行政院國家科學委員會專題研究計畫成果報告

計劃名稱: 花蟳錳型超氧歧化酵素基因之選殖及其在昆蟲細胞之表現

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主持人:林棋財 海洋大學生技所

一. 中文摘要

花蟳(*Charybdis feriatus*)腿肉之粗酵素液經膠体電泳，活性染色，証明有銅鋅型及錳型兩種超氧歧化酶(superoxide dismutase, SD)存在，簡稱為 Cu/Zn-SOD 及 Mn-SOD，兩種酶在pH 2.0~3.0 均失活，在 pH 5.4~11.2 很安定。以 80 °C 加熱 2 分鐘，Cu/Zn-SOD 尚存一半的活性。為了篩選其基因，故取 2 克腿肉，以 Trizol 試劑抽取 total RNA，純化其 mRNA，繼之合成 cDNA。依據魚類 Mn-SOD 較保守區域合成兩段 primers，以 cDNA 當模板，進行 PCR，可得到片段 DNA，經次選殖及定序，証實為 Mn-SOD 之片段，再依據此片斷 DNA 序列，合成二段 primers，分別為 sense 及 antisense，進行 5'RACE 及 3'RACE 進一步予以次選殖及定序，最後得到全長 927 bp cDNA，其表現區含 224 個鞍基酸。將表現區與載体 pVL1392 連接，再與桿狀病毒載体(Bac Vector-1000 triple cut virus DNA)混合並感染昆蟲細胞(sf9) ，經三天培養，由細胞外型可判定外原基因是否與桿狀病毒載体重組並成功地藉由 sf9 細胞產生病毒顆粒，而此病毒帶有該外源基因即可藉昆蟲細胞培養而生產該酵素，唯生產之酵素量無法與大腸桿菌生相比。

關鍵字:manganese superoxide dismutase(Mn-SOD), *Charybdis feriatus*.

Abstract

The crude enzyme from crab (*Charybdis feriatus*) was confirmed by visualization of SOD activities on polyacrylamide gel. The enzyme was labile under pH 2.0~3.0, temperature above 80 C°. In order to screen Mn-SOD, total RNAs were isolated using Trizol reagent , then mRNAs were purified, finally cDNAs were synthesized. Two primers according fish's Mn-SOD cDNA sequence were synthesized and added to a PCR reaction which using cDNAs as templates, a crab Mn-SOD DNA fragment was created. According this fragment sequence and 5', 3' RACE method, a full length crab Mn-SOD cDNA

containing 927 bp was obtained. An open reading frame encoding 224 amino acid residues was found for the sequence starting from an ATG intiation codon located at bp position 72 and terminating just ahead of a TAG stop codon. The coding region subcloned in a Baculovirus transfer vector (pVL1392) was combined with Bac vector-1000 triple cut virus DNA, and then drop-by-drop was added to sf9 cell, after incubated and analyzed, crab Mn-SOD could expressin sf9 cell.

二 緣由與目的

超氧（oxygen free radical；superoxide）即通常的 O_2 帶一個電子形成 anion 的自由基，以 O_2^- 來表示，超氧歧化酶（superoxide dismutase；EC1.15.1.1）取其中三個字母簡寫而成 SOD，是一種酵素，在生物体可以合成，它的作用是排除過量的超氧，其反應如下：

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

SOD 與超氧在維持身体健康上扮演很重要的角色，尤其美國奧克拉荷馬醫學研究中心 Robert A.Floyd 博士的文章指出，人体若產生過多的超氧或排除超氧的抵抗機能有缺失時，超氧便會對人体產生傷害，這種傷害會造成癌症、老化及中風的主因，因為超氧是反應激烈分子，過量時會傷害細胞膜、蛋白質及核酸，使細胞產生病變，因而引起各界對超氧與 SOD 之注意及研究 (1~3)。

SOD 共分為三型（銅鋅型，錳型及鐵型），在真核細胞中只有銅鋅型和錳型。本實驗室致力於具經濟效益或療效的基因產物之開發，目前已從甘藷塊根中篩得一株含全長銅鋅型 SOD cDNA， 此為從非光合作用組織中第一件報告 (4)，並將基因與表現型載体連結，已送入大腸菌中可大量表現，其基因產物具有二種活性型（雙倍体和單体），雙倍体的活性為單体的七倍，尤其雙倍体非常穩定，並推翻過去 SOD 次單元體獨立催化作用之理論 (5)，進而以定位突變法進行結構與功能之研究，證實 Arg-141 改為 Ser 能使次單元體 SOD 活性降低七倍但熱穩定大大提高 (6)，為將來進行基因調控的研究，亦已定出整個基因結構（全長 3950 bp，內有 8 個 exons 和 7 個 introns）(7)。 因為本實驗室亦進行植物老化之研究以木瓜為材料，因有現成之cDNA ，現亦已篩出木瓜銅鋅型 SOD cDNA 並能在大腸菌中表現其活性 (12) ，此為從果實組織中第一篇報告。

據報導錳型 SOD 較具醫療效果，遂進行錳型 SOD 的選殖，目前已從甘藷葉子所誘導的癒傷組織 cDNA 庫中篩到一株錳型 SOD cDNA，全長 1008 bp，可推導出233 個氨基酸，已將表現區與表現型載體連接並成功地能在大腸菌中大量表現 (8) 。

但活性不高且不安定。

1995 年，Westendorp 氏 (9) 指出 HeLa cells 被 HIV-1 病毒感染後，細胞內的 reactive oxygen 增加，並抑制錳型 SOD 而造成細胞的毒害，Kuratko 氏 (10) 指出以1,2-DMH (1，2-dimethylhydrazine) 誘導大白鼠造成大腸癌化後，在細胞亦偵測不到錳型 SOD 的活性，顯示錳型 SOD 對生物的重要性。

據報導1992 年後台灣草蝦的養殖常大量死亡，有報告指出是因受白斑病毒感染 (11)，申請人聯想到是否白斑病毒感染的蝦子是否如同受 HIV-1 感染的細胞亦會造成錳型 SOD 被抑制而引起蝦體抵抗環境壓力的能力降低而引起大量死亡，果真如此，則藉生物技術法將錳型 SOD 基因與含有適當 promoter 的載體連接並轉殖入蝦體，因大量表現錳型 SOD 以增強抵抗環境的壓力而達到預防蝦子大量死亡的目的。

有關蝦子SOD 的研究並無任何報告，因此申請人將藉以往對 SOD 研究之經驗已作部份探討健康草蝦 SOD 的種類，進行 SOD 的純化 (ion exchanger chromatography 、gel filtration and hydrophobic interaction chromatography) 及測試其性質，並與感染白斑病毒的草蝦比較，已証實感染病毒的草蝦能降低 SOD，進而進行草蝦 RNA 抽取，cDNA 合成，冀望能篩選到全長 SOD cDNA，將來進一步將 cDNA 與載體連接進行基因轉殖。

目前本實驗室草蝦 SOD 的研究已有部份數據，其 SOD 有兩種 (被 DE-52吸附及不被 DE-52 吸附)，其活性不被 KCN 抑制應屬錳型或鐵型，純化過程中活性容易降解，不易純化，依據其他生物錳型或鐵型 SOD 最保守區域合成 oilgonucleotides 當 primers ，以 PCR 方法進行片段基因增生，經定序均無法篩到草蝦 SOD ，此一部份工作仍在進行。

1997 年台大動物系郭教授 (13) 指出紅蟳及野生梭子蟹亦會受白點症病毒之感染，並證實對蝦類白點症桿狀病毒對於蟹類的確具有病原性與致病力，並認為兩者為同一類病毒，因此建議蝦蟹混養或以蟹類為生餌餵飼種蝦時都應檢測它們是否為病毒帶原者，亦使人聯想到受白點症桿狀病毒感染的蟹類是否如同前面所提被HIV-1 病毒感染後，亦會抑制錳型 SOD 而造成細胞的毒害，但未聞蟹類如同草蝦般大量死亡，郭教授只指出具有病原性與致病力，或許蟹類養殖環境較佳，或許蟹類錳型 SOD 較不受抑制，同樣兩者均受同一類病毒感染，草蝦會引起大量死亡，但未聞蟹類大量死亡，其機制值得探討。

本實驗室既然從草蝦尚無法篩得 SOD cDNA ，故先嘗試從蟹類 SOD 選殖，目前已從花蟳cDNA 庫中用 PCR 方法增生出全長錳型 SOD cDNA並送入昆虫細胞中進行表現。推測蝦與蟹類之 SOD 同質性應較高，再以蟹類cDNA 當探針進行蝦類 cDNA 庫或基因庫篩選，最終目標是將蝦 SOD cDNA 與適當載體連接送回蝦卵而得到之轉殖蝦能抵抗病毒與環境壓力所引起之大量死亡。

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三. 計劃成果自評

本計劃預定的目標完全達成，並發表一篇文章，茲將發表的文章附上。

Characterization of the Dimer–Monomer Equilibrium of the Papaya Copper/Zinc Superoxide Dismutase and Its Equilibrium Shift by a Single Amino Acid Mutation

Chi-Tsai Lin, Ti-Jung Kuo, Jei-Fu Shaw, and Ming-Ching Kao

Institute of Marine Biotechnology, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 2024, Taiwan, Republic of China, Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, Republic of China, and Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan 100, Republic of China

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Characterization of the Dimer−Monomer Equilibrium of the Papaya Copper/Zinc Superoxide Dismutase and Its Equilibrium Shift by a Single Amino Acid Mutation

Chi-Tsai Lin,*,† Ti-Jung Kuo,† Jei-Fu Shaw,‡ and Ming-Ching Kao§

Institute of Marine Biotechnology, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 2024, Taiwan, Republic of China, Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, Republic of China, and Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan 100, Republic of China

The coding region of the copper/zinc superoxide dismutase (Cu/Zn SOD) cDNA from papaya fruit, *Carica papaya* L. cv. Tainong 2, was cloned into an expression vector, pET-20b(+). The Cu/Zn SOD was expressed in *Escherichia coli* and purified by His-tag technique. Two active forms of the enzyme (30% dimer and 70% monomer) in equilibrium were observed. The activity of the dimeric enzyme was higher than that of the monomeric form. The thermal inactivation rate constant K_d values calculated for the dimer and monomer at 90 °C were −0.0203 and −0.0216 min^{-1}, and the half-lives for inactivation were 41.9 and 31.8 min, respectively. This indicated that the dimeric enzyme was more stable than its monomeric form. The dimerization of the enzyme was inhibited under acidic pH (below 3.0) or imidazole buffer (above 0.5 M), whereas it was not affected under alkaline pH (above 9.0). Both activity and forms of the enzyme were not affected by 1−4% SDS. Furthermore, the dimeric enzyme was much more resistant to proteolytic attack after 3 h of incubation at 37 °C with trypsin or chymotrpsin. In addition, mutation of the papaya Cu/Zn SOD at position 48 from Leu to Phe (L48F) affected the association of monomer, whereas a mutant with Lys substitution (L48K) at the same position tended to dissociate into monomeric form.

Keywords: *Cu/Zn SOD; superoxide dismutase; papaya fruit; Carica papaya L. cv. Tainong 2; dimer−monomer equilibrium shift*

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radical, can cause a lot of deleterious effects in organisms. To prevent damage from oxidative stress, cells maintain these ROS at a steady-state level by a variety of enzymatic and nonenzymatic antioxidant systems. Superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1), which catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen, is a ubiquitous metalloenzyme with antioxidation effect. It is considered to be an important enzyme against oxygen radical-mediated toxicity. According to the metal found in the active sites of SOD, it is classified into three types, namely, Mn, Fe, and Cu/Zn SOD. On the basis of the sequence similarity of the three enzymes, Mn and Fe SOD are likely to evolve from the same ancestor, whereas Cu/Zn SOD may come from a different one. Although several plant Cu/Zn SODs have been studied, only a few studies on expression in an *Escherichia coli* system and no reports on the structure−function relationship of plant Cu/Zn SODs have been demonstrated.

Most reported Cu/Zn SODs from animal species were homodimeric enzymes (Bannister et al., 1987). In bovine, it was suggested that the active sites in each subunit function independently; this suggestion was based on the observation that native subunits exhibited identical activity no matter what was chemically coupled to other native subunits or to chemically modified inactive subunits. However, direct evidence on the effect of functional interaction between subunits was not yet obtained. Because the subunits associated through unusually strong noncovalent interactions, which are not disrupted even in 8.0 M urea and not separated in a way that retains catalytic activity (Fridovich, 1986; Malinowski and Fridovich, 1979).

To the contrary, Battistoni et al. (1996) indicated that the overexpressed SOD from *E. coli* was monomeric even at high protein concentrations, irrespective of pH and ionic strength. This provided a significant evidence of an altered subunit interaction in prokaryotes with respect to the animal SODs.

In our previous study, the overexpressed sweet potato Cu/Zn SOD (SW-SOD) showed two active forms (dimer and monomer): The activity of the dimeric enzyme was 7-fold higher than that of the monomeric enzyme. The dimeric enzyme was more stable than the monomeric form. These suggested that subunit interaction might change the enzyme conformation and enhance the catalytic activity and stability of the enzyme and greatly differ from the observations of animal and *E. coli* SODs (Lin et al., 1995).

In this study, we present further evidence that plant Cu/Zn SOD overproduced in *E. coli* exhibited two active forms in equilibrium. It appears that subunit interaction

* Author to whom correspondence should be addressed (telephone 886-2-2462-2192, ext. 5513; fax 886-2-2462-2320; e-mail ctlin@ntou66.ntou.edu.tw).
† Institute of Marine Biotechnology.
‡ Institute of Botany.
§ Department of Biochemistry.

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also enhances the catalytic activity but is different from SW-SOD. We have noticed that it would be of interest from the comparative biochemical standpoint to study the SOD, and there should be much room left for exploring the physiological roles played by the SOD in the photosynthetic plant fruit. Furthermore, it is known that the dimeric form of SOD may be more stable and active in its enzymatic reaction than the monomeric form of SOD (Lin et al., 1995). We found that the papaya Cu/Zn SOD displayed both dimeric and monomeric forms but favored the monomeric form. To invesigate the equilibrium shift of the Cu/Zn SOD, we used the site-directed mutagenesis approach to understanding its mechanism and further substantiating its applications in medical use.

MATERIALS AND METHODS

Subcloning of the Protein Coding Sequence of the Papaya SOD cDNA. The protein coding region of the papaya (Tainong 2) Cu/Zn SOD cDNA (Lin et al., 1998) was amplified by Polymerase Chain Reaction (PCR) using the previously cloned papaya SOD full-length cDNA. The two synthetic primers (10 pmol each) used in this PCR were the 5′-primer (5′ CCC ATG GTG AAG GCT GTA GCT GTC 3′) and the 3′-primer (5′ GGA ATT CCC TTG GAG ACC GAT GAC GAC TCC 3′). A 0.45 kb fragment of papaya Cu/Zn SOD DNA was amplified and inserted into an expression vector, pET-20b(+), and transformed into *E. coli* AD494(DE3)pLysS. The detailed procedure was described previously (Lin et al., 1998).

Culture and Enzyme Purification. The transformed *E. coli* cells were grown at 37 °C in 200 mL of Luria–Bertani medium (pH 7.4) containing 50 mg/mL ampicillin (Sigma Chemical Co., St. Louis, MO), 30 mg/mL kanamycin (GIBCO BRL, Gaithersburg, MD), and 34 mg/mL chloramphenicol (GIBCO BRL). After cells had grown to 0.9 of OD_{600}, isopropyl β-D-thiogalactopyranoside (IPTG; GIBCO BRL) was added to a concentration of 1 mM. The culture was incubated continuously for 5 h on a rotary shaker (120 rpm), and the bacterial cells were harvested by centrifugation at 7000*g* for 5 min. The cell pellets were suspended in 3 mL extraction buffer (10 mM Tris-HCl, pH 8.0) and vortexed for 15 min before centrifugation at 13000*g* for 5 min. The extraction procedure was repeated two times, and the supernatants were collected and pooled together. The final crude enzyme (6 mL) was loaded on a His-Bind resin column (bed volume = 4 mL), and then the column was washed with 10 volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and 6 volumes of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Finally, the enzyme was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) (1.5 mL/fraction). The purified enzyme (6 mL) was dialyzed against 200 mL Tris-HCl buffer (2 mM, pH 8.0) containing 5% glycerol, 0.5 μM Cu^{2+}, and 0.5 μM Zn^{2+} at 4 °C overnight for two changes and stored at −20 °C for further analysis.

Protein Concentration Measurement. Protein concentration was determined with 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 by using a RANSOD kit (RANDOX, Ardmore, U.K.). 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 unit 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, that fell within the percentage of inhibition that could be transformed into units of SOD by referring to a standard curve according to an instruction manual.

Enzyme Assay by Activity Staining on a Native Gel. The enzyme separated on a 15% native PAGE was assayed by an SOD activity staining method as described previously (Beauchamp and Fridovich, 1971). Proteins were stained with Coomassie brilliant blue. The area and intensity of activity and protein bands were measured by a computing densitometer (Molecular Dynamics Co., Sunnyvale, CA).

SDS–PAGE (Laemmli, 1970). The enzyme samples with 0.2 volume sample buffer (60 mM Tri-HCl, pH 6.8, 25% glyerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) were heated for 5 min and then subjected to a 15% SDS–PAGE. The molecular weight of the enzyme was estimated by comparing the relative distances with molecular weight markers.

Enzyme Characterization. Enzyme sample was 7.5 or 6 μg for the following tests. After treatments as shown below, every sample was divided into two parts, and then each part of the sample was electrophoresed into a 15% native polyacrylamide gel to determine the changes of activity and protein.

(1) Thermal Stability. The enzyme samples in the assay buffer were heated at 90 °C for 10, 20, 30, and 40 min.

(2) pH Stability. Enzyme sample was amended with a half volume of buffer in different pH values: 0.2 M citrate buffer (pH 2.2, 3.0, 4.0, or 5.0), 0.2 M Tris-HCl buffer (pH 7.0 or 9.0), 0.2 M glycine–NaOH buffer (pH 10.0 or 11.0), and 0.2 M KCl–NaOH buffer (pH 12.0). Each sample was incubated at 37 °C for 1 h.

(3) SDS Effect. Enzyme sample was added with SDS to 1.0, 2.0, 3.0, or 4.0% and incubated at 37 °C for 1 h. Both SDS and imidazole are protein denaturing reagents.

(4) Imidazole Effect. The enzyme was added with imidazole to 0.5, 1.0, 1.5, or 2.0 M and incubated at 37 °C for 1 h.

(5) *Proteolytic Susceptibility.* The enzyme was incubated with 1/20 in weight of trypsin or chymotrypsin at pH 8.8 and 37 °C for up to 1, 2, or 3 h. In the chymotrypsin digestion, $CaCl_2$ was added to 20 mM. Aliquots were removed from time to time and analyzed by PAGE.

Site-Directed Mutagenesis of the Papaya Cu/Zn SOD and Preparation of the Mutant Enzymes. According to the amino acid sequence homology among SOD of different organisms, a Phe residue was present at position 48 in animal SOD (human and bovine), whereas Leu and Lys were found at position 48 in plant and *E. coli* SOD, respectively (Figure 1). Inspection of the papaya SOD structure indicated that mutation of Leu 48 with Phe (L48F) or Lys (L48K) could potentially lead to the formation of new hydrophobic–hydrophobic or charge–charge interactions at the interface of the monomer. Therefore, L48F was expected to tend toward the dimer due to stronger interaction, whereas L48K was expected to tend toward the monomer due to charge repulsion. These two mutants (L48F and L48K) were generated by site-directed mutagenesis (Kunkel et al., 1987) and subcloned into the expression vector, pET-20b(+), respectively. Mutant enzymes were produced in *E. coli* strain AD494(DE3)pLysS. Proteins were purified to homogeneity by using the His-tag technique as described before (Lin et al., 1995).

RESULTS AND DISCUSSION

Subcloning and Overproduction of Papaya Cu/Zn SOD. The goal of this study was to clone and express the papaya Cu/Zn SOD coding sequence in *E. coli*. Using papaya cDNA as the template and two specific primers corresponding to the translation initiation and termination sequences, respectively, the 0.45 kb DNA fragment coding for the mature papaya 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 a 15% native PAGE with activity staining or protein staining (Figure 2).

Purification of His-tag Papaya SOD. The papaya SOD was fused in the pET-20b(+)-6His-tag vector and expressed in *E. coli* AD494(DE3)pLysS. The enzyme

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                1                                                  46
                    d d           d  ddd
human     ..ATKAVCVL KGDGPVQGII NFEQKESNGP VKVWGSIKGL TEGLHGFHVH
bovine    ..ATKAVCVL KGDGPVQGTI HFEAK..GDT VVVTGSITGL TEGDHGFHVH
papaya    ...VKAVAVL SSSEGVSGTI FFTQ.AADGP TTVTGEISGL KPGHHGFHVH
Ipomoe    ...VKAVAVL SSSEGVSGTI FFSQ.EGDGP TTVTGNVSGL KPGLHGFHVH
E.coli    ASEKVEMNLV TSQGVGQSIG SVTITETDKG LEFSPDLKAL PPGEHGFHIH
                                                       c  c

               47                                              88
                ddd          dd
human     HFGD..... ..NTAGCTSA GPHFNPLSRK HGGPKDEERH VGDLGNVTAD
bovine    QFGD..... ..NTQGCTSA GPHFNPLSKK HGGPKDEERH VGDLGNVTAD
papaya    ALGD..... ..TTNGCMST GPHFNPAGKE HGAPEDDIRH AGDLGNVNVG
Ipomoe    ALGD..... ..TTNGCMST GPHFNPAGKE HGAPGDDNRH AGDLGNITVG
E.coli    AKGSCQPATK DGKASAAESA GGHLDPQNTG KHEGPEGAGH LGDLPALVVN
                                  c          z         z    z
                                  z

               89                                             138
                                  ddd
human     KDGVADVSIE DSVISLSGDH CIIGRTLVVH EKADDLGKGG NEESTKTGNA
bovine    KNGVAIVDIV DPLISLSGEY SIIGRTMVVH EKPDDLGRGG NEESTKTGNA
papaya    DDGKVSFSII DSQIPLTGPN SIVGRAVVVH ADPDDLGKGG HELSKTTGNA
Ipomoe    EDGTASFTIT DKQIPLTGAN SVIGRAVVVH GDPDDLGKGG HELSKSTGNA
E.coli    NDGKATDAVI APR..LKSLD EIKDKALMVH VGGDNM.... SDQPKPLGGG
                                        c

              139          151
                    ddd  ddd
human     GSRLACGVIG IAQ
bovine    GSRLACGVIG IAK
papaya    GGRVACGVIG LQG
Ipomoe    GGRVACGIIG LQG
E.coli    GERYACGVIK ...
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Figure 1. Amino acid alignment of papaya SOD versus animal and *E. coli* enzymes: papaya, this study (EMBL Y13610); *Ipomoe* (sweet potato, EMBL X73139). Amino acid alignments were performed using the PileUp program developed by the University of Wisconsin Genetics Computer Groups (Devereux et al., 1984). Numbers refer to amino acid residues of the bovine enzyme. "d" indicates residues known to form contacts between subunit in the available eukaryotic three-dimensional structures. The box indicates residues proposed to play a key role in the dimeric form of the animal enzyme/in the monomeric form of the *E. coli* enzyme (Battistoni et al., 1996; Bordo et al., 1994; Carlsson et al., 1996). C indicates copper ligand and Z, zinc ligand.

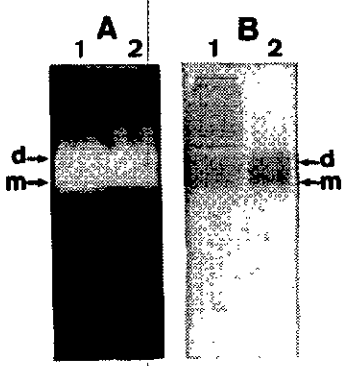

Figure 2. Total soluble protein profiles of IPTG-induced *E. coli* AD494(DE3)pLysS containing the recombinant DNA and one-step purification. Cells were grown in LB medium and induced for the expression of SOD by IPTG as described under Materials and Methods. Seven and a half microliters of induced crude extract (lane 1) and purified enzyme (lane 2) were subjected to a 15% native PAGE followed by activity staining (A, 5.6 μg of purified enzyme) or Coomassie blue staining (B, 11.2 μg of purified enzyme), respectively. "d" denotes the dimeric form and "m", the monomeric form.

containing His-tag in the C terminus was purified by affinity chromatography with nickel chelating resins (His-Bind Resin, Qiagen Co.) according to the instruction manual. The yield was ~25 mg from 1 L of culture. The specific activity of the purified enzyme was 6970 units/mg of protein. The purified enzyme demonstrated two active enzymatic forms (dimer and monomer, Figure 2) on a 15% native PAGE. The result from SDS–PAGE showed that the papaya SOD had an apparent molecular mass of 19 kDa, matching the calculated one from recombinant cDNA (Figure 3, lane 2).

The dimer found in all of the Cu/Zn SODs had caused a considerable debate for a period of time about the importance of subunit–subunit interaction in determining or modulating the catalytic activity and protein stability of these enzymes. In bovine, on the basis of experiments carried out with hybrids of native and chemically modified subunits, it was suggested that the active site of SOD in each subunit acts independently in catalysis (Fridovich, 1986; Malinowski and Fridovich, 1979). However, several other observations supported the possibility of communication between subunits (Rigo et al., 1978).

On the contrary, a monomeric Cu/Zn SOD from *E. coli* was found to have an activity comparable with that of

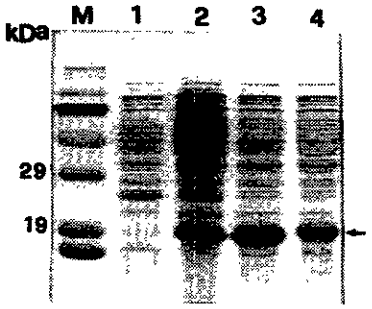

Figure 3. SDS–PAGE analysis of wild type and mutant proteins. Cells (control, wild type, L48F, and L48K) were grown in LB medium and induced for expression of SOD by IPTG as described under Materials and Methods. Each 6 μg of crude enzyme (control, wild type, L48F, and L48K) was amended with 0.2 volume of 5× sample buffer, boiled for 5 min, and then subjected to a 15% SDS–PAGE. M, molecular mass markers; lane 1, AD494(DE3)pLysS carrying pET-20b(+) as control; lane 2, wild type; lane 3, L48F; lane 4, L48K. An arrow denotes Cu/Zn SOD proteins.

a eukaryotic dimer. Thus, it was assumed that communication between subunits was not necessary to ensure efficient catalytic activity (Battistoni et al., 1996).

In our previous work, we found that the purified SW-SOD showed two active forms of the enzyme (dimer and monomer). The subunit interaction resulted in a 7-fold higher catalytic activity in dimer compared with that of monomer. It is important to note that SW-SOD is quite different from other known animal and *E. coli* SODs in having both dimeric and monomeric forms as well as catalytic activity.

In this study, we found that the papaya Cu/Zn SOD showed two active enzymatic forms in equilibrium (30% dimer and 70% monomer), but its subunit interaction was different from that of SW-SOD (dimer is equal to monomer) (Lin et al., 1995).

Characterization of the Purified Papaya Cu/Zn SOD. The enzyme inactivation kinetics at 90 °C fit the first-order inactivation rate equation $\ln(E_t/E_0) = K_d t$, where E_0 and E_t represent the original activity and the residual activity that remained after heating for time t, respectively. The thermal inactivation rate constant K_d values calculated for the dimer and monomer at 90 °C were -0.0203 and -0.0216 min^{-1}, and the half-lives for inactivation were 41.9 and 31.8 min, respectively (Figure 4A–C). Extremely heat stable enzyme as in papaya SOD was not found in all reported Cu/Zn SODs, such as the monomer of SW-SOD at 85 °C that had an inactivation half-life of 28 min (Lin et al., 1995) and fish skin Cu/Zn SOD that when heated at 70 °C was completely inactivated (Nakano et al., 1995).

As shown in Figure 5 (lanes 4–8), papaya SOD was very stable in a broad pH range from pH 5 to pH 11, although the total activity decreased to 80 and 60% at pH 3.0 (lane 2) and pH 2.2 (lane 1), respectively. The decrease of the enzyme activity at acidic pH was due to the dissociation of dimer into monomer (Figure 5B, lanes 1 and 2). Quantitation of proteins by densitometer revealed that the acidic pH favored the monomer formation, whereas the alkaline pH favored the dimer formation. This suggests that the charge interaction could be important for subunit association.

The enzyme activity and the dimer/monomer ratios of the papaya SOD were not significantly changed by SDS (1–4%) (Figure 6A,B). The effect of SDS in papaya was quite different from that of SW-SOD that may be

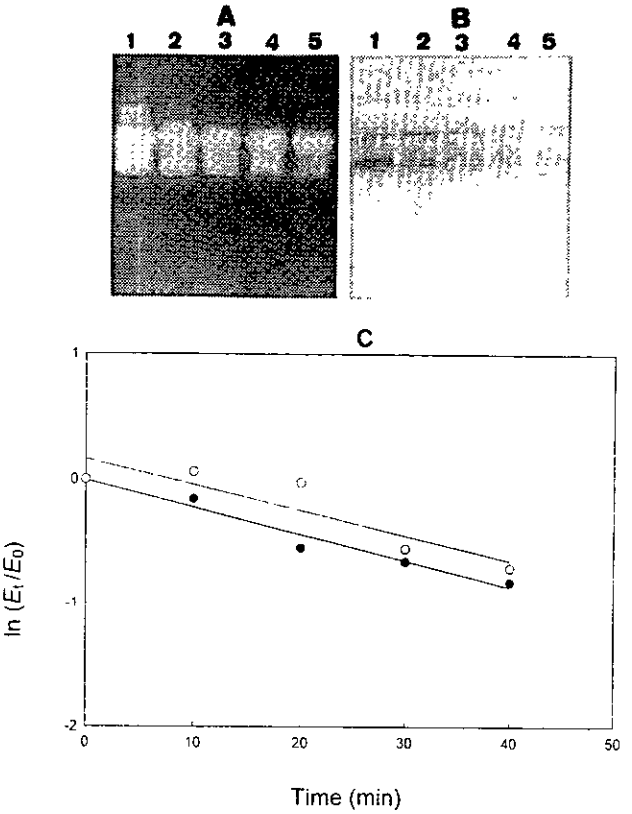

Figure 4. Effect of temperature on the purified papaya SOD. The enzyme samples heated at 90 °C for various times were subjected to a 15% native PAGE: (A) activity staining (3.2 μg each); (B) Coomassie blue staining (4.3 μg each), (lane 1) control, (lane 2) heated for 10 min, (lane 3) heated for 20 min, (lane 4) 30 min, (lane 5) heated for 40 min; (C) plot of thermal inactivation kinetics. The effect of temperature was determined by activity staining (3.2 μg each). The PAGE data were quantitated by a densitometer for calculation. E_0 and E_t are original activity and residual activity after being heated for time t, respectively. ●, monomeric form; ○, dimeric form. Triplicate experiments were done.

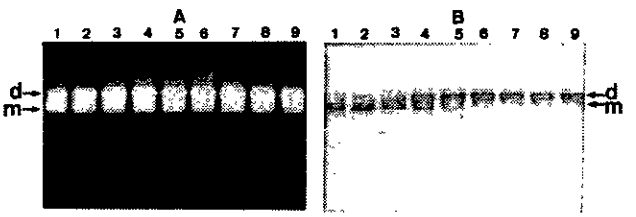

Figure 5. Effect of pH on enzyme stability. The enzyme samples were incubated in buffers with different pH values at 37 °C for 1 h and then subjected to a 15% native PAGE: (A) staining for activity (3.2 μg each well); (B) staining for protein (4.3 μg each well); (lanes 1–4) in citrate of pH 2.2, 3.0, 4.0, and 5.0, respectively; (lanes 5 and 6) in Tris of pH 7.0 and 9.0, respectively; (lanes 7 and 8) in glycine of pH 10.0 and 11.0, respectively; (lane 9) in KCl/NaOH of pH 12.0. The total areas of activity measured by densitometer were 1361.6 ± 86.3 (pH 2.2), 1814.3 ± 23.4 (pH 3.0), 1830.6 ± 44.5 (pH 4.0), and 2268.0 ± 87.5 (pH 5.0–11.0). "d" denotes dimer and m, monomer. Triplicate experiments were done.

due to the easy dissociation from dimer to monomer in the presence of SDS.

The activity of papaya SOD was not significantly affected by imidazole (Figure 6C,D), but most dimers were dissociated into monomers in the presence of imidazole (Lin et al., 1995).

The papaya SOD dimer was much more resistant to proteolytic attack. The enzyme dimer and monomer retained 82 and 30% of original activity, respectively,

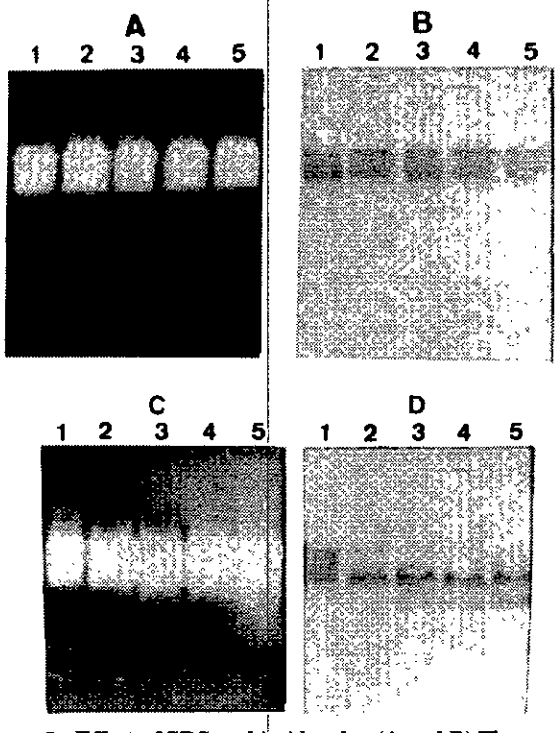

Figure 6. Effect of SDS and imidazole: (A and B) The enzyme samples were treated with various concentrations of SDS at 37 °C for 1 h and subjected to a 15% native PAGE [(A) activity staining (1.5 μg each); (B) protein staining (4.5 μg each); (lane 1) control; (lanes 2–5) in SDS to 1, 2, 3, or 4%, respectively]. The areas of activity measured by densitometer at different concentrations of SDS were almost equal (1566.0 ± 44.4). (C and D) The enzyme samples were incubated in various concentrations of imidazole at 37 °C for 1 h and subjected to a 15% native PAGE [(C) activity staining (2.3 μg each); (D) protein staining (3.7 μg each); (lane 1) control; (lanes 2–5) in imidazole to 0.5, 1.0, 1.5, or 2.0 M, respectively]. The areas of activity measured by densitometer at different concentrations of imidazole were almost equal (1628.8 ± 100.3). Triplicate experiments were done.

after 3 h of incubation with trypsin at 37 °C, whereas the enzyme retained 82 and 47% of original activity, respectively, after 3 h of incubation with chymotrypsin at 37 °C (Figure 7A–D).

Effect of a Single Amino Acid Mutation in the Interface of Subunit. Figure 1 shows the alignment of the amino acid sequence of the papaya SOD with other published animal, plant, and *E. coli* Cu/Zn SODs. Although the secondary structures forming the β-barrel were probably conserved (Bordo et al., 1994), all animal SODs show homodimeric form due to strong hydrophobic interface between the subunits. On the contrary, the *E. coli* SOD retained its monomeric structure even at high protein concentration (Battistoni et al., 1996). This was possibly due to the presence of a charged residue of Lys at position 48. As to plant SODs, a noncharged amino acid residue of Leu was localized at position 48. The animal Cu/Zn SODs contained a noncharged amino acid residue, Phe, at this site. Our results indicated that the plant SODs showed a dimer–monomer equilibrium quite different from that of animal and *E. coli* SODs. Using the site-directed mutagenesis approach, two mutants with a single amino acid change (L48F, L48K) were obtained and analyzed by a 15% SDS–PAGE (Figure 3, lanes 3 and 4). The results demonstrated that the molecular masses of both L48F and L48K were 19 kDa and similar to that of the wild type.

According to the SDS–PAGE analysis, the wild-type papaya SOD displayed 30% dimer and 70% monomer

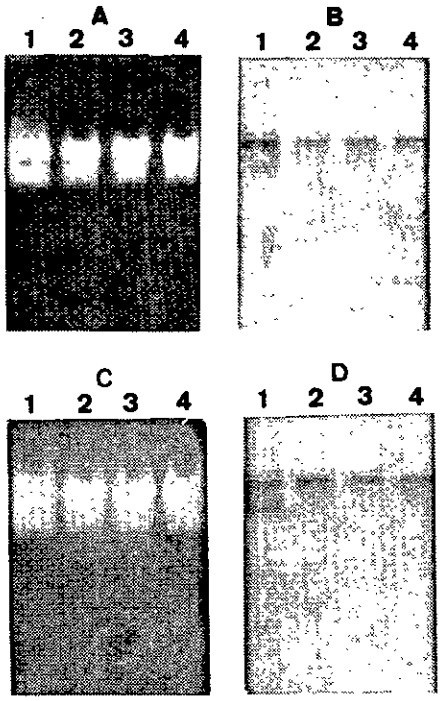

Figure 7. Effect of trypsin (A and B) and chymotrypsin (C and D): (A and B) The enzyme samples were incubated with trypsin at 37 °C for different times and then subjected to a 15% native PAGE [(A) activity staining (3.2 μg each); (B) protein staining (4.3 μg each)]. The enzyme activity after treatment with trypsin was measured by densitomer as lane 1, control ($m = 284.7 \pm 9.4$, $d = 529.7 \pm 25.2$); lane 2, 1 h ($m = 243.4 \pm 14.4$, $d = 400.7 \pm 11.6$); lane 3, 2 h ($m = 195.4 \pm 22.9$, $d = 465.9 \pm 5.6$); and lane 4, 3 h ($m = 87.3 \pm 4.3$, $d = 434.6 \pm 13.1$). (C and D) The purified SOD samples were incubated with chymotrypsin at 37 °C for 1–3 h and subjected to a 15% native PAGE. [(C) activity staining (3.2 μg each); (D) protein staining (4.3 μg each)]. The enzyme activity after treatment with chymotrypsin was measured by densitomer as lane 1, control ($m = 326.2 \pm 23.9$, $d = 560.0 \pm 25.5$); lane 2, 1 h ($m = 320.9 \pm 16.2$, $d = 552.1 \pm 26.3$); lane 3, 2 h ($m = 257.0 \pm 9.4$, $d = 538.8 \pm 18.9$); and lane 4, 3 h ($m = 152.2 \pm 25.8$, $d = 459.5 \pm 21.5$). Data were analyzed by Excel software program. m denotes the area of monomer activity and d, the area of dimer activity.

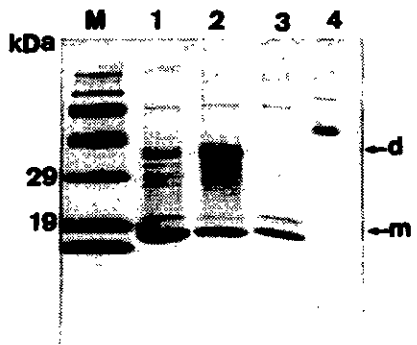

Figure 8. Analysis by 15% SDS–PAGE of wild type and mutant enzymes: (M) molecular mass markers; (lane 1) wild type; (lane 2) L48F; (lane 3) L48K; (lane 4) bovine Cu/Zn SOD; (d) dimeric form; (m) monomeric form. Each of the purified enzymes (6 μg of wild type, 6 μg of L48F, and 3 μg of L48K) and 3 μg of commercial bovine Cu/Zn SOD (RANDOX) were incubated for 20 min at 37 °C in the presence of 1% SDS and loaded into gel without boiling. The electrophoretic migrations of these protein samples (which maintain their initial activity) were compared.

(Figure 8, lane 1). Mutant L48F favored dimeric form (65%; Figure 8, lane 2) like animal Cu/Zn SODs, as we expected due to the noncharged and much more hydro-

phobic Phe residue relative to Leu. In contrast, the mutant L48K (lane 3), like *E. coli* Cu/Zn SOD, demonstrated monomeric form, as we predicted due to the charged Lys residue. This is the first report by genetic engineering to indicate that one amino acid change in the interface of the subunit can influence dimer–monomer equilibrium.

In conclusion, the present work on papaya Cu/Zn SOD suggested that subunit interaction might change the conformation or alter the tertiary structure of the enzyme and enhance the catalytic activity. It was also possible that the intersubunit contacts may stabilize a particular optimal conformation and/or tertiary structure of the enzyme or the dimeric structure to enhance catalytic activity by increasing the electrostatic steering of substrates into the active sites (Lin et al., 1995). These observations did not support the current hypothesis that the Cu/Zn SOD subunit functions independently and provided a new insight for further studies on the structure–function relationship of SOD. From a biotechnological point of view, these results suggest dimer stabilization by protein engineering would greatly enhance the activity and stability of SOD for the purpose of applications such as the protection of the skin against inflammatory reaction and periodontal use (in alveolar pyorrhea) (Nakano, 1989; Wilder and Mass, 1990).

Conclusion. The coding region of Cu/Zn SOD cDNA from papaya fruit and its mutant were successfully overexpressed in an *E. coli* system and provided an easy method for obtaining large amounts of active enzyme for further enzymatic or application studies. Our study has shown that the papaya fruit Cu/Zn SOD is quite unique in that it is stable in a broad pH range and at an elevated temperature as high as 90 °C and resists both detergent (SDS) and proteolytic enzyme treatments. Mutation of the enzyme can affect the association of monomer. These properties will be useful for its applications in the medical field.

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