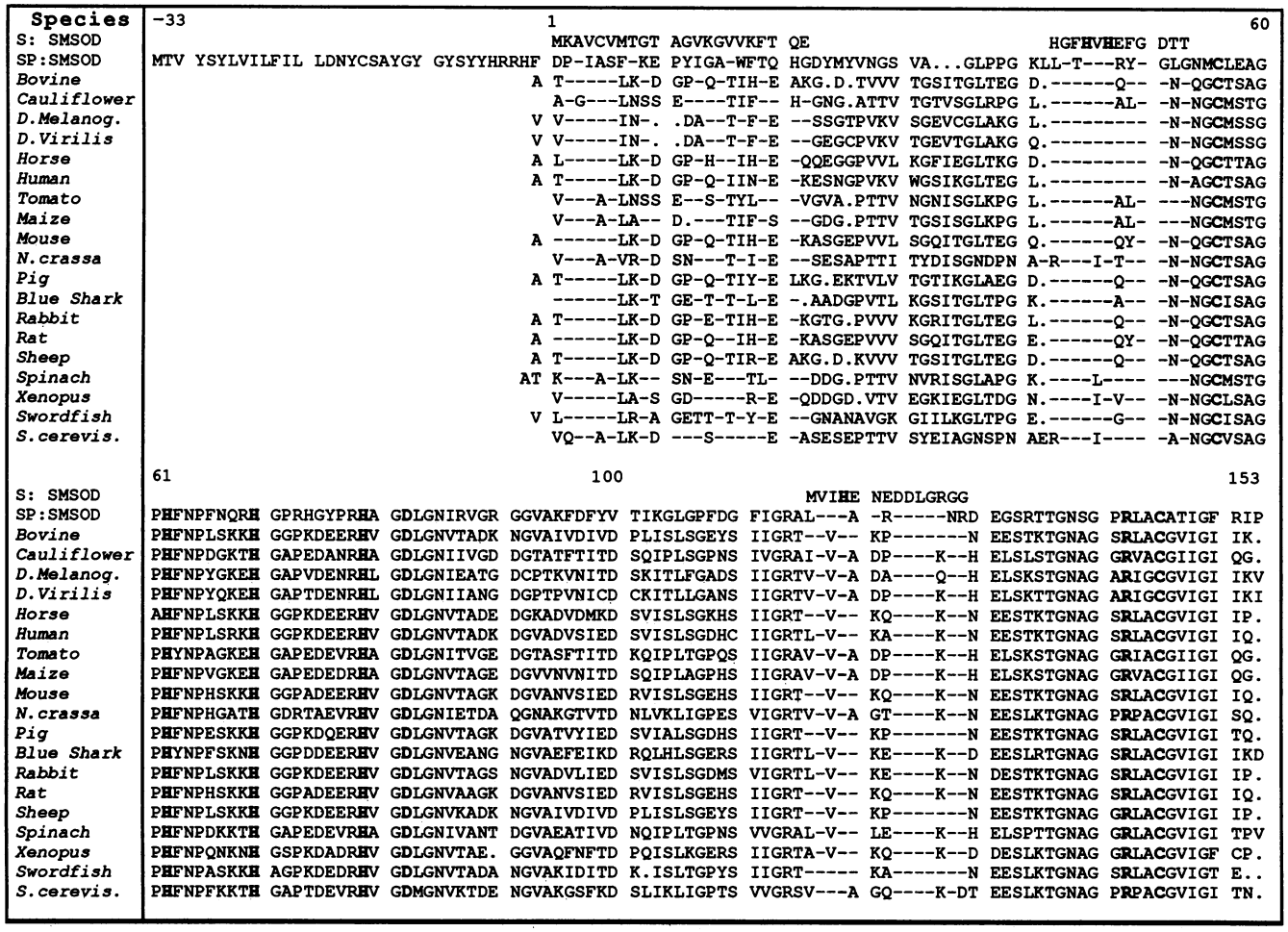

FIG. 9. Peptide alignment with 19 cytosolic Cu/Zn-SODs from various species. The top line shows the peptide sequences of schistosome S Cu/Zn-SOD. The second line is that of schistosome SP Cu/Zn-SOD. The copper-zinc-binding sites (in boldface type) are formed by six His residues (**H**) and one Asp (**D**) residue. The two Cys (**C**) residues (in boldface type) are believed to form a disulfide bond. The Arg (**R**) residue is believed necessary to guide the superoxide to the activity site (45). Identical residues are represented by dashes; deletions are represented by dots.

SOD has, as reported here for *S. mansoni*, been a cytosolic form of the enzyme. The enrichment of S Cu/Zn-SOD activity in a fraction containing tegument components of schistosomes may indicate its role as a defense enzyme against host toxic oxidants. However, there are some questions as to the role of oxidants in killing schistosomula and to the specific role of SOD in protecting schistosomula (47, 51). These questions can be resolved only by direct evidence obtained from in vivo studies by using specific inhibitors or antibodies (5). It is particularly important to know how the SP Cu/Zn-SOD carries out its function in schistosomes, whether it is excreted or membrane associated, and in which tissue it is located in the parasite.

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