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연구보문

Molecular Cloning and Expression of Sequence Variants of Manganese Superoxide Dismutase Genes from Wheat

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Reactive oxygen species (ROS) are very harmful to living organisms due to the potential oxidation of membrane lipids, DNA, proteins, and carbohydrates. transformed *E.coli* strain QC 871, superoxide dismutase (SOD) double-mutant, with three sequence variant MnSOD1, MnSOD2, and MnSOD3 manganese superoxide dismutase (*MnSOD*) gene isolated from wheat. Although all QC 871 transformants grown at 37 ℃ expressed mRNA of MnSOD variants, only MnSOD2 transformant had functional SOD activity. MnSOD3 expressed active protein when grown at 22℃, however, MnSOD1 did not express functional protein at any growing and induction conditions. The sequence comparison of the wheat MnSOD variants revealed that the only amino acid difference between the sequence MnSOD2 and sequences MnSOD1 and 3 is phenylalanine/serine at position 58 amino acid. We made *MnSOD2S58F* gene, which was made by altering the phenylalaine to serine at position 58 in MnSOD2. The expressed MnSOD2S58F protein had functional SOD activity, even at higher levels than the original MnSOD2 at all observed temperatures. These data suggest that amino acid variation can result in highly active forms of MnSOD and the *MnSOD2S58F* gene can be an ideal target used for transforming crops to increase tolerance to environmental stresses.

Key Words: Environmental stress, *Escherichia coli*, Expression, Manganese superoxide dismutase, Multigene family

INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and the hydroxyl radical (OH^-) are very harmful to living organisms because of their high oxidizing potentials in living cells (Bowler et al., 1992). Although ROS are generated on a regular basis in biological pathways as by-products or signal transducers (Wu et al., 1997; Zelko et al., 2002), plants can experience oxidative stress, which can occur under many environmental stresses (Allen, 1995). In oxidative stress, ROS are generatedtoo quickly or inefficiently scavenged by antioxidant systems (Halliwell and Gutteridge, 2007). ROS can attack all basic molecules of living organisms, such as lipids, carbohydrates, proteins, and nucleic acids, resulting in the peroxidation of membranes (Kendall and McKersie, 1989), breakage of polysaccharides (Sato et al., 1993), inactivation of enzymes (Fucci et al., 1983), and "nicking", cross-linkage and scission of DNA strands (Houot et al., 2001). Very efficient antioxidant enzyme systems have evolved in plants to scavenge ROS and resulting in protection from oxidation injury (Allen, 1995; Halliwell and Gutteridge, 2007). Up-regulated ROS scavenging systems usually manifest as increased activities of antioxidant enzymes from *de novo* synthesis (Allen, 1995). One of the most effective antioxidant enzymes is superoxide dismutase (SOD, EC. 1.15.1.1), which catalyzes the dismutation of two superoxide radicals (O_2^-) resulting in the pro-

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duction of H_2O_2 and O_2 (Halliwell and Gutteridge, 2007). Typically, H_2O_2 is then further broken down by catalase. SOD has several isozymes, which can be classified by their cellular location and specific catalytic metals in plants (Halliwell and Gutteridge, 2007; Zelko et al., 2002). MnSOD has Mn (III) at the active site and is localized to mitochondria. FeSOD has Fe(III) at the active site and is found in chloroplasts. Copper-zinc superoxide dismutase has Cu (II) plus Zn (II) at the active site and is generally found in the cytosol and chloroplasts in plants.

Among these isozymes, MnSOD is the only form of SOD essential for the survival of aerobic life (Carlioz and Touati, 1986). Furthermore, increase of MnSOD activity can confer more tolerance to many environmental stresses. Tolerance of chilling stress in *Zea mays* increased significantly when a tobacco MnSOD gene was expressed in the chloroplasts (Breuseman et al., 1999). Both freezing tolerance and recovery from freezing stress were enhanced in *Medicago sativa* transformed with a tobacco *MnSOD* gene targeted to the chloroplasts or mitochondria (McKersie et al., 1993). *MnSOD* transcripts increased significantly in both spring and winter wheat seedlings exposed to 2℃, presumably leading to more MnSOD enzyme production in the mitochondria (Wu et al., 1999; Baek and Skinner, 2003). Transgenic *Brassica* plants overexpressing a wheat MnSOD gene were more tolerant of both oxidative stress and aluminum toxicity (Basu et al., 2001). Ectopic expression of *MnSOD* gene from *Tamarix androssowii* in poplar plants enhanced salt tolerance (Wang et al., 2010). MnSOD enzyme can be used for several beneficial applications, such as an agent treating oxidative damage (McCord et al., 2001), and controlling dermal inflammatory reactions associated with chemical irritation and acne (Wilder et al., 1990), prolonged *in vivo* cell cycle progression and up-regulation of mitochondrial thioredoxin (Kim et al., 2010).

The mass of the MnSOD holoenzyme composed of four subunits in plants is estimated to be around 91 kDa (Streller et al., 1994). Many plants have several copies of *MnSOD* genes (Miao and Gaynor, 1993; Zhu and Scandalios, 1993; Streller et al., 1994 Baek and Skinner, 2006). Members of the MnSOD multigene family have slightly different genomic and amino acid sequences, however, the end products, MnSOD enzymes, have the same function (Zhu and Scandalios, 1993). DNA sequence information on wheat MnSOD genes in GenBank (http://www.ncbi. nlm.nih.gov/), TIGR (www.tigr.org), and GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml) and as well as sequence information obtained in our laboratory indicated that MnSOD in wheat comprised a multigene family with many members differing in nucleotide and amino acid sequence, as well as the 3'untranslated regions. Because both high and low temperature stress can result in ROS formation, MnSOD protein products from some of the sequence variants may be more active than others at specific temperatures.

In this study, we investigated the enzymatic activities of amino acid variants of wheat MnSOD genes expressed in SOD$^-$ *E. coli*. *E. coli* strain QC 871 (*sod*A$^-$, *sod*B$^-$ double mutant Carlioz and Touati, 1986) does not contain any MnSOD gene, therefore, there is no chance of making a complex with *E.coli* original MnSOD with expressed wheat MnSOD in the transformed QC 871 and detected SOD activity in transformed QC 871 solely comes from the newly expressed wheat MnSOD.

Materials and Methods

Molecular cloning of multiple MnSOD genes

Total RNA from the leaves of winter wheat 442 (Storlie et al., 1998) was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). About 200 mg of whole leaves collected from 2 week old wheat plants were ground in liquid nitrogen with a mortar and a pestle; 2 ml Trizol solution was added to the mortar, which froze to the mortar surface. After thawing, the total RNA was extracted according to the instructions provided with the Trizol reagent. The RNA was quantified with a spectrophotometer (Jasco V-530, Jasco Inc, MD, USA) and quality was assessed by running 2 mg on a 1.2% agarose gel under 1 x modified TAE buffer (100 mM Tris-acetate, 1 mM Na_2EDTA). RNA was stored at -80℃ before being used for RT-PCR.

Complementary DNA was made by using the total RNA and oligo dT primers with the ThermoScript kit (Invitrogen, San Diego, USA) according to the manufacturer's instructions. PCR was carried out on a PCR system 9700 (Applied Biosystems, Foster City, CA, USA) using the cDNA template. MnSOD specific primers were used as forward: 5' -

CACACACCAAACACACTATCCATG and reverse: 5' - TCATGCAAGCACTTTCTCATACTCT. The PCR profile was a 3 min denaturation at 95℃, then 32 cycles of 30 s at 95℃, 40 s at 52℃, and 5 min at 72℃. The PCR solution was composed of 1 x reaction buffer and 2 unit of Tag polymerase from Promega (Madison, WI, USA), 2.2 mM $MgCl_2$, 100 nM primers, and 1 μl of cDNA made from 250 ng total RNA in 25 μl reaction solution covered with 20 μl mineral oil. The PCR products were analyzed on 1.2% agarose gel under 1 x modified TAE buffer (100 mM Tris-Acetate, 1 mM Na_2EDTA), purified with a DNA gel extraction kit (Millipore, Bedford, MA, USA), and cloned into Topo-XL vector (Invitrogen, Carlsbad, CA, USA). Identity of the cloned PCR products was confirmed by sequencing, which was performed at the sequencing facility at Washington State University, Pullman. The ClustalW (Larkins et al., 2007) was used for DNA and protein sequence alignments.

Expression of the cloned MnSOD in SOD double mutant *E.coli*

Subclones without the 27 amino acid leader sequence targeted to mitochondria (Schrank et al., 1988; Wu et al., 1999) were generated with PCR using the clones described above as templates and primers (forward: 5' - ACCATGTGGCGACGTTCACG and reverse: 5' - TCATGCAAGCACTTTCTCATACTCT). The PCR product was cloned into pBAD-TOPO vector (Invitrogen, Carlsbad, CA, USA) for expressing protein in *E.coli*. The N-terminal leader in pBAD-Topo vector was removed by *Nco*I restriction digestion (Promega, Madison, WI, USA), followed by self-ligation. These synthetic MnSOD clones in the pBAD-Topo vector were used for transforming *E.coli* strain QC 871 (r^-m^+ derivative of *E.coli* QC 774 *sodA*$^-$, *sodB*$^-$ double mutant Carlioz and Touati 1986), which grew very slowly compared to wild type *E.coli*. Plasmids from the transformed QC 871 were extracted and sequenced to confirm correct configuration.

Cultures were grown in Luria-Bertani (LB) medium (Sambrook and Russell, 2001) for 12 hr at 37℃ with 200 rpm shaking to OD_{600} = 1 (Invitrogen pBAD Technical Manual). Expression of genes in the pBAD-Topo vector was induced by adding L-arabinose (Lee, 1980; Lee et al., 1987), therefore, various concentrations of L-arabinose with $MnCl_2$ (Sigma-Aldrich, St. Louis, MO) with different induction time were tested to induce the MnSOD protein. The cells were collected by centrifugation at 5000 g for 10 min, and resuspended in 0.5 ml of lysis buffer (50 mM Tris-HCl, 24 mM NaCl, 2 mM EDTA, pH 8). Cell lysis solutions were added 30 μl of lysozyme (10 mg/ml) and incubated at 37℃ for 30 min with slow inversion. Two μl of DNase I (10 units/ml, Stratagene, La Jolla, CA, USA) and 2.5 μl of $MgCl_2$ at 1 M were added to the cell lysis solution, and the solution was left at 37℃ for 10 min with slow inversion. The preparations were clarified by centrifugation at 14,000 g for 15 min. The supernatants were filtered through Ultrafree-15 Biomax-50 K (Millipore, Bedford, MA, USA) and the protein amount was quantified using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). In-gel SOD activity assessments were carried out on 10% nondenaturing acrylamide gels (Sambrook and Russell, 2001) for detection of using nitroblue tetrazolium (NBT) oxidation according to Beauchamp and Fridovich (1971).

Site-specific modification in the cloned *MnSOD* and the expression in SOD double mutant *E.coli*

PCR products of 210 bp were generated by PCR reactions using the *MnSOD1* and *MnSOD2* sequences as templates and primers, forward: 5' - ACCATGTG GCGACGTTCACG and reverse: 5' -AGGCTTGAGGT TCTTCCAGA. PCR was carried out as described above as time and conditions. The PCR products, and plasmids of MnSOD1 and MnSOD2 were digested using restriction enzymes, *Hpa*I and *Xho*I. The digested PCR fragment from *MnSOD1* was ligated to the digested *MnSOD2* plasmid, and the digested PCR fragment from *MnSOD2* was ligated to the digested *MnSOD1* plasmid. The ligated plasmids were used to transform Top 10 strain (Invitrogen, Carlsbad, CA, USA). Plasmids were extracted from the transformants, sequenced to confirm strucure, and used to transform QC 871 strain. The QC 871 transformants were examined whether to determine whether they expressed SOD activity using the non-denaturing acrylamide gel assay as described above. An assay of MnSOD activity in liquid buffer was carried out at 0, 10, 20, and 30℃ (Giannopolitis and Ries, 1977). A total of 120 mg of protein were incubated with SOD detection media under 140 umol quanta $m^{-2}s^{-1}$ for 10 min. One unit of SOD activity was defined as the amount of SOD which catalyzes a 50 % inhibition in the rate of reduction of

NBT under the specific conditions (McCord and Fridovich, 1969 Chen and Pan, 1996).Controls included extracts from the original QC 871 and QC 871 carrying only the pBAD-Topo plasmid.

Results

Expression of MnSOD protein in *E.coli*

Three cDNAs were acquired by RT-PCR, cloned into the pBAD-Topo vector without the signal peptides (Fig. 1). The newly cloned wheat MnSOD proteins have all key active-site residues for hydrogen-bonding required for MnSOD enzyme activity (H29, H33, Y39, H77, H97, Q149, D156, H160, Y164), which residues are preserved in human (Greenleaf et al. 2004). All multiple aligned plant MnSOD genes have more than 95 % identities. Especially, wheat MnSOD proteins were very slightly different, only have two or three amino acid difference among 205 amino acids. The cloned wheat MnSOD genes in pBAD-Topo vector were transformed into *E.coli* strain QC 871. The signal peptide of wheat MnSOD is necessary the successful targeting of the enzyme into mitochondria, however, should be removed for successful expression of proteins in *E.coli*. All transformants expressed mRNA for the wheat MnSOD (Fig. 2), and the prepared RNA had no DNA contamination (Fig. 2).

Clones MnSOD1, MnSOD2, MnSOD3, QC 871 Empty, and the original QC 871 were grown in LB medium for 12 hrs at 37℃ and further incubated with 0.004% (w/v) L-arabinose and 1 μM $MnCl_2$ for 6 hr. RT-PCR was performed to confirm the expression of mRNA of MnSOD, and PCR using the total extracted RNA was also performed to check whether there was any contamination of plasmid DNA (Fig. 2). Each of the cDNA clones were expressed as mRNA (Fig. 2), and no contaminating plasmid was found in the extracted total RNA (Fig. 2).

Although QC 871 clones MnSOD1, 2, and 3 expressed mRNA of the wheat *MnSOD* gene when grown at 37℃, only clone *MnSOD2* expressed detectable SOD activity (Fig. 3). The best induction conditions were using LB medium, 0.004% final concentration of L-arabinose, and 1 μM $MnCl_2$. Many attempts were tried to express MnSOD protein from the clone 1 and clone 3 by changing the inducing conditions, such as different temperature (22, 28, 32, 35, 37, and 42℃), different growing time (4, 6, 8, 12, and 24 hrs), different L-arabinose concentrations (0.0004, 0.001, 0.004, 0.008, 0.04, and 4%), various incubation media (LB, SOB, SOC, Terrific broth, 2 x YT, and NZCYM media) (Sambrook and Russell, 2001), different pH (pH 4, 5, 6, 7, 8, 9, and 10). All these treatments were performed to express active wheat MnSOD enzyme. Especially, different temperatures and incubation media were treated to determine the best conditions for active wheat MnSOD enzyme, however, the temperatures and incubation media did not have any

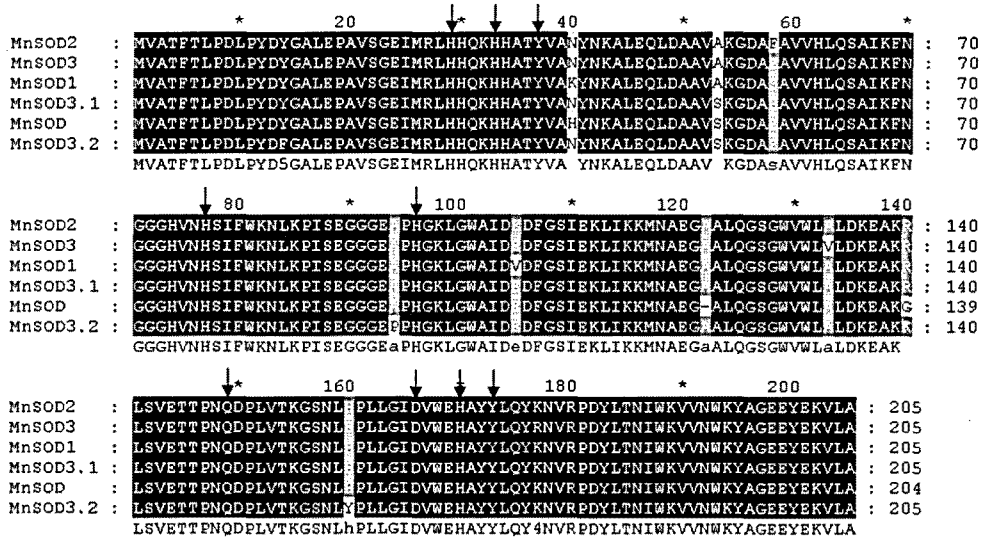

Fig. 1. Amino acid sequences of wheat MnSOD variants. Three wheat MnSOD protein sequences at GenBank [AF092524 (MnSOD), U72212 (MnSOD3.1), and U73172 (MnSOD3.2)] and three MnSOD protein sequences (MnSOD1, 2, and 3) acquired in our laboratory were aligned. Arrows show the key active-site residues for SOD activity in wild-type MnSOD.

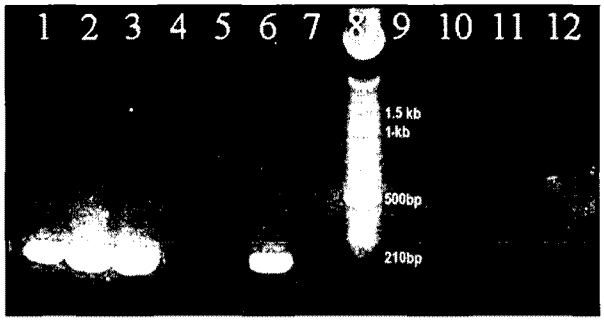

Fig. 2. Photograph of products of RT-PCR (lane 1-6) and PCR (lane 10-12) on 1.2% agarose gel. One-step RT-PCR reaction (Superscript One-Step Kit, Invitrogen, San Diego, USA) was performed using total RNA templates extracted from clone MnSOD1 (lane 1), MnSOD2 (lane 2), MnSOD3 (lane 3), QC 871 carrying empty pBAD-Topo vector (lane 4), and original QC 871 strain (lane 5), or plasmid DNA (lane 6). PCR was also performed using total RNA as the template; clone MnSOD1 (lane 10), MnSOD2 (lane 11), and MnSOD3 (lane 12). One μg of 1 kb plus ladder (Invitrogen, Carlsbad, CA, USA) was loaded on lane 8.

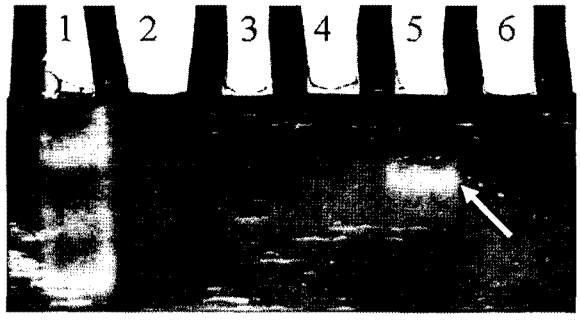

Fig. 3. SOD activity staining of the 10% non-denaturing gel loaded with proteins extracted from clone MnSOD1, MnSOD2, and MnSOD3. Protein extracted from young wheat leaves (lane 1), QC 871 (lane2), QC 871 Empty (lane3), clone MnSOD1 (lane 4), clone MnSOD2 (lane 5), and clone MnSOD3 (lane 6) was loaded on 10% native acrylamide gel and stained for visualization of the SOD activity. The arrow on lane 5 shows the inhibition of NBT oxidation by the expressed MnSOD enzyme.

effect on the protein expression. Various L-arabinose concentrations were tested for the best working concentration for inducing highest MnSOD proteins. QC 871 carrying clone MnSOD3expressed SOD activity only when the transformants was grown at 22℃. MnSOD activities were never detected in the clone MnSOD1 protein extracts at any grown temperatures and conditions.

Western blotting was performed to confirm no *E.coli* MnSOD was expressed in QC 871 after transformation with wheat *MnSOD* genes (not shown). All of the clones lacked *E.coli* MnSOD enzymatic activity (Fig. 3). SOD activity staining of the nondenaturing gels pretreated with 5 mM H_2O_2 revealed that the expressed SOD was insensitive to H_2O_2, characteristic of MnSOD protein (Rubio et al., 2001).

Amino acid swapping test of MnSOD

The sequences of wheat MnSOD1, 2, and 3 were 98~ 99% homologous, however, only clone MnSOD2 had SOD activity at 37℃ expression conditions. The sequence comparison of the MnSOD1, 2, and 3 revealed that the only amino acid difference between thesequence MnSOD2 and sequences MnSOD1 and 3 was phenylalanine (F) at position 58 amino acid (sequence MnSOD2: F and sequences MnSOD1, 3:serine (S)) (Fig. 1). We swapped the F to S at position 58 amino acid in sequence MnSOD2 (Sequence MnSOD2S58F) (Fig. 4), and transformed QC 871 with sequence MnSOD2S58F to make clone MnSOD2S58F, which was expected not to have the SOD enzymatic activity. However, clone MnSOD2S58F still had SOD activity. Sequence N4 was made by changing F at position 58 amino acid (SF) and R at position 62 (H R) in sequence MnSOD1. The transformed QC 871 with the sequence N4 did not show any SOD enzymatic activity (data not shown).

Measurement of the enzymatic activity of the expressed MnSOD

The SOD activities of the protein extract from MnSOD2 or MnSOD2S58F at 37℃ expression conditions composed of LB liquid medium, 0.004% final concentration of L-arabinose, and 1 μM $MnCl_2$ were measured by the oxidation hindrance of NBT at different temperatures (Fig. 5). Due to unsuccessful induction of other sequence variants, only SOD enzymatic activities of MnSOD2 or MnSOD2S58F were determined. The clone MnSOD2 had SOD activity ranging from 0.65 to 0.75 units and clone MnSOD2S58F had SOD activities ranging from 0.85 to 1.05 units when they were incubated from 0 to 30℃. The highest activities for SOD were observed when the protein extracts from clones MnSOD2 and MnSOD2S58F were incubated at 30℃ (0.75 and 1.05 units, respectively). The biggest difference in the SOD enzymatic activitiesbetween protein extract from clone MnSOD2 and that from clone MnSOD2S58F was observed at 10℃ reaction condition, in which condition

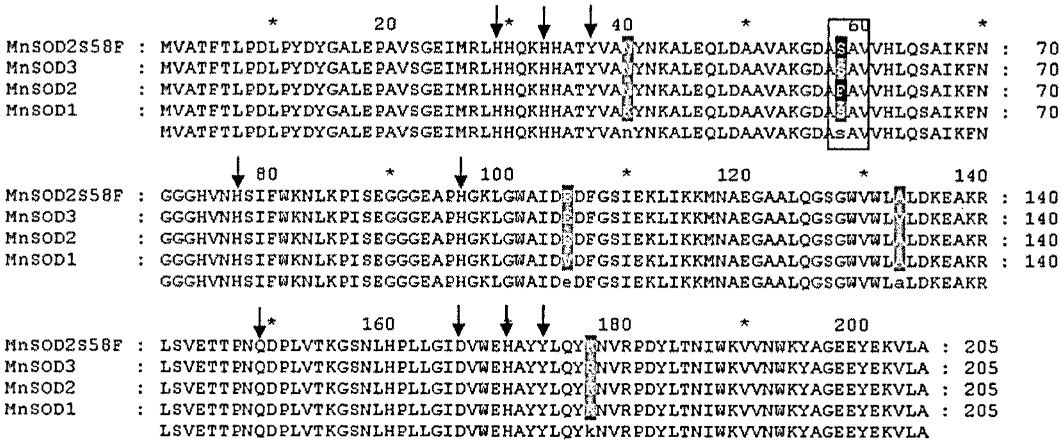

Fig. 4. Alignment of the amino acid sequences for the wheat MnSOD clones using ClustalW program. The MnSOD amino acid sequences were compared between MnSOD2 and MnSOD2S58F. The sequence of MnSOD2S58F was artificially made by changing one amino acids of MnSOD2. Arrows show the key active-site residues for SOD activity in wild-type MnSOD.

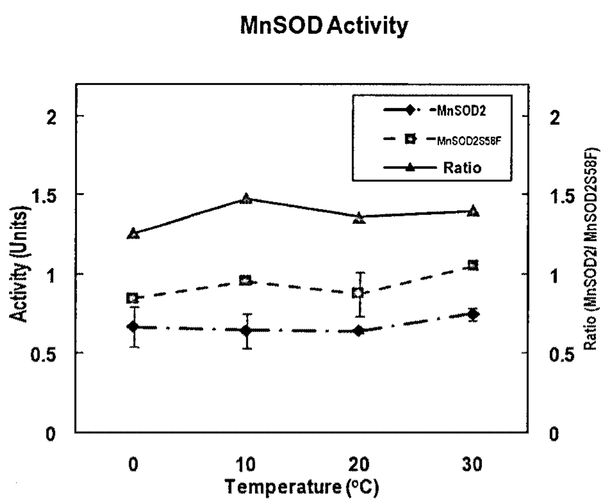

Fig. 5. Measurement of SOD activities from the protein extracts of clone MnSOD2 and clone MnSOD2S58F at different temperatures. 120 μg of protein extracts were incubated with SOD detection media for 10 min under 140 μmol quanta·m^{-2}·s^{-1} for 10 min. One unit of SOD activity was defined as the amount of SOD which catalyzes a 50% inhibition in the rate of reduction of NBT under the specific conditions. The protein extract of original QC 871 was used for the blank control for detecting the SOD activity.

protein extract from clone MnSOD2S58F had 48% more activity than that from MnSOD2 (Fig. 5).

Discussion

All QC 871 transformants carrying wheat *MnSOD* clone *MnSOD1*, *2*, or *3* expressed the wheat mRNA, however, only MnSOD2 expressed SOD activity when expression was induced at 37℃. MnSOD3 expressed SOD activity only when induced at 22℃ and MnSOD1 did not express activity under the conditions we tested. Heterologous expression of plant proteins in *E.coli* frequently results in the protein being expressed in inactive inclusion bodies (Fisher et al., 1993; Rudolph and Lilie, 1996). We do not know whether MnSOD1 and MnSOD3 formed inclusion bodies under the conditions we tested or whether position 58 amino acid, the only different amino acid between MnSOD2 and MnSOD1 and 3, prevented expression. When this amino acid in MnSOD2 sequence was swapped from F to S, the newly made variant (MnSOD2S58F) expressed SOD activity. In fact, the protein extract from clone MnSOD2S58F had greater enzymatic activities than clone MnSOD2 at all temperatures tested (Fig. 5). In human MnSOD, formation of a tetrameric interface by two identical four-helix bundles stabilizes the active sites (Borgstah et al., 1996). There is Ile58Thr human MnSOD, naturally occurring polymorphic MnSOD variant which causes two packing defects in each of the two four-helix bundles of the tetrameric interface. In solution, Ile58Thr MnSOD is primarily dimeric, and is significantly less thermostable than the normal enzyme. The sequence variances embedded in MnSOD1 and 3 might be a reason for the no sign of SOD enzymatic activity in solution. Furthermore, as shown in human

Ile58Thr MnSOD, which had decreases of 15℃ in the main melting temperature and 20℃ in the heat-inactivation temperature, MnSOD2S58F might have more stability than MnSOD2 by the single amino acid change. The stability of our newly characterized MnSOD protein products emphasizes again that MnSOD is an ideal scavenging enzyme protecting cells from ROS in various temperature conditions.

These data showed that even a single amino acid modification in MnSOD significantly changed SOD enzymatic activities. Although these experiments were done with heterologous expression of wheat *MnSOD* in *E.coli*, the results suggest that single amino acid variants in wheat plants also may have significantly different levels of expression. Since it now is known that wheat MnSOD genes comprise a multigene family, it is possible that highly efficient forms of MnSOD may be identified and utilized through a molecular breeding approach. Furthermore, the active wheat MnSOD sequence can be used for transforming monocot crops to make them more tolerant to environmental stresses relevant to producing oxidative stresses. Many environmental stresses, e.g., heat, chilling, freezing, salt, metal, ultraviolet light, etc., produce high amounts of superoxide anion directly and indirectly. As shown in transgenic corn or alfalfa, MnSOD can be the most appropriate SOD for increasing tolerance for environmental stresses, therefore, selection for the highest enzymatic activity for MnSOD will be one of the most important step for plant transformation.

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