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Expression and Characterization of Rice Manganese Superoxide Dismutase in *Escherichia coli*

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ABSTRACT: The corrected cDNA coding for rice mature MnSOD protein was made by PCR and inserted into pGEX-4T-1 expression vector. The recombinant DNA was transformed to *E. coli* BL21 and expression of GST-MnSOD fusion protein was induced by addition of IPTG to bacterial cultures. The homogeneous GST-MnSOD was purified by the one-step GST-glutathione affinity system. Both purified GST-MnSOD and recombinant MnSOD (rMnSOD) remained MnSOD activity, which showed an insensitivity towards KCN and H_2O_2. The molecular size of monomer of GST-MnSOD or rMnSOD was 50 kDa and 23 kDa respectively, and the functional form of both was dimer. The isoelectric point of rMnSOD was 4.64, but that of GST-MnSOD was in the range of pH 4.74 - 4.97. The SOD activities of GST-MnSOD and rMnSOD declined to 30 % after incubation at 60 ℃ for 20 minutes; but they were more stable in an alkaline pH environment.

KEY WORDS: Superoxide dismutase (SOD), MnSOD, *Oryza sativa*, *E. coli*.

INTRODUCTION

Superoxide dismutases (E.C. 1.15.1.1; SOD) are a group of metaloproteins that catalyze the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide (Beyer *et al.,* 1991). Superoxide, hydrogen peroxide and hydroxyl radicals are active oxygen species (AOS) and can be generated in a number of pathways (Fridovich, 1978). Accumulation of these AOS may cause deleterious effects on the different levels of organisms, such as lipid peroxidation, DNA strand breakage, and enzyme inactivation (Baum and Fridovich, 1981; Imlay and Linn, 1988). Defense systems including enzymatic and nonenzymatic antioxidants in living organisms can minimize the deleterious effects of AOS (Pell and Steffen, 1991). SOD is the first antioxidant enzyme to scavenge AOS, then catalases and ascorbate peroxidases are used to remove the toxic product H_2O_2 in plants. SODs may be classified into three types: CuZnSOD, MnSOD, and FeSOD forms. CuZnSOD, the most abundant form of SOD in higher plants, is located either in cytosol or in chloroplasts (Kanematsu and Asada 1989). MnSOD is primarily located in mitochondria (Jackson *et al.,* 1978) of higher plants. MnSOD has also been detected in the peroxisomal matrix (Del Rio *et al.*, 1983; Sandalio *et al.*, 1987) and the external side of the glyoxysomal membrane of higher plants (Sandalio and Del Rio, 1987).

SOD activity in plant increases differentially in response to various environmental stresses (Bowler *et al.* 1989; Tsang *et al.,* 1991); MnSOD activity has been proposed to correlate with the severity of stress (Bowler *et al.,* 1992). Transgenic plants having the

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elevated levels of SOD activity enhance the tolerance towards oxidative stress (Bowler *et al.*, 1991; Perl *et al.*, 1993; Sen Gupta *et al.*, 1993) and other stresses (Mckersie *et al.*, 1993; Van Camp *et al.*, 1994). Therefore, SOD is considered to be one of gene candidates to improve stress tolerance in plants by gene transfer (Holmberg and Bulow, 1998).

The catalytic function of SOD is well known, and the other role of SOD as a stabilizer of calcineurin was also reported (Wang *et al.*, 1996). However, the physiological roles of SOD in the growth and development of whole plants still need to study. The invasion of environmental stresses to organisms is often injury to specific membranes or defined organelles, so it may be desirable to locate free radical-scavenging enzymes as SOD as a stress marker for the subcellular damage. In our laboratory, the effect of stress on SOD activity of rice was studied (Wei *et al.*, 1995; Chen, 1994), cDNAs of CuZnSOD and MnSOD from rice were cloned (Pan *et al.*, 1995; Chen *et al.*, 1997) and several CuZnSOD- or MnSOD-overexpressors of *Aarabidopsis* were generated (Chen, 1998). We also identified MnSOD was as being the major form of SOD in *Ganoderma* species and it was purified to homogeneity (Pan *et al.*, 1997). Some detail immunological studies regarding the cellular localization of MnSOD in animal systems were reported (Oberley *et al.*, 1993; Tannahill, 1995), but no similar immunological study to locate MnSOD in plant cell was reported. In order to identify and locate MnSOD protein in the plants during the development or in response to various stresses, the antibody specific to MnSOD is required. For this purpose, we described the expression and characterization of rice MnSOD in *E. coli* in this report. And the homogenous MnSOD will be used for raising the antibody against MnSOD.

MATERIALS AND METHODS

Construction of the recombinant expression vector and transformation

The vector pGEX-4T-1 was purchased from manufacture (Pharmacia Biotech). The expression vector used for construction of the fused gene was glutathione-S-transferase gene Fusion System (GST Gene Fusion System, Pharmacia). The full-length cDNA clone for mature MnSOD was isolated from a rice cDNA library (Chen *et al.*, 1997). The cDNA sequence encoding mature MnSOD protein was made by PCR, with *Eco*RI and *Not*I sites at the ends for ligating with the vector cleaved with *Eco*RI and *Not*I. The recombinant plasmid (pMSF1) was transformed into competent *E. coli* BL21 cells by heat shock method (Sambrook *et al.*, 1989). More than ten transformed clones were picked and cultured in LB broth for mini-scale preparation. The selected clone was named mn101. It was sequenced to confirm the insert DNA identity and the fusion protein expression was checked as protocol described by manufacture (Pharmacia Biotech).

Induction and purification of the fusion protein

Induction and purification of the fusion protein from culture of mn101 clone were followed the protocol described by manufacture (Pharmacia Biotech). Overnight culture cells were harvested by centrifugation at 5,000xg, 10 min at 4℃. Pellets were resuspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3) and cells were sonicated for 15 min at 4℃. After centrifugation at 12,000xg, 15 min at 4℃, the supernatant was collected. The fusion protein was batch-purified in one step using bulk glutathione Sepharose 4B. The purified fusion protein was cleaved overnight by

thrombin to produce the recombinant MnSOD (rMnSOD). The reaction mixture was analyzed on a 10% SDS-PAGE to check the purity or on a 10% native-PAGE to detect the SOD activity of GST-MnSOD or rMnSOD.

Electrophoresis and SOD activity stain

Electrophoresis was conducted on a 10% native acrylamide gel in standard Tris-glycine buffer, pH 8.3. An appropriate amount of protein extract was applied and run at 12-15 mA for 2 hrs. After electrophoresis, the protein was stained with CBR reagent, or the gel was stained for SOD activity. It was first soaked in 1.225 mM nitroblue tetrazolium solution for 15 min, briefly washed, then soaked in 100 mM potassium phosphate buffer, pH 7.0 containing 28 μM riboflavin and 28 mM TEMED for another 15 min. After briefly washed, the gel was illuminated on a light box with intensity of 30 $\mu Es^{-1}m^{-2}$ for 15 min to initiate the photochemical reaction. For the identification of MnSOD, the gels were first soaked in 8 mM hydrogen peroxide in 100 mM potassium phosphate buffer, pH 7.0, for 30 min, followed by SOD activity staining. All procedures were carried out at room temperature and the two soaking steps were shaken at 75 rpm. The tested gel was scanned with a laser densitometer (Molecular Dynamics) after the gel was stained for SOD activity. To estimate the total SOD activity, the area of each sample corresponding to each SOD activity was framed and scanned and the activity was calculated by comparing each sample with that of standard SOD, which was also run in the same gel (Chen and Pan, 1996).

Assay for the stability of rMnSOD activity

The GST-MnSOD or rMnSOD protein after thrombin digestion was taken as the enzyme sample. The concentration of protein from 3.5 to 6.5 μg of each sample was used for the following tests. After treatments, every sample was electrophoresed on 10% native PAGE to detect the SOD activity. For thermal stability, the enzyme samples were heated at 25℃, 40, 50, 60, 80 and 100 ℃, respectively for 20 min. For pH stability, each enzyme sample was added into equal volume of buffer in different pH values: 0.2M sodium citrate buffer (pH 2.2, 3.0, 4.0, 5.0, and 7.0), 0.2M glycine-NaOH (pH 10.4). Each sample was incubated at 37℃ for 1 hr.

RESULTS AND DISCUSSION

Expression of inducible GST-MnSOD fusion protein

The cDNA sequence encoding for the rice mature MnSOD was made by PCR and inserted into the pGEX-4T-1 expression vector (Fig. 1). Total cell lysates of the transformed clone mn101 were prepared at hourly after the induction of IPTG in the cell culture and analyzed by SDS-PAGE. The maximal levels of expression was obtained after the 3hr of 0.1 mM IPTG addition (Fig. 2). This concentration of IPTG was lower compared to 1.0 mM of that used in other expression system. And after the single one-step purification by glutathione affinity gel chromatography, the homogenous GST-MnSOD was obtained. The molecular size of GST (Smith and Johnson, 1988) and rice MnSOD (Chen, *et al.*, 1997) was 26 kDa and 22 kDa, respectively. So, the predicted molecular weight of this fusion protein was 50 kDa and it was confirmed by SDS-PAGE (Fig. 2). Because the inclusion body-like precipitate was formed during the extraction of fusion protein, only 1.5-3.0 mg of purified fusion protein was obtained from the soluble, cell extracts of one-liter culture of clone

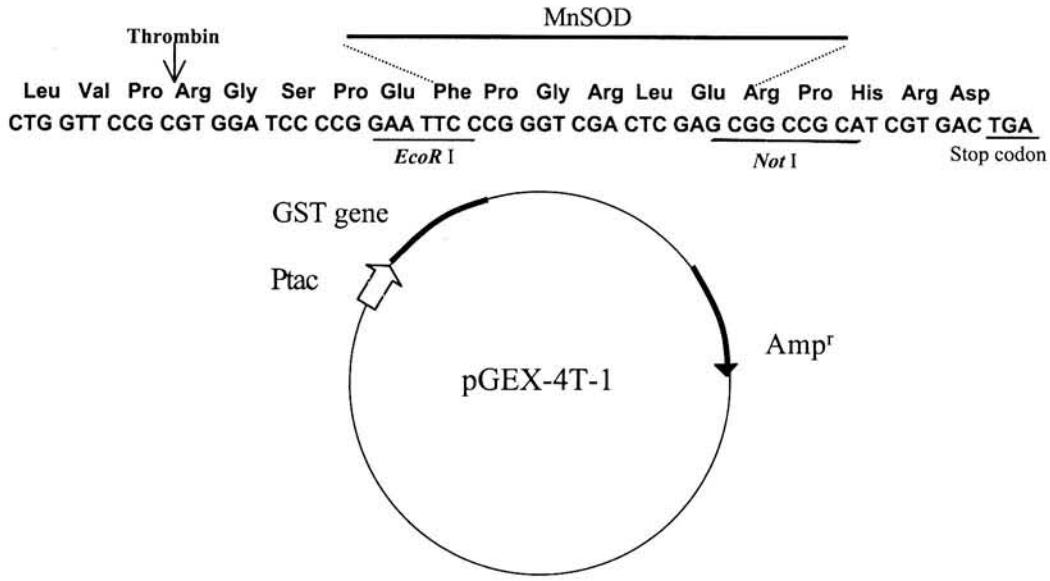

Fig. 1. The construct of GST-MnSOD fusion gene.

mn101 cells. The insoluble fraction was collected after centrifugation of the cell extract at 10,000xg. It could be partially dissolved in 6.0 M urea, and the dissolved fraction contained MnSOD activity. The dissolved, active SOD proteins from inclusion body fraction was further purified to homogenous GST-MnSOD by glutathione affinity gel to obtain another 1-2 mg from 1 liter of bacterial culture. The purified GST-MnSOD protein showed a single protein band on SDS-PAGE, and it could be cleaved to 90% completion with thrombin overnight to produce rMnSOD. Both GST-MnSOD and rMnSOD showed insensitivity towards H_2O_2 and KCN (Fig. 3), similar to the native MnSOD isolated from plant tissues (Kanematsu and Asada, 1979). The functional form of GST-MnSOD or rMnSOD was analyzed on a gradient PAGE to show its dimeric property (Fig. 4). The dimeric form of functional GST-MnSOD or rMnSOD was confirmed by gel filtration analysis (Tzeng, 1997). Therefore, the enzymatically active rice MnSOD could be obtained by such a cloning expression and purification procedure. However, the yield was only one tenth as that of rice CuZnSOD expressed in the transformed *E. coli* by the similar procedure (Pan *et al.*, 1999). The expression of functional rice MnSOD in *E. coli*, although the yield was not high, still could facilitate the preparation of antibody specific for rice MnSOD protein.

The effect of thrombin digestion on the purified GST-MnSOD

Time course of thrombin digestion on the purified GST-MnSOD was carried out for three days. It could be completely digested within 16 hr, however, only 70% of GST-CuZnSOD cleaved with thrombin for three days was obtained (Liu, 1997). The pI of the rMnSOD was determined to be at pH 4.64 and that of MnSOD-GST was in the range of 4.74 - 4.97 by the isoelectric focusing and activity staining on the IEF gel. The acidic pIs of rMnSOD and GST-MnSOD were comparable to those native forms isolated from other plants (Steller *et al.*, 1994).

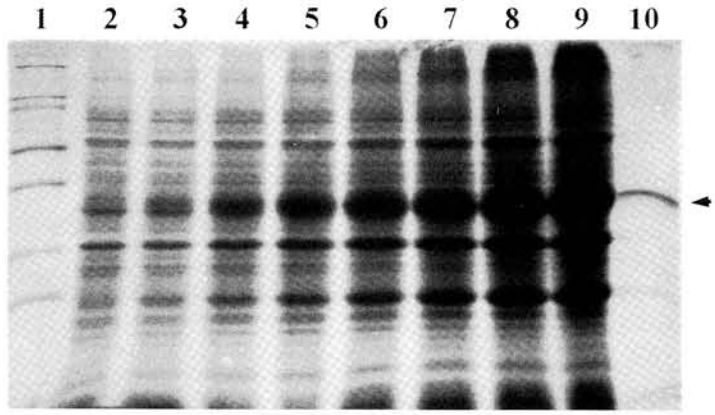

Fig. 2. Expression of GST-MnSOD fusion protein in the transformed cells. Lane 1: the low molecular weight markers (94, 67, 43, 30, 24, 14 kD, respectively), lane 2: the cell extract of untransformed cells, lane 3-9: the cell extract of transformed cells harvested at hourly intervals to 6 hr and 8 hr after 0.1 mM IPTG induction of fusion protein; lane 10: the purified fusion protein.

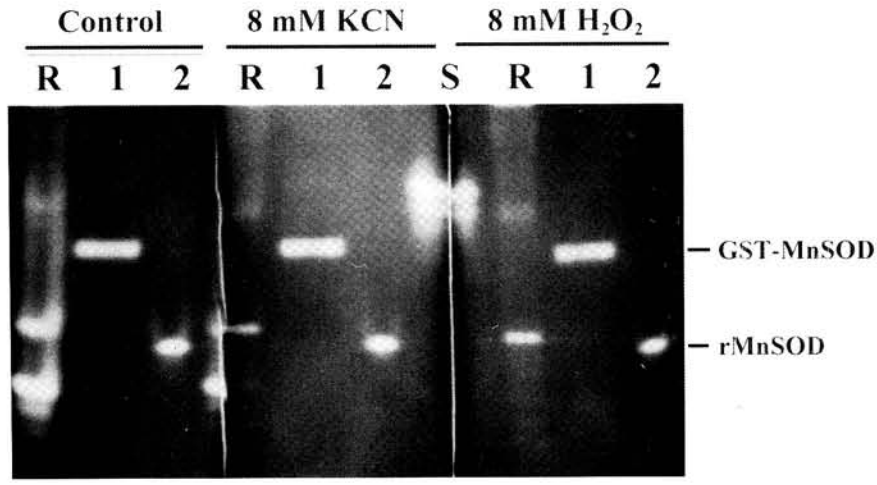

Fig. 3. The effect of inhibitors on the SOD activity of GST-MnSOD and rMnSOD. 8 mM KCN and 8 mM H_2O_2 as inhibitors to differentiate the MnSOD activity; R: 80 μg of rice leaf extract; 1: 3.5 μg of the purified GST-MnSOD; 2: 6.5 μg of the purified GST-MnSOD digested with thrombin overnight to generate rMnSOD; S: bovine liver MnSOD.

Properties of the GST-MnSOD and rMnSOD

The thrombin-digested GST-MnSOD mixture was heated at the various temperatures, both GST-MnSOD and rMnSOD remained complete SOD activity at 50℃ heat treatment and lost 70% of activity at 60℃. Both forms showed the similar trend to various temperature treatments. Both GST-MnSOD and rMnSOD also showed the similar response to various pH tests, they were stable at alkaline pH (Fig. 5).

The characteristics of purified rMnSOD compared with MnSOD isolated from other plant materials was showed in table 1, in terms of the tolerance to KCN or H_2O_2 inhibition, response to pH and temperature. (Table 1). The heat labile property of rMnSOD of rice or other plant MnSOD was observed, but rCuZnSOD had contrarily high thermostability as the

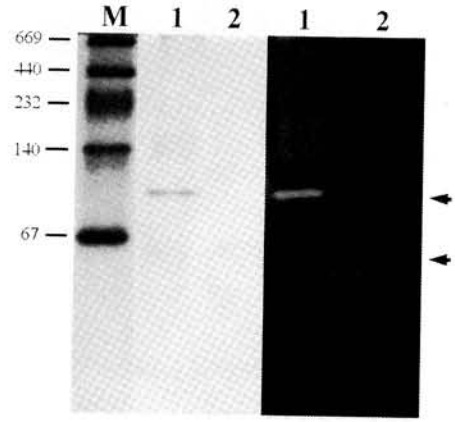

Fig. 4. SOD activity analysis of GST-MnSOD on the 5 to 20% acrylamide gradient PAGE. M: the molecular markers; 1: 3.5 μg of the purified GST-MnSOD; 2: 6.5 μg of the purified GST-MnSOD digested with thrombin overnight to generate rMnSOD. The left-half gel was stained protein with CBR reagent and the right-half gel was stained with SOD activity.

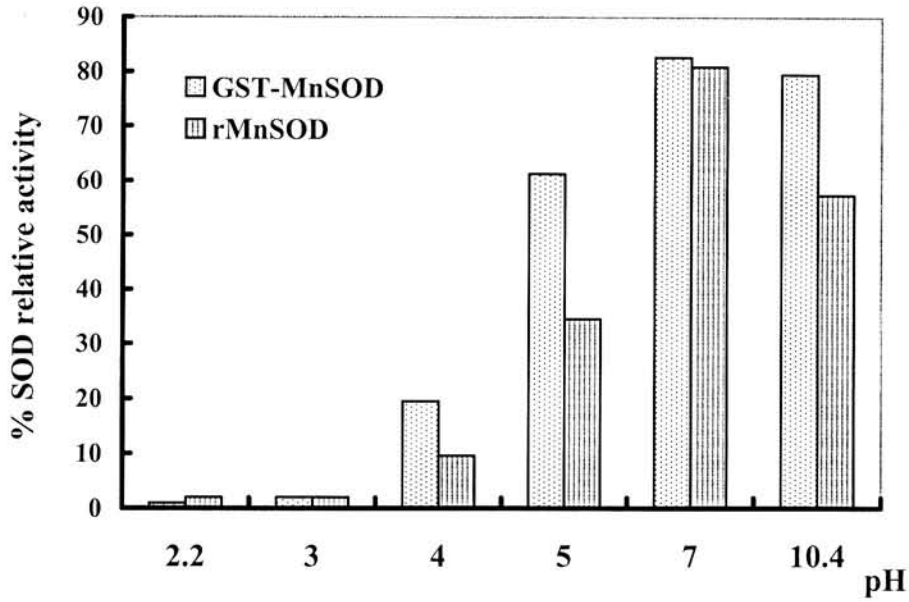

Fig. 5. Effects of pH on the activity of GST-MnSOD and rMnSOD protein. The thrombin-digested GST-MnSOD was treated with various pH buffer at 37℃ for 1 hr, then 3.5 μg of each sample were loaded onto a 10 % native/PAGE. The effects of pH were determined by activity staining.

unique property of plant CuZnSODs (Pan *et al.*, 1999). The SOD specific activity of GST-MnSOD or rMnSOD in the transformed *E.coli* was estimated and showed several-fold lower than that of native forms isolated from various plant tissues (Kono *et al.*, 1979; Baum, *et al.*, 1983; Kanematsu and Asada, 1989). Similarly, drastically decreased enzyme specific activity of the recombinant SOD in transformed *E.coli* was observed in that of rCuZnSOD (Pan *et al.*, 1999). One possibility of lower enzyme specific activity for the recombinant SOD protein is the misplaced metal as cofactor in transformed *E. col.* Since MnSOD is a metalloenzyme, the right incorporation of manganese ion into each GST-MnSOD apoprotein must be crucial for its enzyme activity. Although there was no report to detect the Mn

chaperone protein in any organisms, it could incorporate manganese properly to its responsive apoprotein. One of Cu chaperone proteins was identified in yeast and humans, and this chaperone protein was demonstrated to mediate the delivery of copper to SOD *in vivo* (Culotta *et al.*, 1997). Possibly, *E. coli* might not have the correct molecular machinery, such as metal chaperone proteins, to correctly incorporate the metals in the recombinant rice apoSOD. Therefore, the lower specific activity of rCuZnSOD and rMnSOD in the transformed bacteria was observed. Or, the availability of the free manganese ions in the proper subcellular site of the transformed bacteria becomes a limiting factor. Alternatively, the presence of five extra amino acids, glycine, serine, proline, glutamate and phenylalanine in the N-terminal amino acids of rMnSOD derived from the pGEX-4T-1 expression vector may have some effects on the catalytic activity of rMnSOD or GST-MnSOD. The similar explanation was reported in the study of GST (Marr, 1996).

Table 1. Comparison of MnSOD in the various plants.

Plant species	M.W. of subunit	Native form	pI	pH optimal	Thermostability
Rice[a] (Transform *E. coli*)	23 kDa	Dimer 45.5 kDa	4.64	More stable at alkaline pH	Activity remain completely at 50℃ and lost 70% at 60℃
Norway spruce[b] (Seed)	22 kDa	Tetramer 86 kDa	5.5	Activity is stongly pH dependent	—
Pine[c] (Seed)	23 kDa	Tetramer 91 kDa	6.5	—	—
Carnation[d] (Petal)	—	75 kDa	4.85	—	—
Pea[e] (Leaf)	—	94 kDa	—	—	More labile at high temperature
Kidney bean[f] (Leaf)	—	44 kDa	—	—	—
Yeast[g]	—	Tetramer 96 kDa	—	Rate of reaction with O^{2-} falls as the pH is raised above 7.8	Add SDS at 100℃ to dissociate into dimer

a: Tzeng, 1997; b: Kroniger *et al.*, 1995; c: Streller *et al.*, 1994; d: Droillard and Paulin, 1990; e: Sevilla *et al.*, 1980; f: Yasuhisa *et al.*, 1979; g: Ravindranath and Fridovich, 1975.

Comparison of rMnSOD and rCuZnSOD

In our laboratory, two transformed *E. coli* to express rice MnSOD and CuZnSOD were generated, respectively (Tzeng, 1997; Liu, 1997). The properties of the recombinant SODs purified from these two recombinant bacteria were compared (Table 2). Both GST-MnSOD and rMnSOD showed the similar trend to various temperature or pH treatments, they were stable at alkaline pH. But the GST-CuZnSOD and rCuZnSOD showed the differential response to temperature, and rCuZnSOD had contrarily high thermostability as the unique property of plant CuZnSODs (Liu, 1997). The lowered SOD enzyme specific activity of GST-MnSOD or rMnSOD was similar as observed in that of rCuZnSOD (Pan *et al.*, 1999). For considering the application of recombinant SODs to medicinal use, rCuZnSOD seems superior to rMnSOD in terms of availability, yield, molecular size and thermostability.

Table 2. The comparison of GST-CuZnSOD and GST-MnSOD.

Characterics	Source	Native form	pI	Heat (>70℃)	SDS treatment	Others
GST-CuZnSOD[a] (cDNA of rice)	GST-fusion system	Dimer	5.2-5.6	unstable	unstable	unstable at acidic pH; more insensitive to H_2O_2
RCuZnSOD[a] (cDNA of rice)	GST-fusion system	Dimer	5.2-5.6	stable	stable	stable at pH 2-10; more insensitive to H_2O_2
Rice CuZnSOD[b] (cytosolic)	Rice leaves	Dimer	5.0	stable	—	—
Rice CuZnSOD[b] (chloroplastic)	Rice leaves	Dimer	5.3	stable	—	—
Sweet potato CuZnSOD[c] (cDNA of sweet potato)	His-tag system	Dimer, monomer	—	stable (dimer) unstable (monomer)	dimer dissociated to monomer	dissociated to monomer at acidic pH
Ganoderma sp. MnSOD[d,e]	Hyphae	Tetramer	5.06-8.06	unstable	dissociated to monomer	unstable at acidic pH
GST-MnSOD and rMnSOD[f] (rice MnSOD cDNA)	GST-fusion system	Dimer	4.64	unstable	—	unstable at acidic pH

a: Liu, 1997; b: Kanematsu and Asada, 1989; c: Lin, *et al.*, 1995; d: Yeh, 1996; e: Pan *et al.*, 1997; f: Tzeng, 1997.

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水稻超氧歧化酶在大腸桿菌中的表現及生化性質研究

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摘　　要

本文報告水稻超氧歧化酶 (MnSOD) 在大腸桿菌中的表現及生化性質的分析。利用 PCR 將對應到水稻 MnSOD 成熟蛋白質之正確 cDNA 序列接入 pGEX-4T-1 表現載體中，將建構好的質體再轉型至大腸桿菌 BL21 品系中。融合蛋白質 GST-MnSOD 可被 IPTG 誘導表現，而經由 glutathione 親和膠體層析可純化均質之 GST-MnSOD 融合蛋白質。

重組之 MnSOD (rMnSOD) 及 GST-MnSOD 均對 KCN 和 H_2O_2 不敏感，此即保有生物中原態 MnSOD 的一特性。兩者的單體分子量分別為 23 kDa 和 50 kDa，而在原態上均以二元體的形式存在。rMnSOD 的等電點為 4.64，GST-MnSOD 的等電點則介於 pH 4.74－4.975 之間。rMnSOD 及 GST-MnSOD 兩蛋白質處理 60℃， 20 分鐘後，SOD 活性下降至 30%；兩者在鹼性環境中均較穩定。

關鍵詞：超氧歧化酶，含錳超氧歧化酶，水稻，大腸桿菌。

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