Unusual stability of manganese superoxide dismutase from a new species, *Tatumella ptyseos* ct: its gene structure, expression, and enzyme properties

Chuian-Fu Ken a,1, Chuing-Chi Lee c,1, Kow-Jen Duan b,1, Chi-Tsai Lin c,*,1

a Institute of Biotechnology, National Changhua University of Education, Changhua 500, Taiwan
b Department of Bioengineering, Tatung University, Taipei 104, Taiwan
c Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung 202, Taiwan

Received 5 July 2004, and in revised form 1 October 2004
Available online 28 December 2004

Abstract

A genomic DNA of 1416 bp containing an open reading frame encoding a manganese superoxide dismutase (Mn-SOD) from *Tatumella ptyseos* ct was cloned. Sequence analysis of this new gene revealed that it translates 205 amino acid residues. The deduced amino acid sequence showed variable identities (41–91%) with sequences of Mn-SODs from other species. The residues required to coordinate the single trivalent manganese ion and the 11 residues putatively involved in the active center are conserved as they are in other reported Mn-SODs. In addition, the gene was introduced into the expression vector, pET-20b(+), and transformed in *Escherichia coli* BL21(DE3). The Mn-SOD was purified by a His-tag technique. The yield was 0.9 mg from 0.5 L of culture. The specific activity was 6540 U/mg. A dimer is the major form of the enzyme in equilibrium. The half-life of dimer is approximately 50 min and its thermal inactivation rate constant *k* d was 0.015 min⁻¹ at 80 °C. The dimerization of the enzyme was inhibited under an acidic pH (below 4.0), or in the presence of SDS (above 1%) or imidazole (above 0.5 M), whereas it was not affected under an alkaline pH (above 9.0). Furthermore, the dimeric enzyme was much more resistant to proteolytic attack after 3 h of incubation at 37 °C with trypsin and chymotrypsin. This unusually stable enzyme can be used as cosmetic to the protection of skin against the unaesthetic effects caused by free radicals.

© 2004 Elsevier Inc. All rights reserved.

Keywords: *Tatumella ptyseos* ct; Manganese superoxide dismutase; Cosmetic; Expression; His-tag purification

Superoxide dismutases (SODs) form the first-line defense system in various organisms against reactive superoxide radicals and are vital to the survival of cells [1]. SODs can be classified into three types, Cu/Zn, Mn, and Fe-SOD, depending on the metal bound at the active site [2–4]. Cu/Zn-SOD is predominantly associated with the cytosolic fraction of eukaryotes, Mn-SOD is insensitive to cyanide and hydrogen peroxide. Fe-SOD is found in prokaryotes and is not sensitive to cyanide but is inhibited by hydrogen peroxide. SOD plays an essential role in allowing organisms to survive in the presence of O₂. It catalyzes the dismutation of the highly reactive O₂⁻ to O₂ and hydrogen peroxide in all oxygen-metabolizing organisms. Mn-SOD has been found in the cytosolic fractions of prokaryotes and in the mitochondrial matrix of eukaryotes. In eukaryotic cells, Mn-SOD is synthesized in the cytosol and imported post-translationally into the mitochondrial matrix [5,6]. In eukaryotes, the mitochondrial matrix is the intracellular site of the final combustion of nutrients and the reduction of O₂ to water. It is also an important site for the single-electron reduction of O₂ to O₂⁻.
Mn-SOD is thought to be a major scavenger of damaging reactive oxygen metabolites in the mitochondrial matrix.

Inactivation of Mn-SOD and Fe-SOD genes in *Escherichia coli* increased mutation frequency when grown under aerobic conditions [7]. Elimination of the Mn-SOD gene in *Saccharomyces cerevisiae* increased its sensitivity to O$_2$ [8,9]. Expression of Mn-SOD from maize in SOD-deficient strains of *S. cerevisiae* protects the cells against oxidative stress [10] and its over-expression increases the mean life span of *Caenorhabditis elegans* and *Drosophila melanogaster* [11,12]. Expression of human Mn-SOD genes in transgenic mice protected the mice against oxygen-induced pulmonary injury [13]. Thus, the expression of Mn-SOD is essential for the survival of aerobic life and for the development of cellular resistance to oxygen radical-mediated toxicity.

SODs also have been associated with the pathogenicity and viability of some microbes pathogenic to animals [14,15] and plants [16,17]. Furthermore, SODs make it possible in particular to protect the skin and hair especially by maintaining the integrity of the natural keratinous structure; see for example, French Patent Application No. 75.31354. SODs improve cutaneous cell respiration and maintain or improve the skin characteristics such as the soft feel, the suppleness, and the elasticity.

A species of microbe was screened from a ripe guava grown in central Taiwan, which gave off a special fragrance when cultured in medium at 28°C for 8 h. Preliminary study found this microbe to be similar to *Tatumella ptyseos* (ATCC 33301) [18]. But the *T. ptyseos* from commercial agent did not produce a special fragrance when cultured under the same conditions. To identify this microbe and since no Mn-SOD has been characterized from this *T. ptyseos*, we clone the Mn-SOD gene from this microbe and study the characteristics of the enzyme expressed from this Mn-SOD gene. This gene structure can provide a reference for comparisons of the differences between *T. ptyseos* species in the future, and this enzyme can be used for several beneficial applications such as an agent against inflammatory reactions associated with chemical irritation and acne [19], and in cosmetic with porphyrin [20] or even melanin [21] for the protection of the skin, hair, and/or mucosae against the harmful and/or unaesthetic effects caused by oxygen-containing free radicals.

### Materials and methods

#### Identify bacteria and culture condition

A ripening guava was obtained from Nantou, central Taiwan, and we used a loop to stick into the guava and then streaked it on a MY plate (4 g/L yeast extract, 10 g/L malt extract, and 4 g/L glucose) at 28°C for 12 h. After this, many single colonies appeared, and we selected each colony in 15 mL falcon containing 5 mL MY medium at 28°C for 8 h. One of those colonies produced a special fragrance. This colony was sent to the Industry Research and Development Institute, where it was analyzed using the Vitek identification system (BioM'erieux Vitek, USA), the API 20NE system [22], a MicroStation System, release 4.0. 1999 (Biolog, Hayward, CA, USA), and a MicroSeq500 16S rDNA bacterial sequencing kit (Perkin–Elmer, USA). Based on reported data, the microbe was possibly *T. ptyseos*. However, we cultured commercial *T. ptyseos* under the same conditions, no special fragrance was produced. This indicated that they were different, so we defined this new microbe as *T. ptyseos* ct.

#### Genomic DNA preparation

Five milliliters of culture (28°C for 8 h) of this fragrance-producing colony was centrifuged at 10,000g for 5 min, and the bacterial cells were suspended in 567 μL TE; 3 μL of solution (10% SDS, 20 mg/mL protease K) was added and incubated at 37°C for 1 h, and then 80 μL CTAB/NaCl (1 g/L CTAB and 0.41 g NaCl) was added and heated for 10 min. Finally one volume of phenol:chloroform (1:1) was added, and the mixture was centrifuged at 13,000g for 10 min. Phenol:chloroform extraction was repeated once, the aqueous phase was transferred to a new tube, and 0.6 volumes of isopropanol was added at −20°C for 30 min; a DNA pellet was obtained by centrifugation at 10,000g for 5 min, after which it was washed with 0.3 mL of 70% ethanol twice. The DNA pellet was finally dissolved in 100 mL TE and was ready for use.

#### Subcloning and DNA sequence analysis

In accordance with the amino acid sequences of Mn-SOD from *Salmonella typhimurium* (EMBL Accession No. U20645) and *E. coli* (EMBL Accession No. X03951), two primers (5’GCT TAT GAT GCG TTA GAG CC3’ and 5’TAA GCG TGT TCC CAA ACG TC3’) were synthesized. Using 0.2 μg of the genomic DNA as a template, 10 pmol of each of the two primers was added. One 0.5-kb fragment of DNA was amplified by polymerase chain reaction (PCR) techniques (25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s). The 0.5-kb DNA fragment was subcloned into a pCR2.1 cloning vector (Invitrogen, Grand Island, NY, USA) using TOPO10 as a host. The nucleotide sequence of the insert was determined in both directions according to autosequencing (ABI PRISM 377-96 DNA sequencer, Perkin–Elmer). Based on this DNA sequence, a TMn-1 primer (5’ACC GGA ACG ACT CTG CAG GG3’) and a TMn-2 primer (5’CTA CCG AAA TCT
TTC TCA AT3’) were synthesized. One microgram of blunted genomic DNA (the genomic DNA was digested with Sau3A) and then treated with T4 DNA polymerase) was ligated with 30 pmol of Marathon cDNA adaptor (Clontech, Palo Alto, CA, USA) at 4°C for 16 h for the following experiments. To a 0.5-mL microtube containing 0.02 μg of the ligated DNA as a template, we added 10 pmol of Clontech adaptor primer and 10 pmol of TMn-1 primer. Ten picomole Clontech adaptor primer and 10 pmol of TMn-2 primer were added to another 0.5-mL microtube containing 0.02 μg of the ligated DNA. One 0.5-kb DNA fragment (3’-RACE; 3’-DNA end) and one 0.5-kb DNA fragment (5’-RACE; 5’-DNA end) were amplified by the PCR technique (25 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s). Both 0.5-kb DNA fragments were subcloned into a pCR2.1 cloning vector using TOPO10 as a host. The nucleotide sequences of these inserts were determined in both directions according to the dideoxy technique using autosequencing. Sequence analysis revealed that 5’-RACE and 3’-RACE covered an open reading frame of Mn-SOD DNA (1.4 kb). Using the 5’-RACE, 0.5 kb of DNA, and 3’-RACE as templates, one fused DNA fragment of Mn-SOD was assembled by the PCR technique (EMBL Accession No. AF317618).

Recombinant DNA preparation for transformation

According to the 1.4-kb DNA sequence, a 5’ upstream primer (5’GAA TTC GAT GAG TTA TTC ACT GCC ATC C 3’) and a 3’ downstream primer (5’CTC GAG TTT TGC AGA AGC AAA ACG GGC CGC 3’) were synthesized. Using 0.02 μg of genomic DNA as a template, 10 pmol of each 5’ upstream and 3’ downstream primer was added, a 0.6-kb fragment was amplified by PCR (25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s), digested with EcoRI and XhoI, and then transformed into an E. coli TOPO10 host. A positive clone was selected by hybridization with 32P-labeled Mn-SOD DNA as the probe, and plasmid DNA was prepared. A suitable amount of plasmid DNA was digested with EcoRI and XhoI, and then run on a 0.8% agarose gel. A 0.6-kb insert DNA containing EcoRI and XhoI sites was recovered and subcloned into the pET-20(+) expression vector (pretreated with EcoRI and XhoI) from Novagen (Madison, WI, USA). The recombinant DNA was then transformed into E. coli BL21(DE3). The recombinant DNA was expressed in E. coli BL21(DE3), and its protein was identified by activity staining.

Culture and enzyme purification

The transformed E. coli was grown at 32°C in 500 mL of Luria–Bertani medium containing 50 μg/mL ampicillin until A600 reached 0.9. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated at 32°C for 4 h at 150 rpm, and then the bacterial cells were harvested by centrifugation at 6000g for 5 min. Cells were suspended in 4 mL of 10 mM Tris 8.0 buffer containing 0.1% glycerol and 1 g glass beads, then vortexed for 5 min and centrifuged at 10,000g for 5 min. The extraction procedure was repeated two times, and the supernatants were pooled together. The final crude enzyme (10 mL) was loaded on a Ni2+-nitrilotriacetic acid Sepharose superflow (Qiagen) column (with a bed volume of 2 mL), and then the column was washed with 12 mL of 10 mM Tris 8.0 buffer containing 5 mM imidazole. The enzyme was eluted with 6 mL of 10 mM Tris 8.0 buffer containing 100 mM imidazole (at a flow rate of 0.4 mL/min and 1.5 mL/fraction at room temperature). The purified enzyme (3 mL) that was dialyzed against 200 mL of 10 mM Tris 8.0 buffer containing 0.1% glycerol at 4°C for 4 h twice was used for analysis or stored at −20°C for further analysis.

Protein concentration measurement

The protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

Enzyme assay in solution

The SOD activity was measured using a RANSOD kit (RANDOX, Ardmore, UK). One milliliter of the assay solution contained 40 mM CAPS at pH 10.2, 0.94 mM EDTA, 0.05 mM xanthine, 0.025 mM INT[2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride], and 0.01 U of xanthine oxidase. The amount of SOD added was adjusted to obtain a rate of INT reduction at 25°C over the first 3-min time interval, measured as the absorbance at 505 nm, which fell within the percentage of inhibition that could be transformed into units of SOD by referring to a standard curve according to the instruction manual.

Enzyme assay by activity staining on a native gel

Samples of the enzyme were electrophoresed on a 10% native gel for 2.5 h at 100 V. The slab acrylamide gel was then cut into parts. One part was assayed, as previously described [23], for Mn-SOD activity staining. The gel was soaked in 0.6 mg/mL nitro blue tetrazolium (NBT) solution for 15 min in the dark with gentle shaking, followed by immersion with illumination in a solution containing 0.45% tetramethylbenzidine and 10 μg/mL riboflavin. During illumination, the gel became uniformly blue except at positions containing SOD, which showed achromatic zones revealing insolubility of the blue reduction product of NBT by the
superoxide anion. The other part was stained with Coo-
massie blue. The area and intensity of activity and the
protein bands were measured by a computing densitom-
eter (Molecular Dynamics, CA).

**Enzyme characterization**

Each enzyme sample underwent the following tests. After the treatments shown below, each sample was
divided into two parts, and then each part of the sample
was electrophoresed onto a 10% native polyacrylamide
gel to determine the changes in activity and protein levels.

1. **Thermal stability.** Enzyme samples were heated to
80 °C for 10, 20, 40, or 80 min.
2. **pH stability.** Enzyme samples were amended with
half a volume of buffer at different pH values:
0.2 M citrate buffer (pH 2.2, 3.0, 4.0, or 5.0), 0.2 M

---

**Fig. 1.** The structure of the *T. pyseos* ct Mn-SOD gene and the deduced amino acid sequence. Numbers to the left refer to nucleotide and amino acid residues. The asterisk denotes the stop signal.
Tris–HCl buffer (pH 7.0, 8.0, or 9.0), or 0.2 M glycine–NaOH buffer (pH 10.0, 11.0, or 12.0). Each sample was incubated at 37 °C for 1 h.

3. SDS effect. SDS was added to the enzyme sample to the level of 1, 2, or 4% and incubated at 37 °C for 1 h. Both SDS and imidazole are protein-denaturing reagents.

4. Imidazole effect. Imidazole was added to the enzyme sample to the level of 0.5, 1 or 2 M and incubated at 37 °C for 1 h.

5. Proteolytic susceptibility. The enzyme was incubated with one-twentieth its weight of trypsin or chymotrypsin at pH 8.0, and 37 °C for up to 1, 2, or 3 h. In the chymotrypsin digestion, CaCl₂ was added until it reached 20 mM. Aliquots were removed from time to time and analyzed.

Results and discussion

Cloning and characterization of a gene coding for Mn-SOD

Fig. 1 shows the nucleotide and deduced amino acid sequence of one T. ptyseos ct Mn-SOD clone. Sequence analysis found that the DNA comprised a complete open reading frame coding 205 amino acid residues.

Table 1 shows higher identity with the amino acid sequence of Mn-SOD from other bacterial species (60–91%) than with Mn-SOD sequences from other organisms including mammals (mouse, 44%; human, 45%), sweet potato (43%), and yeast (41%).

Fig. 2 shows that the four residues (H-27, H-81, D-167, and H-171 of the enzyme) which are putatively required to coordinate the single trivalent manganese are conserved as they are in other reported Mn-SODs (S. typhimurium, E. coli, mouse, sweet potato, and yeast). Residues H-27 through Y-35 containing four histidines, as well as the helix (residues P-162 through F-177 which contain H-171, a patch of four aromatic residues) are conserved and presumably form a trivalent manganese binding region. In addition, the putative active center involving 11 residues (H-27, H-31, Y-35, H-81, F-84, W-85, W-128, Q-146, D-167, W-169, and H-171 in this study) is also conserved in this sequence. Stallings et al. [24] suggested that the observed helical conformation is required to juxtapose the three residues (H-27, H-31, and Y-35), and that this arrangement is crucial for Mn-SOD catalysis based on the structure of Mn-SOD from Thermus thermophilus at 2.4-Å resolution.

Transformation and expression of T. ptyseos ct Mn-SOD

One goal of this study was to clone and express the T. ptyseos ct Mn-SOD coding sequence in E. coli. Using genomic DNA as the template and two specific primers corresponding to the translation initiation and termination sequences, respectively, the 0.6-kb DNA fragment coding for the T. ptyseos ct Mn-SOD was amplified by PCR and successfully subcloned into the expression vector, pET-20b(+). Positive clones were verified by DNA sequence analysis. The transformants were induced with IPTG, and their total cellular proteins were analyzed by 10% native PAGE with activity staining or protein staining (Fig. 3, lane 2).

Purification of T. ptyseos ct Mn-SOD

The T. ptyseos ct Mn-SOD was fused in the pET-20b(+)–6His-tag vector and expressed in E. coli BL21(DE3). The enzyme containing the His-tag in the C terminus was purified by affinity chromatography with nickel chelating Sepharose (Qiagen) according to the instruction manual. The yield was 0.9 mg from 0.5 L of culture. The specific activity was 6540 U/mg. The purified enzyme showed two active enzymatic forms (a dimer and monomer, Fig. 4, lane 6) on 10% native PAGE.

Characterization of the purified T. ptyseos ct Mn-SOD

The enzyme was heated at 80 °C for 10, 20, 40 or 80 min to investigate the thermal stability. Some of both

<table>
<thead>
<tr>
<th>Name</th>
<th>EMBL No.</th>
<th>Species name</th>
<th>Percent identity</th>
<th>Percent similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tatumella ptyseos ct</td>
<td>AF317618</td>
<td>Tatumella ptyseos ct</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>U20645</td>
<td>Salmonella typhimurium</td>
<td>91</td>
<td>95</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>X03951</td>
<td>Escherichia coli</td>
<td>88</td>
<td>94</td>
</tr>
<tr>
<td>Bacillus stearothermophilus</td>
<td>M81188</td>
<td>Bacillus stearothermophilus</td>
<td>60</td>
<td>69</td>
</tr>
<tr>
<td>Human</td>
<td>X14322</td>
<td>Homo sapiens</td>
<td>43</td>
<td>61</td>
</tr>
<tr>
<td>Mouse</td>
<td>X04972</td>
<td>Mus musculus</td>
<td>44</td>
<td>58</td>
</tr>
<tr>
<td>Rice</td>
<td>L19436</td>
<td>Oryza sativa</td>
<td>43</td>
<td>55</td>
</tr>
<tr>
<td>Maize</td>
<td>L19461</td>
<td>Zea mays</td>
<td>43</td>
<td>55</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>L77078</td>
<td>Impomea batatas</td>
<td>43</td>
<td>55</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>X02156</td>
<td>Saccharomyces cerevisiae</td>
<td>41</td>
<td>56</td>
</tr>
</tbody>
</table>
the dimer and monomer of SOD smeared to lower molecular weight as analyzed from native PAGE after treatment of heat (Fig. 5B). It is interesting to note that only the smeared dimer retains activity as shown in Fig. 5A (between dimer and monomer). The enzyme inactivation kinetics for dimer (not including smeared dimer) fit the first-order inactivation rate equation $\ln \left( \frac{E_t}{E_0} \right) = k_d t$, where $E_0$ and $E_t$ represent the original activity and the residual activity that remained after...

**Fig. 2.** Optimal alignment of *T. ptyseos* ct Mn-SOD from other organisms. TatMnSOD (this study); SalMnSOD, *S. typhimurium* SOD; EscMnSOD, *E. coli* SOD; BacMnSOD, *Bacillus stearothermophilus* SOD; HumMnSOD, human SOD; MouMnSOD, mouse SOD; SweMnSOD, sweet potato SOD; RicMnSOD, rice SOD; MaiMnSOD, maize SOD; and SacMnSOD, yeast SOD. A dot refers to identity with TatMnSOD. A dash denotes a deletion. Dark squares refer to residues required for binding the single trivalent manganese or involving the enzyme active center.

**Fig. 3.** Total soluble protein of IPTG-induced *E. coli* BL21(DE3) containing the *T. ptyseos* ct Mn-SOD. Five milliliters of culture was extracted and subjected to 10% native gel. Lane 1, 15 μL crude extract of pET-20b(+) in BL21(DE3) as the control; lane 2, 15 μL crude extract of recombinant DNA in BL21(DE3) (IPTG-induced) (A) Activity stain; (B) Coomassie blue stain. "d" denotes the area of the dimer and "m" denotes the area of the monomer.

**Fig. 4.** Ten percent native gel analysis of *T. ptyseos* ct Mn-SOD purification. Crude extract at 12 mL was obtained from 500 mL culture. (A) Activity staining (15 μL/lane); (B) Coomassie blue staining (15 μL/lane). Lanes 1–7, crude extract, pass through, wash, and fractions 1–4. The volume of each fraction was 1.5 mL.

Fig. 2. Optimal alignment of *T. ptyseos* ct Mn-SOD from other organisms. TatMnSOD (this study); SalMnSOD, *S. typhimurium* SOD; EscMnSOD, *E. coli* SOD; BacMnSOD, *Bacillus stearothermophilus* SOD; HumMnSOD, human SOD; MouMnSOD, mouse SOD; SweMnSOD, sweet potato SOD; RicMnSOD, rice SOD; MaiMnSOD, maize SOD; and SacMnSOD, yeast SOD. A dot refers to identity with TatMnSOD. A dash denotes a deletion. Dark squares refer to residues required for binding the single trivalent manganese or involving the enzyme active center.

Fig. 3. Total soluble protein of IPTG-induced *E. coli* BL21(DE3) containing the *T. ptyseos* ct Mn-SOD. Five milliliters of culture was extracted and subjected to 10% native gel. Lane 1, 15 μL crude extract of pET-20b(+) in BL21(DE3) as the control; lane 2, 15 μL crude extract of recombinant DNA in BL21(DE3) (IPTG-induced) (A) Activity stain; (B) Coomassie blue stain. "d" denotes the area of the dimer and "m" denotes the area of the monomer.

Fig. 4. Ten percent native gel analysis of *T. ptyseos* ct Mn-SOD purification. Crude extract at 12 mL was obtained from 500 mL culture. (A) Activity staining (15 μL/lane); (B) Coomassie blue staining (15 μL/lane). Lanes 1–7, crude extract, pass through, wash, and fractions 1–4. The volume of each fraction was 1.5 mL.
Fig. 5. Effect of temperature on purified T. ptyseos ct Mn-SOD. The enzyme samples heated at 80 °C for various times were analyzed by 10% native PAGE. (A) Staining for activity (3.2 μg/lane). (B) Staining for protein (4.3 μg/lane). Lanes 1–5, control, 10, 20, 40, or 80 min. (C) Plot of thermal inactivation kinetics of dimer. The effect of temperature was determined by activity staining. PAGE data were quantitated by a densitometer for calculation. $E_0$ and $E_t$ are original activity and residual activity of dimer, respectively, after being heated for different times. The total areas of activity (dimer) measured by a densitometer were 460.23 ± 5.19 (control), 407.73 ± 28.24 (10 min), 335.2 ± 4.36 (20 min), 288.67 ± 25.48 (40 min), and 142.77 ± 7.88 (80 min). “$d$” denotes the area of the dimer and “$m$” denotes the area of the monomer.

Fig. 6. Effect of pH on T. ptyseos ct Mn-SOD stability. The enzyme samples were incubated in buffers with different pH values at 37 °C for 1 h and then analyzed by 10% native PAGE followed by activity staining (A panel, 2 μg/lane), Coomassie blue staining (B panel, 2 μg/lane). Lanes 1–10, pH 2.3, 3.0, 4.0, 5.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0. The total areas of activity (of the dimer) measured by a densitometer were 562.85 ± 81.3 (pH 2.3), 570.85 ± 89.8 (pH 3.0), 594.35 ± 41.5 (pH 4.0), and 619.9 ± 77.1 (pH 5.0–12.0). “$d$” denotes the area of dimer activity and “$m$” denotes the area of monomer activity.

Fig. 7. Effect of SDS on T. ptyseos ct Mn-SOD. The purified SOD samples were incubated with various concentrations of SDS at 37 °C for 1 h and analyzed by 10% native PAGE. (A) Staining for activity (2 μg/lane). (B) Staining for protein (2 μg/lane). Lanes 1-4, control, 1, 2, or 4%. The enzyme activity after treatment with different concentrations of SDS was measured by a densitometer as lane 1, control ($d = 1259 ± 110.9$, $m = 147.8 ± 29.8$); lane 2, 1% ($d = 523.95 ± 21.6$, $m = 728.95 ± 28.0$); lane 3, 2% ($d = 600.3 ± 34.1$, $m = 772.15 ± 14.1$); and lane 4, 4% ($d = 300.75 ± 19.2$, $m = 709.9 ± 13.1$). “$d$” denotes the area of dimer activity and “$m$” denotes the area of monomer activity.
heating for various times (Fig. 5C). The half-life of dimer is approximately 50 min and the thermal inactivation rate constant $k_d$ was 0.015 min$^{-1}$ at 80°C as calculated from Fig. 5C.

As shown in Fig. 6 (lanes 4–10), SOD was very stable in a broad pH range from pH 5 to 12, although the total activity decreased to 4–10% at pH 4.0 (lane 3) to pH 2.2 (lane 1). The decrease in the enzyme activity at acidic pH values was due to dissociation of the dimer into the monomer (Fig. 6B, lanes 1–3). Quantitation of proteins with the densitometer revealed that an acidic pH favors monomer formation. This suggests that the charge interaction could be important for subunit association [25].

The enzyme activity showed a decrease in both SDS (Figs. 7A and B) and imidazole (data not shown) due to dissociation of the dimer into the monomer. The enzyme was resistant to digestion by trypsin and chymotrypsin even at a high enzyme/substrate (w/w) ratio of 1/20 (Figs. 8A–D). Trypsin cuts preferentially to the carboxylate side of basic amino acid residues like lysine or arginine, whereas chymotrypsin acts most strongly if a hydrophobic residue (phenylalanine, tyrosine, leucine, isoleucine, valine, tryptophan, and histine at high pH) is in this position. As shown in Fig. 1, there are several such amino acids in these positions, why this enzyme was not acted by such protease possibly due to its rigid conformation.

Neither the new Mn-SOD gene of *T. ptyseos* ct nor the properties of recombinant Mn-SOD were reported, the results from the experiments illustrate that the new gene from this microbe not only overexpressed stable Mn-SOD in *E. coli*, but also this microbe produced a special fragrance, which might be applied in other fields. These results indicate that the enzyme was highly stable under pH 5–12, high temperature, or proteases. These stable properties are beneficial for applications in cosmetic, hygienic or dermopharmaceutical composition as described in Introduction.

**Acknowledgments**

This work was partially supported by the National Science Council of the Republic of China under Grant NSC 92-2313-B-019-037 to C-T.L. and supported by the Council of Agriculture, Executive Yuan under Grant 92AS-4.2.3-FD-Z4 to C-T.L.

**References**


