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Copper, Zinc-Superoxide Dismutase (Cu/Zn SOD) Gene During Embryogenesis of *Bombyx mori*: Molecular Cloning, Characterization and Expression

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BmCu/Zn SOD was isolated from early embryo of *Bombyx mori* using microarray analysis. The BmCu/Zn SOD gene was observed during the early embryonic stage with the strongest signal found at the unfertilizaion, fertilization and blastoderm stages. The BmCu/Zn SOD gene encodes a protein of 154 amino acids with a calculated Mr of 15 kDa. The deduced amino acid sequence of BmCu/Zn SOD indicated that the residues that form on the Cu/Zn binding site are conserved and that the sequence is a 60% identity to that of *M. domestica*. In a phylogenetic tree, Bm SOD was also close to Drosophila SODs rather than other insect SODs. The BmCu/Zn SOD gene exists as a single copy in the genome. Transcripts of BmCu/Zn SOD cDNA were identified by northern blot analysis. The expression of the BmCu/Zn SOD gene was observed weakly in most of larvae, pre-pupae, pupae and adult tissues. Also, the BmCu/Zn SOD gene was observed in early embryonic stage. Although the roles of SODs remains to be further elucidated, the high expression of Bm Cu/Zn SOD gene at before 24 h post fertilization suggests that this gene is of general importance during early embryogenesis in the *Bombyx mori*.

Key words: Silkworm, Superoxide dismutases (SOD), Embryogenesis, Microarray, Gene expression

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Introduction

Reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) are inevitably formed in all aerobic organisms and can cause damage to a wide range of biomolecules, such as DNA molecules and proteins (Imlay and Linn, 1988; Li, Huang, Zhou, Liu, Li, Nomura and Zhao, 2002). Both O_2^- and H_2O_2 must be removed promptly in order to avoid cellular damage. They use enzymes and nonenzymatic antioxidants to remove ROS. The most critical enzymes in removing superoxide anion are superoxide dismutases (SODs), which carry out the following reaction: $2O_2^- + H^+ \rightarrow H_2O_2 + O_2$ (Bermingham-McDonogh, Gralla and Valentine, 1988). The H_2O_2 formed is removed by catalases or peroxidases. SOD is classified into Mn, Fe and Cu, Zn SOD, depending on the metal in the active site. Cu, Zn SODs are found in the cytosol of all eukaryotic cells (Puget and Michelson, 1974; Li, Huang, Zhou, Liu, Li, Nomura and Zhao, 2002) but they are not generally in prokaryotic cells, with a few exceptions. The SODs all participate in protecting cellular molecules from damage caused by the ROS (Fridovich, 1995; Choudhury, Lee, Davidson, Yim, Bose, Sharma, Kang, Cabelli and Maroney, 1999). On the other hand, the ROS plays a significant role in the innate immunity system of insects.

SODs have been implicated in human disease (Rosen, Siddique, Patterson, Figlewicz, Sapp, Hentati, Donaldson, Goto, O'Regan, Deng and *et al.*, 1993) and its possible role in aging has been elucidated (Taniguchi, 1992). Aging is largely caused by damage to cells, particularly by reactive oxygen, but it is also under genetic control. Genes that can increase life span have been identified in *C. ele-*

ganse and Drosophila and they may act by increasing the animals' resistance to oxidative stress (Lewis. W, 2002). Hence, their genes are necessary for aerobic organisms to control ROS. SODs are a ubiquitous metalloenzyme in aerobic organisms. The role of SODs in Drosophila aging found two different wild type strains with the same level of SOD activity.

Here, we isolated the cDNA encoding the Cu, Zn SOD gene from the early embryo of the silkworm, *B. mori*. Also, we present an expression of different levels of the SOD gene at each development stage and identify the expression of the SOD deficiency in segments and in egg-shaped malformation mutants.

Materials and Methods

Insects

Silkwrom larvae (*kl20*, Korean native strain) were originally obtained from by the College of Agriculture and Life Science, Kyungpook National University. Larvae were reared on mulberry leaves at 25°C.

Construction of the embryo cDNA library

In order to generate a *B. mori* embryo of the cDNA library, we modified Maruyama and Sugano's method (1994). The total RNA was isolated from the eggs of a germ-band formation stage using the Total RNA Isolation Kit (Promega, USA). The total RNA was pre-treated by using BAP (Bacterial alkaline phosphatase) and TAP (Tabacco acid pyrophosphatase) and then, it was ligated with 5'oligo (5'-AGC AUC GAG UCG GCC UUG UUG GCC UAC UGG-3'). Poly(A)$^+$RNA was subsequently purified using the mRNA Isolation Kit (Qiagen, USA). The synthesis of the first-strand cDNA and amplification were performed as described by Maruyama and Sugano (1994). The amplified cDNAs were ligated into the pCNS vector (GenBank Accession No. AF416744) and transformed. Then, we constructed two libraries, non-normalized library by oligo-capping and a normalized library by using abundant cDNAs in the non-normalized library as a driver. The titers following the amplification of the non-normalized and the normalized cDNA libraries were both 3.0×10^6.

Microarray hybridization

In this study, we employed both normalized and non-normalized *B. mori* embryo cDNA libraries. The cDNA inserts were amplified via PCR using Top-Taq^{TM} DNA polymerase (Corebio, Korea) in a 30 μl reaction mixture. The PCR products, confirmed by gel electrophoresis, were cleaned by a precipitation wash with ethanol, and resuspended in distilled water. The DNA clones were printed on to glass slides which had been precoated with amino silane (Corning, USA). The microarrays were constructed according to the instructions of the Genomictree Company (www.GenomicTree.com). The cDNAs that were obtained from 100 micrograms of total RNA were synthesized and labeled using a cDNA post-labeling kit (Amersham Pharmacia, USA), in accordance with the manufacturer's instructions, with the minor exception that we used Superscripts (Invitrogen, USA) for reverse transcription. Also, a PCR purification of the labeled cDNAs was conducted. We utilized reference RNA, which was the total RNA that was obtained from the eggs, during the germ-band formation stage. The cDNAs obtained from the RNAs of the embryos at each time point were then labeled with Cy5, and those from the reference RNA were labeled with Cy3. A hybridization buffer, including 1 × SSC, 0.2% SDS, formamide, probe, and distilled water, was incubated for 16 hrs at 42°C. The microarrays were then washed in 1 × SSC and 0.1% SDS for 5 minutes at 42°C; 0.1 × SSC, and 0.2% SDS for 5 minutes at 42°C; then in 0.1 × SSC for 1 minute, and this was repeated. The microarrays were then scanned using an Axon 4000B array scanner and the signal intensity levels of the spots were acquired using GenePix Pro software, ver 4.0 (Axon, USA). In order to substantiate the accuracy of the data, the experiments were independently conducted twice. Both experiments revealed the same results.

Statistical analyses were conducted using GeneSpring 6.2 (Agilent, USA). The data were divided into two ranges, UFE-BDF and SF-AAF. According to the selected experimental conditions, we set the significance values at >2.0 for the up-regulated genes, and <0.5 for the down-regulated genes. Then, genes that exhibited more significant perturbation were isolated via an ANOVA analysis.

Isolation of full length cDNA clones

A cDNA clone of Cu/Zn SOD, which was expressed at an early embryo by microarray analysis, was selected. The clone that contained full-length sequences was sequenced from both strands by using an ABI Prism BigDyeTM Terminatior Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) on an ABI Model 377 Prism DNA Sequencer (PE Applied Systems). The clone was identified by a search of a cDNA or genomic database.

Sequence analysis

DNAstar (ver. 5.0) was used for the analysis of the DNA sequences. Alignments and phylogenetic trees of the SOD sequences were made by CLUSTASW and MEGA3, respectively. Referring to the sequence of a cDNA that encoded other species, SODs were searched in the NCBI

database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi).

Expression analysis of SOD mRNA

Total RNAs were extracted from fat-bodies, mid-gut, silk-glands, skin, brain, ovaries, testes and hemocytes at the each development stages. The total RNA was prepared using the RNAgents total RNA isolationsystem (Promega). The inserts of the cDNA clone were PCR amplified by using vector primers and then labeled with [α-^{32}P] dATP using a Random Primer Labeling Kit (Stratagene). Northern blotting was performed as described by Sambrook *et al.* (1989). 5 μg of total RNA was fractionated on a 1.2% agarose gel containing formaldehyde and then transferred to a nylon membrane (Schleicher and Schuell Bioscience). The membranes were hybridized with [α-^{32}P]-labeled probes at 65°C overnight in 5 × SSC, 5 × Denhardt's solution, 0.5% SDS with 20 μg/m*l* of denatured salmon sperm and washed in 2 × SSC supplemented with 0.1% SDS at RT for 10 minutes. This was followed by a final wash in 0.1 × SSC containing 0.1% SDS at 65°C for 20 minutes.

Southern analysis

Five micrograms of *B. mori* DNA were digested separately with *BamHI*, *EcoRI*, *HindIII* and *XhoI* that can't be mapped to a Bm SOD sequence and with *SacI* and *SwaI* that can be a mapped to Bm SOD sequence. The digests were separated on a 0.8% agarose gel and blotted on to a nylon membrane (Schleicher and Schuell Bioscience). The membrane was hybridized with 0.9 kb PCR product. The probe was labeled using a Random Primer Labeling Kit (Stratagene). The membranes were hybridized with [α-^{32}P]-labeled probes at 60°C overnight in 5 × SSC, 5 × Denhardt's solution and 0.5% SDS with 20 μg/m*l* denatured salmon sperm.

Results

Identification of Cu/Zn Superoxide dismutase (SOD) gene

Data from 2,446 ESTs were obtained from an embryo library. Of them, 246 ESTs showed an expression level that was at least two-fold higher before the germ-band formation stage, and 37 ESTs were at least two-fold higher, after a germ-band formation stage. Among the genes that displayed higher expression at an early embryo stage included Cu/Zn SOD, cecropin, serpin and hemolin binding protein and yippee related with immune. Among the early embryo-upregulated genes, a gene that showed a higher expression at the blasterm stage was found to have amino acid sequence similar to that of the Cu/Zn SOD gene, and it was designated Cu/Zn SOD (Fig. 1A).

Bm Cu/Zn SOD

Cu/Zn SOD was isolated by DNA microarray analysis and appeared to be an up-regulated gene the at unfertilization, fertilization and blastoderm stages (Fig. 1B). Cu/Zn SOD has an open reading frame of 937 nucleotides, which encodes a protein of 154 amino acid residues. The sequence encoding BmCu/Zn SOD was deposited in GenBank (Accession no AY461705). The molecular mass and Pi of the putative SOD were evaluated to be 15,840 and

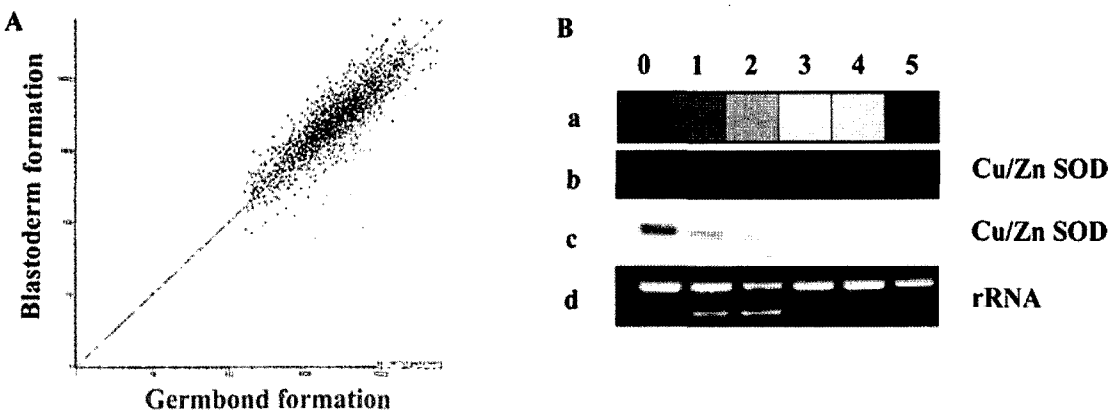

Fig. 1. Analysis showing the expression pattern of the Cu,Zn-superoxide dismuatse gene. A, Scatter plot of fluorescent signal intensity from a hybridaztion of Germband and Blastoderm formation with Cy3 and Cy5 labeled proves respectively. Lines show a two-fold expression in both channels; B, Expression of Bm Cu/Zn SOD; a and b, Cluster analysis showing expression patterns by Cu,Zn SOD at an early embryonic development. Red cells, high levels of expression (positive): Green cells, low levels of expression (negative) : Gray cells, moderate expression levels (missing) and Black cells, unexpression (zero); 0, unfertilization: 1, fertilizatioin: 2, blastoderm: 3, germband: 4, spatula: 5, abdominal leg appendages formation; c, Northern blot hybridization showing the pattern of expression of Cu,Zn SOD at the egg stage; d, rRNA is shown as a control.

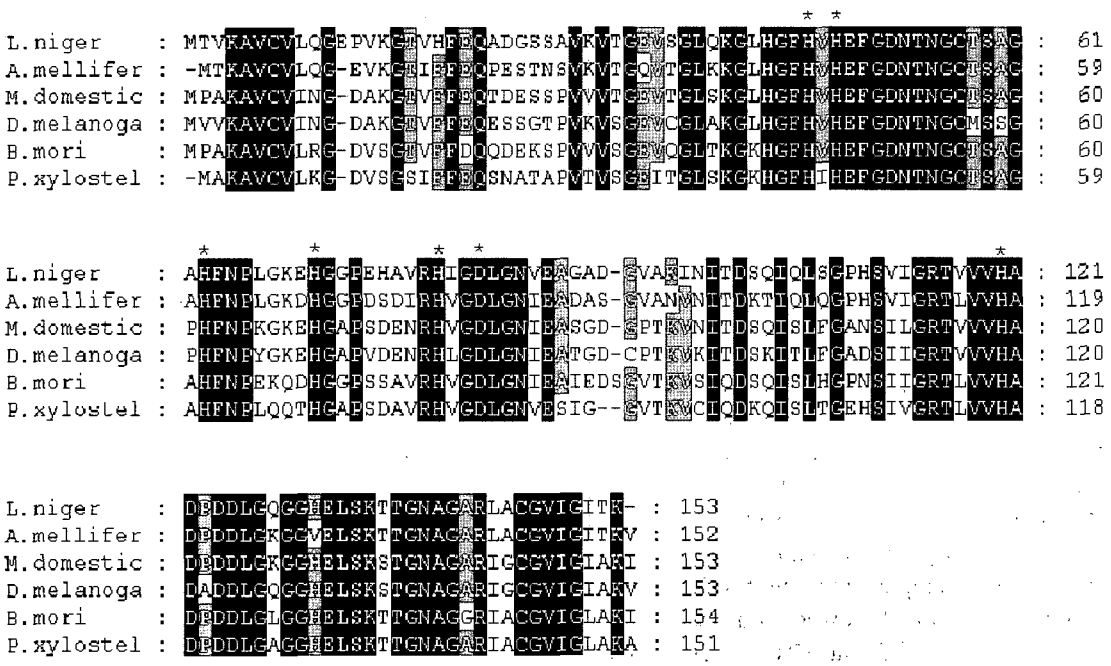

Fig. 2. Multiple alignment of amino acid sequences of 5 Cu,Zn SODs, from *Musca domestica* (AAR23787), *Drosophila melanogaster* (CAA35210), *Lasius niger* (AAQ81639), *Apis mellifera* (AAP93581), *Plutella xylostella* (BAD52256) and *B. mori* (AAR97568). The black shaded boxes refer to fully conserved and the light gray shaded boxes refer to highly conservered. Asterisks refer to cupper and zinc binding amino acids. Putative amino acid for Cu-binding (His 43, His46, His 63 and His 120) putative amino acid for Zn-binding (His 63, His 71, His 80 and Asp 83): glycine conserved in all species.

6.04, respectively. The deduced amino acid sequence of the protein displays a high similarity to the sequences Cu/Zn SOD from *M. domestica* (77%), *A. mellifera* (71%), *D. willistoni* (70%), *D. virilis* (69%), *D. teissieri* (68%) and *D. orena* (68%). Conceptual translation provided a protein sequence with all functionally important metal binding sites and two Cu/Zn SOD signature sequences (Fig. 2). As shown in Fig. 2, there were seven residues for the Cu or Zn binding sites in the sequence. The insect Cu/Zn SODs were annotated as possible Cu/Zn SODs based on the presence of the Cu/Zn SOD signature sequences. The novel BmCu/Zn SOD has Cu/Zn SOD signature sequences (Fig. 2).

In a phylogenetic analysis, BmCu/Zn SOD was clustered with insects rather than with other vertebrates (Fig. 3). Within the insect clade, the BmCu/Zn SOD was grouped with the Drosophila Cu/Zn SODs rather than with the other insect Cu/Zn SODs (Fig. 3).

The gene-containing fragments were identified by Southern blot hybridization using 0.3 kb probe, the sequences from position 151 to 433. Restriction digests were carried out according to standard procedures. Southern blot hybridizations were done on total silkworm genomic DNA (6 μg per reaction) cut with six restriction enzymes. *BamHI*, *EcoRI*, *HindIII* and *XhoI* enzymes don't have any recognition sites within the Cu/Zn SOD gene and *ScaI* and *SwaI* have each one recognition site within the gene. *ScaI* has one recognition site within the sequence used as a probe (position 300) and *SwaI* enzyme (position 579) has no recognition site within probe sequences. We can observe, in fact, only one band produced by each enzyme hydrolysis used for the construction of the restricted genomic DNA (Fig. 4). Only a single band of the size is obtained for experiment (Fig. 4), indicating the presence of a single copy gene.

Development and tissue distribution of the BmCu/Zn SOD

BmCu/Zn SOD was detected by northern blot analysis in each tissue of each development. BmCu/Zn SOD mRNA could be detected by northern blotting in larvae, prepupae, pupae and adults (Fig. 5), indicating that it is constitutively expressed at a low level. The product corresponding to BmCu/Zn SOD showed a high in intensity in the ovaries and testes of the pupae stage. The SOD gene is expressed constitutively in all tissues and stages.

Also, BmCu/Zn SOD m RNA was detected by northern blot analysis in early embryogenesis (Fig. 6). Northern analysis indicated that BmCu/Zn SOD is a rare transcript in all embryo stage. Its level was expressed clearly in the

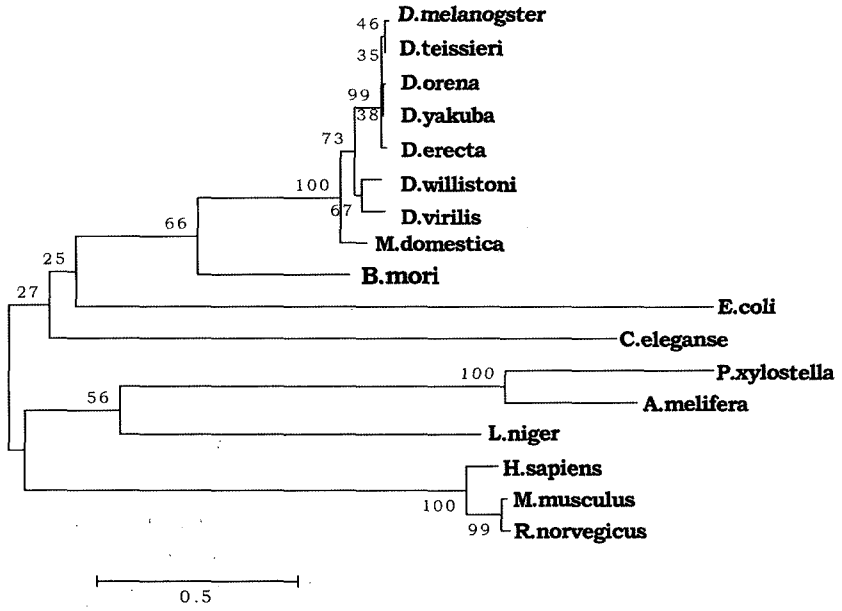

Fig. 3. Phylogenitic trees of twelve insects (eight species of the melanogaster subgroup, two species of the Hymenoptera and one specie of the Lepidoptera), *E. coli*, *C. eleganse* and three mammals based Cu,Zn SOD sequences. A phylogenetic tree was built on the alignment of the SOD sequences obtained from MEGA3: *Drosophila melanogaster* (CAA35210), *Drosophila teissieri* (AAF23599), *Drosophila orena* (AAF23594), *Drosophila yakuba* (AAF23598), *Drosophila erecta* (AAF23595), *Drosophila willistoni* (AAA57250), *Drosophila virilis* (CAA32060), *Musca domestica* (AAR23787), *Escherichia coli* (ZP_00722777), *Caenorhabditis elegans* (NM_062378), *Plutella xylostella* (BAD52256), *Apis mellifera* (AAP93581), *Lasius niger* (AAQ81639), *Homo sapiens* (AB05661), *Mus musculus* (NP_035564) and *Rattus norvegicus* (AP036734). The tree was made with Neighbour-joining plot software. The number represents the bootstrap values.

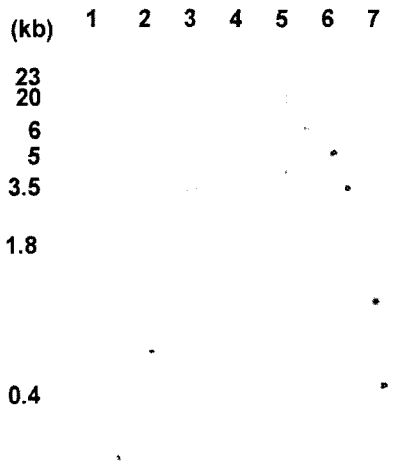

Fig. 4. Southern analysis of silkworm genomic DNA. Six μg/lane of digested genomic DNA (using five different restriction endonucleases: 1, not digested DNA; 2, *BamHI*; 3, *EcoRI*; 4, *HindIII*; 5, *XhoI*; 6, *SacI*; 7, *SwaI*) was loaded on gel. Southern blot membrane was probed with 300 bp PCR product to the gene of the silkworm Cu/Zn SOD.

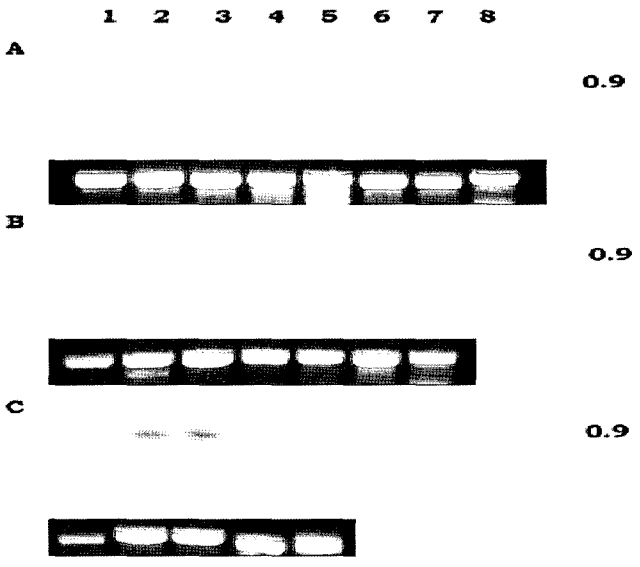

Fig. 5. Northern blotting of the *Bm* Cu,Zn-SOD transcript in each development stage. (A) larvae stage, (B) pre-pupa (C) pupa and adult; (A)1-8: pre-molting of 3th instar, molting 3th instar, post-molting of 3th instar, fatty body, mid-gut, ovaries, testes and hemocyte; (B) 1-7: fatty-body, mid-gut, ovaries, testes, brains, skin and silkglands; (C) 1-5: fatty-body, ovaries, testes, adult female and male.

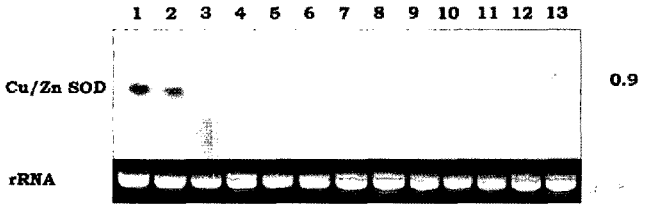

Fig. 6. Expression profile of *Cu/Zn SOD* transcript in different developmental stages. 1, unfertilization eggs; 2, fertilization eggs (1-2 h After Oviposition); 3, blastoderm formation eggs (8-10 h A.O); 4, germband formation eggs (1 day A.O); 5, spatula stage eggs (2 day A.O); 6, abdominal legs appendage formation stage eggs (3 day A.O); 7, Blastokinesis (4 day A.O); 8, Blasotkinesis terminate (5 day A.O); 9, Blistle and caudal legs (6 day A.O); 10, organ fiber (7 day A.O); 11, blue spot state (8 day, A.O); 12, before hatching stage (9 day, A.O); 13, hatching stage (10 day, A.O). rRNA is shown as a control. The molecular sizes are shown to the right and left of the figures.

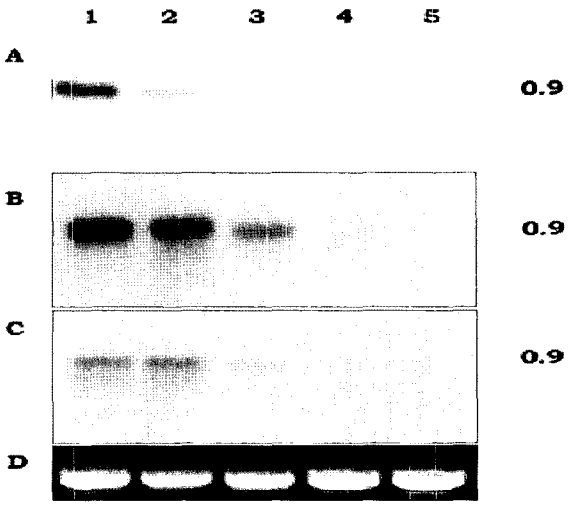

Fig. 7. The expression of the *Cu/Zn SOD* profile from mutants. A, *kl20* (normal, korean native strain); B, *malk•malp16* (segment malformation strain); C, *kd4140* (egg malformation strain); 1, unfertilization egg (0 h After oviposition); 2, blastoderm formation eggs (8-10 h AO); 3, germband formation eggs (24 h AO); 4, spatula stage eggs (48 h AO); 5, abdominal legs appendage formation stage (72 h A0). rRNA is shown as a control. The molecular sizes are shown to the right of the figures.

unfertilization, fertilization and blastoderm stages. Its level, however decreased significantly at 24 h post-fertilization (Fig. 6). There was no difference in the size from tissue to tissue and development stage.

Expression of the BmCu/Zn SOD in mutants

In order to know whether additional BmCu/Zn SOD affects embryonic development in silkworms, we investigated the BmCu/Zn SOD expression by northern blot analysis using mutants, segment malformation and egg-shape malformation strains during early embryogenesis (Fig. 7).

Northern analysis revealed that the BmCu/Zn SOD gene was expressed at the early embryonic stage in mutants and the correct size BmCu/Zn SOD mRNA was produced in each the mutant. The level of BmCu/Zn SOD-specific mRNA was standardized relative to normal transcripts between the unfertilization (0 h After Oviposition) and abdominal appendage leg-formation stage (72 h AO). After comparing the transcription to the normal strain, the BmCu/Zn SOD mRNA levels in the segment malformation mutants were higher (Fig. 7B), but levels in the egg-shape malformation were lower (Fig. 7C). Differences in the SOD expression between the mutants may be implicated as an important development of the early embryo.

Discussion

The result of applying a microarray analysis to the screening of genes associated with early embryo development was that 246 ESTs showed expression levels that were at least two-fold higher before the germ-band stage (24 h after oviposition, HCl treatment time point for artificial hatching). On the other hand, 37 ESTs were expressed at least two-fold lower after the germ-band stage. We found that the BmCu/Zn SOD among the genes showed a higher expression at an early embryonic stage. This supported the idea that microarray analysis could be adapted to the screening of developmentally regulated genes.

The expression of BmCu/Zn SOD was observed between the unfertilization (Oh AO) and the germ-band formation (24 h AO) stages of total embryo development. SODs is a ubiquitous metalloenzyme in aerobic organisms that plays a crucial role in protecting organisms against toxic effects caused by ROS (Bannister, Bannister and Rotilio, 1987). The SOD gene is expressed constitutively in all tissues (Seto, Hayashi and Tener, 1990). We discovered a BmCu/Zn SOD in the early embryo of the silkworm, *B. mori.* This gene exhibits 77% sequence homology to the housefly, *M.domestica.* Phylogenetic analysis also showed that the BmCu/Zn SOD is closely related to the Drosophila Cu/Zn SOD, which was already reported. In the amino acid sequence of the BmCu/Zn SOD, seven of the residues interacting with Cu/Zn were conserved. Important roles for SOD function and structure are played by histidine residues 45, 47, 62, 70, 79 and 120, as well as

Asp 82, that contribute to the metal binding. Thus, His45, His47, His62, and His120 and His62, His70, His79 and Asp 82, the crucial amino acids for Cu and Zn binding, respectively, are conserved for all species examined (Arhontaki, Eliopoulos, Goulielmos, Kastanis, Tsakas, Loukas and Ayala, 2002). The conservation rate is also high around the active site, at the positions where the amino acids interact with amino acids that are linked to the metals. All residues that are hydrogen-bound to the Cu-linked amino acids, such as His42, Gly43, Thr116, Val118, Asp121, and Gly138, are conserved. Furthermore Asn64, Arg77 and His78, which are found in the Zn binding region and make hydrogen bonds to the Zn-linked amino acids, are also conserved. SODs is a highly conserved enzyme and the critical residues in the enzyme active site were found to be invariant in many known insect sequences, including *B. mori*.

For a better understanding of the tissue distribution of the BmCu/Zn SOD, we examined the expression of its mRNA at the larvae, pre-pupae, pupae and adult stages. The BmCu/Zn SOD was widely distributed in all tissues tested at the larvae stage but BmCu/Zn SOD of the pre-pupae, pupae and adult stages was distributed in the ovaries, testes, brain and skin (Fig. 4). We also examined the distribution of the BmCu/Zn SOD of mutant embryo stage. Considering the role of BmCu/Zn SOD, the embryo distribution of the transcript in the silkworm mutants is to be expected. Mutant strains, segment malformation and egg-shape malformation used experiments that have a malformation factor from the embryonic stage. The distribution of mutant BmCu/Zn SOD was similar to that of the normal strain. BmCu/Zn SOD is constitutively expressed in a relatively high level at the early embryonic stage more than in the other development stages. A number of enzymes exist whose function appears to be the protection of cells from oxidative damage (Fridovich, 1978). Oxygen free radicals are generated as intermediates during normal metabolism (Halliwell and Gutteridge, 1984), and even more so when organisms are exposed to such environmental conditions as active compounds, radiation, and other stress agents. Increased levels of oxygen radicals can damage cells by oxidizing many bio-molecules, which can result in several diseases, such as mutation, carcinogenesis, aging and cell death (Harman 1981; Fleming, Miquel, Cottrell, Yengoyan and Economos, 1982). Judging by the widespread occurrence of Cu/Zn SOD in silkworms, it seems clear that a type of protection is essential. It is less clear what exactly causes the damage.

In conclusion, by using ESTs and microarray analysis, we have found strong evidence for the existence of BmCu/Zn SOD in the early embryonic stage. Sequencing of the gene revealed extensive homology with other insect Cu/Zn SODs. Expression analysis indicated that the BmCu/Zn SOD gene is transcribed in most of tissues of the silkworm and in the early embryonic stage. With the presence of BmCu/Zn SOD in early embryonic stage, the possibility remains that the BmCu/Zn SOD plays some other role in dioxygen metabolism. We believe that this is an important step in elucidating the specific functions of Cu/Zn SOD and in elucidating the role of superoxides in embryonic development.

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