The cDNA Sequences of Equine Antioxidative Enzyme Genes Cu/Zn-SOD and Mn-SOD, and These Expressions in Equine Tissues

Nobushige ISHIDA, Yoshinari KATAYAMA, Fumio SATO, Telhisa HASEGAWA and Harutaka MUKOYAMA
Equine Research Institute, Japan Racing Association, 321-4 Tokami-cho, Utsunomiya, Tochigi 320-0856, Japan
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ABSTRACT. The entire cDNA sequences were determined by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) techniques for equine copper/zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) through the use of total RNA extracted from the testis of an adult Thoroughbred. The results revealed a protein coding region for equine Cu/Zn-SOD with bases totaling 465 bp, accompanied by an estimated 154 residues of amino acids. As for equine Mn-SOD, its coding region contained a total of 669 bp and an estimated 222 residues of amino acids. Further, the expression of Cu/Zn-SOD and Mn-SOD genes were confirmed in the equine tissues by RT-PCR and *in situ* hybridization. —KEY WORDS: equine, *in situ* hybridization, RT-PCR, SOD.

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Organisms are inevitably exposed to active oxygen, which may denature their cell elements, proteins, lipids, and enzymes, leading to a variety of disorders, including inflammation [8], cancer [24], arteriosclerosis [25], myocardial injury [14], and aging [19]. To protect tissue from active oxygen, organisms are equipped with a multi-stage biological defense system. Cellular defense against superoxide anion is provided by a family of metalloenzymes, the superoxide dismutases, which catalyze the following reaction: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ [7].

In mammals, the presence of the following three types of SOD has been confirmed: Cu/Zn-SOD found in the cytoplasm [17], Mn-SOD in the mitochondria [20], and EC-SOD in serum [16]. The cDNA sequences of these enzymes in humans have already been determined [2, 6, 23]. In this study, to allow us to carry out our basic research and enable clinical application regarding the role of active oxygen and antioxidative enzymes in equine biological functions, the cDNA sequences of Cu/Zn-SOD and Mn-SOD genes were first determined. Based on this data, the sequences of amino acids were then deduced. At the same time, the expressions of SOD genes in equine tissues were investigated by RT-PCR and *in situ* hybridization.

Total RNA from the testis of an adult Thoroughbred was extracted by guanidinium/cesium chloride ultracentrifugation [4, 21], and used in cDNA synthesis with RNA PCR Kit (Takara, Kyoto) using random primer. For both Cu/Zn-SOD and Mn-SOD, RT-PCR was performed using primers designed by referring to highly conserved regions of cDNA sequences between humans [2, 23] and mice [3, 9]. The PCR products were cloned to T-vector (pCRTMII, Invitrogen, San Diego), and these cDNA sequences were determined by the dideoxy chain termination method [22]. Based on the cDNA sequences identified using the RT-PCR products, new PCR primers were designed. The entire cDNA sequences of equine Cu/Zn-SOD and Mn-SOD genes were then determined by 5' RACE and 3' RACE methods [8] using a 5'/3' RACE Kit (Boehringer Mannheim, Tokyo). The expressions of SOD genes in equine tissues were

confirmed by RT-PCR and *in situ* hybridization. Total RNAs for RT-PCR were extracted from the nine tissues; testis, adrenal gland, spleen, cerebrum, cerebellum, heart, thyroid gland, kidney, and liver. For *in situ* hybridization, the clones of equine Cu/Zn-SOD and Mn-SOD genes were digested with the restriction enzyme *Spe* I. *In vitro* transcription was conducted to produce RNA probes using a DIG-RNA Labeling Kit SP6/T7 (Boehringer Mannheim, Tokyo). *In situ* hybridization was conducted using a deparaffinated pieces of spleen tissue and these probes. Colorization was then performed using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Tokyo) [15].

Figures 1 and 2 show the cDNA sequences of equine Cu/Zn-SOD and Mn-SOD, together with deduced amino acid sequences. The 648-bp nucleotide sequence of Cu/Zn-SOD cDNA contains 79 bp of the 5' flanking untranslated sequence, and a total of 465 of open reading frame coding 154 amino acids, 153 of which are considered to be mature peptides based on a report on humans [23]. The result was identical with the previously reported equine cDNA sequence [5]. The 971-bp nucleotide sequence of Mn-SOD cDNA, on the other hand, contains 69 bp of the 5' flanking untranslated sequence, and a total of 669 of open reading frame coding 222 amino acids. Comparing the deduced amino acid sequence with the known amino-terminal of mature human Mn-SOD, the first 24 amino acids of the primarily translated precursor may constitute the leader peptide for transport to the mitochondria [1].

Compared with other mammalian species, an 83% homology was found in the Cu/Zn-SOD cDNA sequence for both equine genes with humans [23] and bovines [10], while an 81% homology was found with mice [3] and rats [11]. In amino acids, the homology was approximately 81% with humans [23], bovines [10], mice [3], and rats [11]. For Mn-SOD, on the other hand, an 89% homology was found in the cDNA sequence with humans [2], while an 84% homology was found with mice [9] and rats [12]. In amino acids, the homology was 92% with humans [2] and approximately 88% with mice [9] and rats [11]. These

CTAGTTTG 8
 CCTTCGCTCGCGCTCTTCTGCTGCTGTGGTCCCTTCGGATCTCGAGCAGAGCGCGACCCCGCAGTC 79
 1 10
 Met Ala Leu Lys Ala Val Cys Val Leu Lys Gly Asp Gly Pro Val His Gly Val
 ATG GCG CTG AAG GCC GTG TGC GTG CTG AAG GGC GAT GGC CCG GTG CAC GGC GTC 133
 20 30
 Ile His Phe Glu Gln Gln Gln Glu Gly Gly Pro Val Val Leu Lys Gly Phe Ile
 ATC CAC TTC GAG CAG CAG CAG GAA GGT GGC CCC GTC GTT CTG AAG GGA TTC ATT 187
 40 50
 Glu Gly Leu Thr Lys Gly Asp His Gly Phe His Val His Glu Phe Gly Asp Asn
 GAA GGA TTA ACC AAA GGC GAT CAC GGA TTC CAC GTC CAC GAG TTT GGA GAT AAT 241
 60 70
 Thr Gln Gly Cys Thr Thr Ala Gly Ala His Phe Asn Pro Leu Ser Lys Lys His
 ACA CAA GGC TGT ACC ACT GCT GGT GCT CAC TTT AAT CCT CTG TCG AAA AAA CAC 295
 80
 Gly Gly Pro Lys Asp Glu Glu Arg His Val Gly Asp Leu Gly Asn Val Thr Ala
 GGT GGA CCA AAG GAG GAA GAG AGG CAT GTT GGA GAC CTG GGC AAT GTG ACT GCT 349
 90 100
 Asp Glu Asn Gly Lys Ala Asp Val Asp Met Lys Ser Ser Val Ile Ser Leu Ser
 GAT GAA AAT GGT AAG GCT GAT GTG GAT ATG AAA GAT TCT GTG ATC TCA CTC TCG 403
 110 120
 Gly Lys His Ser Ile Ile Gly Arg Thr Met Val Val His Glu Lys Gln Asp Asp
 GGA AAG CAT TCC ATC ATT GGC GCG ACA ATG GTG GTC CAC GAG AAA CAA GAT GAC 457
 130 140
 Leu Gly Lys Gly Gly Asn Glu Glu Ser Thr Lys Thr Gly Asn Ala Gly Ser Arg
 CTG GGC AAA GGT GGA AAT GAA GAA AGT ACA AAG ACG GGC AAC GCT GGC AGT CGT 511
 150 153
 Leu Ala Cys Gly Val Ile Gly Ile Ala Pro ***
 CTG GCT TGT GGT GTG ATT GGG ATC GCT CCG TAA ACATCCCCGTGCTGGTCTGAGTCC 571
 TAGTAACATCATCTGTTATCTTCTGCTAGTTGTAAGAAATTTAACTTGATAACATTAACGCTGTAACTTAA 641
 AAAAAAA 648

Fig. 1. Nucleotide sequence and deduced amino acid sequence of equine Cu/Zn superoxide dismutase. The amino acid sequence corresponding to the entire mature protein is numbered 1–153.

findings demonstrate that both genes are conserved highly among mammals.

The expressions of two SOD mRNAs were confirmed in all of the nine tissues by RT-PCR. From this result, it was suggested that two SOD mRNAs would be widely expressed in equine tissues. Using the probe RNA transcribed from SOD cDNA separated in our experiment, we performed *in situ* hybridization of the spleen tissue. The SOD distribution thus obtained revealed the notable presence of Cu/Zn-SOD in the germinal center of the spleen tissue. Mn-SOD expressed itself in this region, although only slightly. This confirms the effectiveness of the SOD cDNA clones as probes for SOD gene distribution in tissue, enabling us to employ it in examining the degree of SOD expression in damaged tissues.

It should be noted that the 190th of mature equine Mn-SOD peptides differs from that found in humans [13]. Since this region is regarded as the antigen recognition site in humans [13], we have already begun developing a quantitative analysis method for Mn-SOD in equine serum by the antipeptide antibody method, through the use of an antipeptide antibody obtained from the cDNA sequence identified in our study.

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GACGCGCTCCGCGCGGCGCGTGTGCGCGCGATCTCCGAGTGCTTTCGCTTTCGACGGTCACC 69
 -24 -20 -10
 Met Leu Cys Arg Ala Ala Cys Ser Thr Ser Arg Lys Leu Val Pro Ala Leu Gly
 ATG TTG TGC CGG GCG GCG TGC AGC ACG AGC AGG AAG CTG GTG CCA GCT CTG GGG 123
 1 10
 Ser Leu Gly Ser Arg Gln Lys His Ser Leu Pro Asp Leu Gln Tyr Asp Tyr Gly
 TCT CTG GGG TCC CGG CAG AAG CAC AGC CTC CCC GAC TTG CAG TAT GAT TAT GGC 177
 20 30
 Ala Leu Glu Pro Tyr Ile Asn Ala Gln Ile Met Gln Leu His His Ser Lys His
 GCT CTG GAG CCC TAC ATC AAC GCC CAG ATC ATG CAG CTG CAC CAC AGC AAG CAC 231
 40
 His Ala Ala Tyr Val Asn Asn Leu Asn Val Thr Glu Lys Tyr Gln Glu Ala
 CAC GCG GCC TAT GTG AAC AAC CTG AAC GTC ACC GAG GAG AAG TAC CAG GAG GCG 285
 50 60
 Leu Ala Lys Gly Asp Met Thr Ala Gln Thr Ala Leu Gln Pro Ala Leu Lys Phe
 CTG GCC AAG GGG GAC ATG ACA GCT CAG ACT GCT CTG CAG CCT GCA CTC AAG TTC 339
 70 80
 Asn Gly Gly Gly His Ile Asn His Thr Ile Phe Trp Thr Asn Leu Ser Pro Asn
 AAT GGT GGA GGC CAT ATC AAT CAT ACC ATT TTC TGG ACA AAC CTG AGC CCC AAT 393
 90 100
 Gly Gly Gly Glu Pro Lys Gly Glu Leu Leu Asp Ala Ile Lys Arg Asp Phe Gly
 GGC GGG GGA GAA CCC AAA GGG GAA TTG CTG GAC GCC ATC AAA CGT GAC TTT GGT 447
 110 120
 Ser Phe Asp Lys Phe Lys Glu Lys Leu Thr Ala Val Ser Ala Gly Val Gln Gly
 TCC TTC GAC AAA TTT AAA GAG AAG TTG ACT GCT GTA TCG GCT GGT GTC CAA GGC 501
 130
 Ser Gly Trp Gly Trp Leu Gly Phe Asn Lys Asp Gln Gly Arg Leu Gln Ile Val
 TCG GGT TGG GGT TGG CTT GGT TCT AAT AAG GAC CAG GGA GCG CTC CAG ATT GTT 555
 140 150
 Ala Cys Pro Asn Gln Asp Pro Leu Gln Gly Thr Thr Gly Leu Ile Pro Leu Leu
 GCC TGT CCT AAC CAG GAT CCC CTG CAG GGA ACA ACA GGT CTT ATT CCC CTG CTG 609
 160 170
 Gly Ile Asp Val Trp Glu His Ala Tyr Tyr Leu Gln Tyr Lys Asn Val Arg Pro
 GGG ATT GAC GTG TGG GAG CAC GCT TAT TAC CTT CAG TAT AAA AAT GTC AGG CCT 663
 180 190
 Asp Tyr Leu Lys Ala Ile Trp Asn Val Ile Asn Trp Glu Asn Val Ser Glu Arg
 GAT TAT CTG AAA GCC ATT TGG AAT GTG ATC AAC TGG GAG AAT GTA TCT GAG AGA 717
 198
 Tyr Met Ala Cys Lys Lys ***
 TAC ATG GCT TGC AAA AAG TAA AGCATTATCGTTACACCGAGTGCAATTAAGCAATTTCCAATAT 781
 TTTGTAGTAGCTCGAAGTACCACAGTATACCAAGCTGCTAGGATAGCATTTCTGAATGTGGCTTA 852
 TTCAGATATTTGATAAACGTAATGCTATGAATAATTTCTGTTTACAATTTTGTATTGGCAACTGTTT 923
 GAAAAATTAATGCTTTGTATGATTCGTTAAAAAATAAAAAAAAAAAAAA 971

Fig. 2. Nucleotide sequence and deduced amino acid sequence of equine manganese superoxide dismutase. The amino acid sequence corresponding to the entire mature protein is numbered 1–198.

Fig. 3. RT-PCR in equine tissues. (A): Cu/Zn-SOD. (B): Mn-SOD.

Fig. 4. ISH for the SODs mRNA in equine spleen. Magnification is X60. A: Positive hybridization signal (arrow head) with an anti-sense Cu/Zn-SOD RNA probe. B: Negative control with sense Cu/Zn-SOD RNA probe. C: Positive hybridization signal (arrow head) with an anti-sense Mn-SOD RNA probe. D: Negative control with sense Mn-SOD RNA probe.

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