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นางสาววันวิสา มูลอ้าย

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PURIFICATION AND CHARACTERIZATION OF SUPEROXIDE DISMUTASE FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS

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A Thesis Submitted in Partial Fulfillment of the Requirements

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วันวิสา มูลอ้าย : การทำให้บริสุทธิ์และลักษณะสมบัติของซูเปอร์ออกไซด์ดิสมิวเทสจากเหง้าของพืชวงศ์ขิง (PURIFICATION AND CHARACTERIZATION OF SUPEROXIDE DISMUTASE FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.อภิชาติ กาญจนทัต , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.พลกฤษณ์ แสงวณิช, 53 หน้า.

ซุปเปอร์ออกไซด์ดิสมิวเทส (SOD, EC 1.15.1.1) เป็นเมทัลโลเอนไซม์ที่เร่งปฏิกิริยากำจัดสารอนุมูลอิสระในระยะปฐมภูมิ ช่วยปกป้องการเกิดภาวะอนุมูลอิสระที่มีมากเกินสมดุล โดยทำหน้าที่กำจัดแอนไอออนของซุปเปอร์ออกไซด์ซึ่งเป็นสารตั้งต้นของอนุมูลอิสระ โดยประโยชน์ของซุปเปอร์ออกไซด์ดิสมิวเทส ได้มีการนำมาประยุกต์ใช้ทางการแพทย์ อุตสาหกรรมเครื่องสำอางและอุตสาหกรรมเคมี เป็นต้น งานวิจัยนี้ได้ทำการคัดกรองซุปเปอร์ออกไซด์ดิสมิวเทสจากส่วนสกัดหยาบและโปรตีนหยาบจากเหง้าของพืชวงศ์ขิง 15 ชนิด พบว่าส่วนสกัดหยาบ และโปรตีนหยาบจากเหง้าของว่านมหาเมฆมีกิจกรรมของซุปเปอร์ออกไซด์ดิสมิวเทสสูงสุด จึงได้นำโปรตีนหยาบจากเหง้าของว่านมหาเมฆมาทำให้บริสุทธิ์ โดย การตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต และทำให้บริสุทธิ์เพิ่มขึ้นโดยใช้เทคนิค โครมาโตกราฟี ได้แก่ โครมาโตกราฟีแบบแลกเปลี่ยนประจุ คอลัมน์ DEAE cellulose และ โครมาโทกราฟีแบบเจลฟิลเตรชัน คอลัมน์ Superdex 75 พบว่าซุปเปอร์ออกไซด์ดิสมิวเทสมีความบริสุทธิ์เพิ่มขึ้น 4.36 เท่าและมีผลผลิตเท่ากับ 2.51 เปอร์เซ็นต์ จากนั้นศึกษาลักษณะสมบัติของซุปเปอร์ออกไซด์ดิสมิวเทสบริสุทธิ์ที่ได้ เช่น หาน้ำหนักโมเลกุลซึ่งได้เท่ากับ 31.5 กิโลดาลตัน โดยใช้เทคนิคโพลีอะคริลาไมด์ เจลอิเล็กโตรฟอริซิส และแอคติวิตีของซุปเปอร์ออกไซด์ดิสมิวเทสสูงสุดที่ค่าความเป็นกรด – ด่างเท่ากับ 4.0 และเอนไซม์มีความเสถียรที่อุณหภูมิ -20 จนถึง 45 องศาเซลเซียส นอกจากนี้ได้ศึกษาชนิดของเอนไซม์ พบว่าเป็นเอนไซม์ชนิด MnSOD ซึ่งไม่มีความไวต่อไซยาไนด์และไฮโดรเจนเปอร์ออกไซด์ จากการศึกษาถึงค่าจลนพลศาสตร์ของเอนไซม์โดยใช้ NBT เป็นสับสเตรท พบว่าค่า K_m และ V_{max} เป็น 57.31 โมลาร์ และ 333.7 ไมโครโมล/นาที/มิลลิกรัมของโปรตีนและเมื่อศึกษาถึงค่าจลนพลศาสตร์ของเอนไซม์โดยใช้riboflavin เป็นสับสเตรท พบว่าค่า K_m และ V_{max} เป็น 1.51 โมลาร์ และ 254.1 ไมโครโมล/นาที/มิลลิกรัมของโปรตีน

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5272528523 : MAJOR BIOTECHNOLOGY
KEYWORDS : SUPEROXIDE DISMUTASE / ZINGIBERACEAE PLANTS / PURIFICATION / CHARACTERIZATION

WANWISA MOON-AI: PURIFICATION AND CHARACTERIZATION OF SUPEROXIDE DISMUTASE FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS. ADVISOR: APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., 53 pp.

Superoxide dismutase (SOD, EC 1.15.1.1) was metalloenzyme or antioxidant enzyme. It catalyzes the disproportionation of harmful superoxide anionic radical to hydrogen peroxide and molecular oxygen. Due to its antioxidative effects, SOD has long been applied in medicinal treatment, cosmetic and other chemical industries. In this study, aqueous extracts and crude proteins from the rhizomes of fifteen Zingiberaceae plants were tested were tested for SOD activity. The *Curcuma aeruginosa* showed a highest SOD activity. SOD from the rhizomes of *C. aeruginosa* was purified by ammonium sulphate precipitation, DEAE cellulose and Superdex 75 column chromatography. A 4.36 fold purification and on overall yield of 2.51% were achieved. The purified enzyme was 31.5 kDa as judged by SDS-PAGE. The enzyme has a pH optimum of 4.0. It was stable over a wide range of temperatures -20 – 45 ºC. It belongs to the MnSOD category due to the fact that it was insensitive to KCN or hydrogen peroxide inhibitor. The $Mn^{2+}$and Fe^{2+} ions stimulated SOD inhibition activity when increased metal ions concentrations. Maximum activity was observed to 333.7 and 254.1 µmol/min/mg for NBT, and riboflavin respectively. The K_m of enzyme was found to be 57.31, and 1.51 M for NBT, and riboflavin respectively. This is the first report for SOD from *C. aeruginosa*, a traditionally used Thai medicinal plants.

Field of Study : Biotechnology Student's Signature .....
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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
μg	microgram
μl	microlitre
A	absorbance
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CNS	central nervous system
cm	centimeter
Da	Dalton
EDTA	ethylenediamine tetraacetic acid
ESI/MS/MS	Electrospray ionisation/Mass spectrometry/Mass spectrometry;
g	gram
hr	hour
IC_{50}	The half maximal inhibitory
kDa	kilodaton
K_m	Michaelis-Menten constant
l	litre
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
M	molar
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
N	normal
nm	nanometer
NaCl	sodium chloride

NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
rpm	revolution per minute
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SOD	superoxide dismutase
TEMED	*N*,*N*,*N'*,*N'*-tetramethy ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
U	Unit activity
V	volt
V_{max}	maximum velocity
V/V	volume by volume
W/V	weight by volume

CHAPTER l

INTRODUCTION

In the plant system, reactive oxygen species (ROS) such as superoxide radical ($^{\bullet}O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) are generally formed the electron transport activities of chloroplast, mitochondria and plasma membranes (Foyer 1997; Allen, 1995). In addition, ROS production has been stimulated by various environmental stresses (Foyer *et al*., 1997; Boo and Jung, 1999) as well as by some biotic factors such as invasion of various pathogens. The ROS are highly toxic to cell as they cause lipid peroxidation of biomembranes, mutations, protein denaturation and enzyme inactivation (Regoli and Winston, 1999). However, antioxidants such as ascorbate and glutathione and antioxidation enzyme namely superoxide dismutase, catalase, ascorbate peroxidase etc., are present in plants to scavenge ROS and give protection against oxidative stress (Foyer *et al*., 1994).

Of these antioxidative enzymes, SOD ($^{\bullet}O_2^-$: $^{\bullet}O_2^-$ oxidoreductase EC 1.15.1.1) is of immense physiological importance as it catalyses the dismutation of superoxide radical to molecular oxygen and H_2O_2, which is the first toxic product generated during oxidative stress (Fridovich, *et al*., 1969) and should be decomposed to prevent generation of other harmful ROS. SOD has been purified and characterized from a number of organisms such as plant (Ishikawa, 1995; Zaki, 1999; Pruthi, 2002), animals (Weisiger, 1973), and microorganisms (Kanematsu and Asada, 1978; Pavlina *et al*., 1999; Barkely and Gregory, 1990). It is a metalloenzyme and based on the prosthetic group, three different forms have been characterized: CuZn-SOD, Mn-SOD, and Fe-SOD. CuZn-SOD is sensitive to cyanide and H2O2 but not to chloroform plus ethanol, Mn-SOD is denaturated by chloroform plus ethanol, whereas Fe-SOD is insensitive to chloroform plus ethanol as well as cyanide but in activated by H_2O_2 (Bray *et al*., 1974; Fridovich, 1978).

The Zingiberaceae is a well-known plant family in Southeast Asia and many of its species are used in traditional folklore medicine for the effective treatment of several diseases. They are perennial herbs that are widely cultivated in Thailand and the tropical regions of Asia, and have been commonly used as medicinal plants and spices in Thailand. The rhizomes of these plants possess diverse biological activities,

including anti-microbial (Yamada *et al*., 1992; Hiserodt *et al*., 1998), anti-ulcer (Al-Yahya *et al*., 1990; Matsuda *et al*., 2003), anti-inflammatory (Araujo and Leon, 2001), anti-oxidant (Selvam *et al*., 1995), cytotoxic and anti-tumor (Itokawa *et al*., 1987; Murakami *et al*., 1995, 2000; Pal *et al*., 2001), vasorelaxant (Othman *et al*., 2002), anti-spasmodic (Ammon and Wahl, 1991), anti-hepatotoxic (Hikino *et al*., 1985) and anti-depressant activities (Noro *et al*., 1983; Yu *et al*., 2002).

The demand for natural products which promote a good health status is growing considerably. Antioxidants are important in human nutrition for their portective role against different pathological situations and the aging processes (Halliwell and Gutteridge, 2000). Besides the low molecular weight antioxidants (ascorbate, glutathione, vitamin A and E, etc.), there are several enzymatic systems, inculding superoxide dismutase which is an important defence line against oxidative stress (Vang *et al*., 2001; Alscher *et al*., 2002). Zingiberaceae plants are one of the most consumed and popular herbs, and the knowledge of its nutritional properties, including its antioxidative content is important for human nutrition. Although there have been many reports concerning the active chemical constituents of these plants including the rhizomes, and some biological activities of these species, these have largely been reported on the non-proteinaceous compounds. Indeed, only a few reports have focused on the antioxidation proteins such as SOD from these plants. Consequently, the aims of this study were to purify and characterize SODs from the rhizomes of selected Zingiberaceae plants.

CHAPTER ll

LITERATURE REVIEWS

2.1 Active Oxygen Species and General Scavenging Systems

In the plant system, the levels of reactive oxygen species (ROS) inside the cell are maintained at their lowest by the relevant protective mechanisms. However, excesses of ROS are produced during particular periods of development as well as in response to various types of stress. Plant cells posses both enzymic and non-enzymic mechanisms which can overcome oxygen toxicity and delay the deleterious effects of free radicals. The enzymic antioxidant defense systems include enzymes capable of removing, neutralizing, or scavenging free radicals and oxyintermediates (Scandalios, 1993). Without these defenses, plants could not efficiently convert solar energy to chemical energy.

2.1.1 Reactive oxygen species (ROS) formation and activity

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. Reactive oxygen species are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation. ROS contain two free radical species, the superoxide anion ($O_2^{\bullet-}$), the perhydroxyl radical ($HO_2^{\bullet-}$), the uncharged, non-radical species hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\bullet}$). ROS also include singlet oxygen ($^1O_2$), and the primary source of this is the chlorophyll pigments relevant to the electron transport system. Reactive oxygen species are produced by several diverse mechanisms: through the interaction of ioinizing radiation with biological molecules, as an inevitable by-product of cellular respiration, and through being synthesized by dedicated enzymes like NADPH (nicotinamine adenine dinucleotide phosphate) oxidase (NOX) and cell wall peroxidases (Bolwell *et al.*, 2002). Intense oxidants, like the diverse ROS, can damage other molecules and the cell structures, and this may result in metabolic

destruction and disrupt cellular structures. It is important to note, however, that attempts to limit the production of ROS would not be desirable, because ROS have some positive functions in plant physiology beyond their role as agents of cellular damage. ROS, at low concentrations, have the function of centrally co-ordinating cell biology and the responses of the latter to a number of environmental stimuli.

2.1.2 Superoxide production

Superoxide is produced in plants in diverse ways, and in several cellular compartments. These ways include non-enzymatic mechanisms, such as electron transfer to molecular oxygen during photosynthesis and respiration in chloroplasts and mitochondria. Plant cell can also increase their generation of superoxide through specific enzymes, such as NADPH oxidases and cell wall peroxidases.

2.1.2.1 Superoxide production in plasma membranes

The initial steps in many of the processes in plants, such as signalling, nutrient uptake, stress response, growth, and development, are achieved via activated oxygen and other radical species, some of them accompanied by an externally measurable oxidative burst considered being a component of the plant defence responses. The production of extracellular activated oxygen species during such an oxidative burst in plants may be mediated by the activity of enzymes located in the plasma membrane. The involvement of a plasma membrane oxidase, catalysing the reduction of molecular oxygen to superoxide at the expense of NAD(P)H, similar to the inducible NADPH oxidase in the plasma membrane of mammalian phagocytes was observed as an early response to pathogen attack. The second mechanism for apoplastic production in plants, which can be discriminated from the phagocyte-type oxidase by high sensitivity to inhibition by KCN, is provided by the NAD(P)H oxidizing activity of peroxidase localized in the cell wall (or at the plasma membrane). The extremely reactive $^{\bullet}OH$ radical can be generated in a Fenton reaction when and H_2O_2 are available. However, it has recently been demonstrated that $^{\bullet}OH$ radicals are released by maize coleoptiles, and that this is mediated by an enzymatic reaction, suggested to be a cell wall peroxidase. Plants, like animals, generate ROS when they are attacked by pathogens (the oxidative burst), as this can provide a direct defence by damaging the pathogen. ROS have also been proposed as having a role in programmed cell death (PCD) and in senescence. Plasma membrane localized NADPH oxidase (NOX) enzymes, producing extracellular superoxide, have been shown to be related to these

processes of plant development, pathogen defence, PCD and senescence (Jones and Smirnoff 2005). It can be seen, therefore, that ROS have a crucial function as signalling molecules in cell growth, development, PCD and an important source of these signals is the superoxide ($O_2^{\bullet-}$) generating plasma membrane (PM) NOX complex. This is because $O_2^{\bullet-}$ can easily generate other ROS, including H_2O_2 and OH•, and because H_2O_2 generation is catalysed by SOD (Halliwell and Gutteridge 1999). Plant NOXs are inherent proteins in the plasma membrane that catalyse the production of $O_2^{\bullet-}$ from molecular oxygen using reduced NADPH as an electron donor. Regular NOX-mediated $O_2^{\bullet-}$ production in the PM of plants is regulated by ROS, small GTPase (ROP GTPase), Ca^{2+}, phosphoatidic acid and PI3P (phosatidylinositol 3- phosphate). NADPH oxidase, containing six transmembrane domains, predicts N- and C-terminal cytoplasmic domains, which are activated by Ca^{2+} *in vitro* because the N-terminus has one or two Ca^{2+} binding motifs. Therefore, Ca^{2+}may be a major factor in regulating NOX-mediated $O_2^{\bullet-}$ production in plants. This oxygen reduction occurs on the apoplastic side.

2.1.2.2 Superoxide in plant cell walls

Superoxide production in plant cell walls is controlled by the developmental programme, for example, the lignification of vascular tissue in spinach hypocotyls. It can be also regulated by internal and external stimuli such as mechanical stress in potato tubers. Most external stimuli have been shown to induce increased superoxide production, and pathogen and pathogen-related stimuli have been particularly widely studied. In addition, $O_2^{\bullet-}$ production in maize coleoptiles has been shown to be controlled by hormonal regulation. Experimentation showed that $O_2^{\bullet-}$ production in coleoptile segments was enhanced by the addition of exogenous auxin. The life-cycle of $O_2^{\bullet-}$ production is brief because SOD is the major scavenger of $O_2^{\bullet-}$ in the apoplast. Most SOD found in cell wall is the Cu/Zn SOD, which catalyses the dismutation of $O_2^{\bullet-}$ to produce O_2 and H_2O_2. It has also been proposed that, to dismutate the $O_2^{\bullet-}$ yielded by NADPH oxidases, SOD is localized near membrane-bound enzymes. $O_2^{\bullet-}$ can also be scavenged through reactions with phenolics, ascorbate and glutathione (Vreeburg and Fry, 2005).

2.1.2.3 The production of superoxide radicals

The production of superoxide radicals, via immune responses and normal metabolism, is a substantial contributor, if not the primary cause, of pathology associated with neurodegenerative diseases, ischemia reperfusion injury,

atherosclerosis and aging. Superoxide Dismutases (SODs) catalyze the dismutation of the superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2) which diffuses into the intermembrane space or mitochondrial matrix (Figure 2.1), and thus, SODs provide an important defense against the toxicity of superoxide radicals.

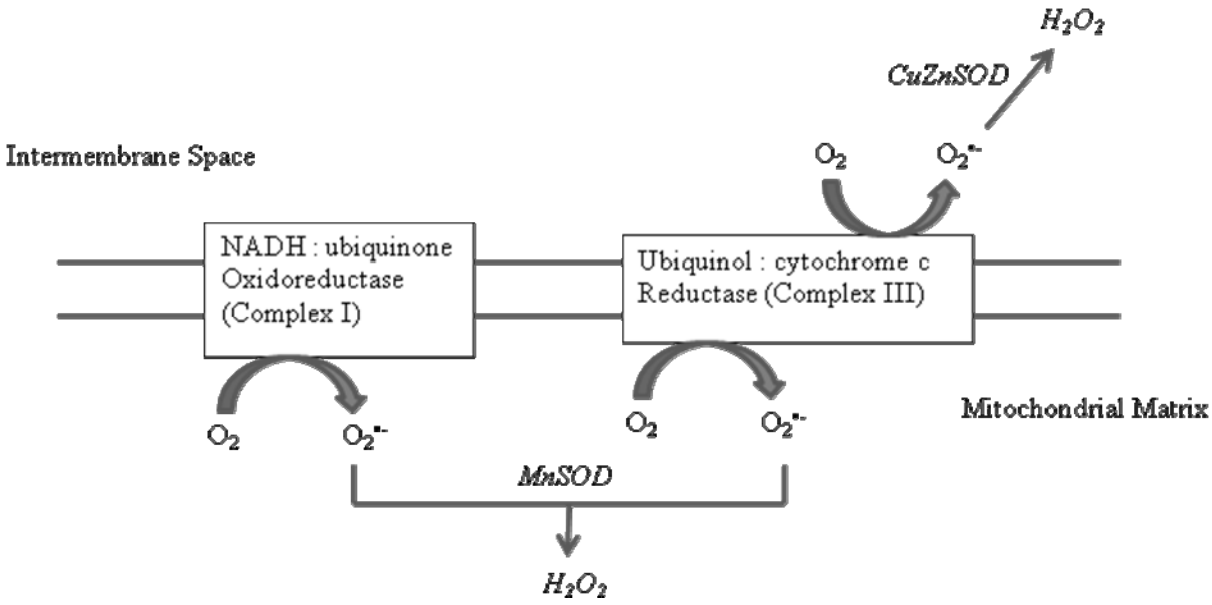

Figure 2.1 Hydrogen peroxide production by SODs.

The SOD activity was determined using the riboflavin-nitroblue tetrazolium (NBT) assay, with slight modifications from the Lai method (Lai *et al*., 2008). NBT generates blue formazan deposits after reduction by $O_2^{\bullet-}$, and this change of colour can be measured by a spectrometer. The appearance of blue formazan deposits, due to the reaction of NBT with superoxide, can also indicate the areas of superoxide formation. For example, superoxide has been found in leaves undergoing photo-oxidative stress by its reduction of NBT to formazan deposits. In this study, the tip region of the leaf, which had been exposed to strong photo oxidative stress, exhibited stronger staining than non-stress regions, with the majority of the staining being related to the mesophyll tissue. Superoxide radical production in the tissues of

mechanically stressed potato tubers has also been directly measured using this NBT method (Johnson *et al*., 2003).

2.2 Superoxide dismutase (SOD)

Superoxide is eliminated by dismutation to H_2O_2 catalyzed by superoxide dismutase (SOD) (Fridovich, 1978) and accumulation of H_2O_2 is prevented by the action of catalases and peroxidases (Hassan and Fridovich, 1978). SODs are essential for aerobic survival and are ubiquitous among aerobic and aerotolerant organisms (Hassan, 1989) and even some anaerobic organisms. Fulghum and Worthington (1984) presented data that show that some species of ruminal bacteria contain measurable levels of SOD activity. Four SOD isoenzymes have been discovered in prokaryotic and eukaryotic organisms. Unlike most other living organisms, plant SODs exist in multiple forms (isozymes). Baum and Scandalios (1979) first demonstrated the existence of SOD isozymes in plants and established their genetic basis. SOD is an intracellular enzyme that is found in every cell and remains comparatively stable. SODs increase when plants are under environmental stresses, while they are also known, in animals and humans to decrease with age. A number of studies have also indicated that oxidative stress increases SOD activity in both prokaryotes and eukaryotes (Scandalios, 1993).

2.2.1 Classification of SOD

Based on the property that SODs are metalloenzymes, SODs have been classified into three common isozymes distinguished by their prosthetic metal, namely copper and zinc (Cu/Zn SOD) and manganese (Mn SOD), or iron (Fe SOD). Cu/Zn SODs are commonly found in the cytosol of eukaryotic cells and chloroplasts and are the most abundant SOD in higher plants. As a result, most Cu/Zn SODs have been purified from the tissues of higher plants, for example spinach leaves (Asada and Kiso 1973), tobacco leaves (Sheng *et al*., 2004) and camellia pollen (Xiao-hong 2005). Almost all of the Cu/Zn SOD enzymes purified so far from eukaryotic cells are homodimers, and the molecular weight of these is around 32,000 Daltons (Da). Each subunit is 151 amino acids long. The active site, which contains one copper and one zinc ion, is made of two large loops that connect the strands. The Mn SOD isozyme is also present in both eukaryotes and prokaryotes. Although Mn SOD is known as the mitochondrial enzyme of all eukaryotic cells, Mn SOD containing has been located in the peroxisomes as well. The presence of one peroxisomes and mitochondrial Mn SOD was shown by using immunolocilazation assays in watermelon (Rio *et al*.,

1992). In green algae and cyanobacteria, Mn SOD is found in the thylakoid membrane (Kanematsu and Asada, 1979; Li *et al*., 2002). In pea leaves, Mn SOD is localized in mitochondria as well as peroxisome (Rio *et al*., 2003). In higher plants, Mn SODs are mainly present in mitochondria from different plants (Rio *et al*., 1992, Halliwell and Gutteridge, 2000). On the other hand, in the fruit and leaves of most plants, Mn SOD has been shown to be only 3-5% of the total SOD activity (Fridovich, 1986). The subunit weight of Mn SOD is around 23,000 Da and the enzyme may be dimeric or tetrameric. Fe SODs are generally found in prokaryotes and have also been found in three families of higher plants *Ginkoaceae, Cruciferae* and *Nymphaceae*. Up to now, in all plant species examined, it is inferred that Fe SOD is located in chloroplasts (Alscher *et al*., 2002). Support for this theory comes from the existence of several conserved regions that are present in plant and cyanobacterial Fe SOD sequence, but absent in non-photosynthetic bacteria (Bowler *et al*., 1994) .As with Mn SOD, the subunit weight of Fe SOD is close to 23,000 Da and the enzyme may be a dimer or tetramer. The amino acid sequences of Fe SODs are very similar to those of Mn SODs, but quite distinct from the sequences of Cu/Zn SODs.

2.2.2 SOD inhibitors

Cyanide is an extremely powerful inhibitor of Cu/Zn SODs, and H_2O_2 also inhibits these enzymes irreversibly. This is because that both cyanide and H_2O_2 interact with the Cu of the enzymes (Scandalios, 1993). As a result, this susceptibility of Cu/Zn SODs to cyanide has been utilized as an important tool to distinguish Cu/Zn SODs from Fe SOD and Mn SOD, as cyanide has no effect on these latter two types of enzyme. Mn SOD is not inhibited by cyanide and H_2O_2, but it can be removed through treatment with chloroform plus ethanol. Fe SOD is similar to Mn SOD in that it is not affected by cyanide, while it is distinguishable in that it is irreversibly inactivated by H_2O_2 (Halliwell and Gutteridge, 1985).

2.3. Studies of SOD in plants

2.3.1 SOD activities

There have been a number of papers published in this area. In the published research, cDNA clones were used as probes for choroloplast Cu/Zn SOD (clone T1) and cytosolic Cu/Zn SOD (clone P31). When this was performed, the two genes presented relatively different expression patterns. The T1 transcript was rare or absent

in roots, stems and ripening fruits, whereas the P31 transcript was abundant. The shoot tips, seedlings, flower buds, and young leaves, however, presented high levels of the two mRNAs at all developmental stages. In tomatoes (Perltreves and Galun, 1991), it was found that the expression of the two Cu/Zn SOD genes present depended on the organs and the developmental stage of the plant and on the levels ofenzyme activity. In Norway spruce, the effect of developmental changes on SOD activity, and on the presence or absence of isozymes, were also studied (Kroniger *et al*., 1993). It was found that there were two Cu/Zn SODs (SOD I and SOD II) and one Mn SOD (SOD III) in the spruce spruce seeds, seedlings and foliar buds. With these SODs, the highest activities were found in the buds and germinating seeds. The highest SOD activities occurred in young differentiating tissues of Norway spruce, implying that younger tissues need higher protection against oxidative stress than do photosynthetically active needles. It seems that the appearance of the three SOD isozymes was independently regulated with regard to developmental stages and seasons. In the seedlings and developing kernels of maize, the steady-state levels of cytosolic (Cu/Zn) and mitochondrial (Mn) SOD mRNAs were investigated by means of an RNA blot analysis, which used Cu/Zn and Mn cDNA encoding these isozymes as molecular probes (White *et al*., 1990).

2.3.2 SOD in seeds

The generation of active oxygen species (AOS) is incorporated in aspects of seed physiology, such as germination and aging, and may result in oxidative stress and cellular damage. The activities of antioxidant enzymes and other antioxidants during the first stages of germination have been studied in *Chenopodium rubrum* seeds (Ducic *et al*., 2003). In this study, SOD activity was highest during radicle protrusion and seedling development, while SOD and CAT activities were highest before radicle protrusion. The principal difference was that SOD specific activity in oats increased rapidly during the first days of germination, while in peas it remained at the initial level at this stage. In peas, it was found that the most significant increase in SOD specific activity occurred during greening and hook opening. It was also found that concentrations of oxidized and reduced glutathione remained constant during germination, with the highest levels detected at the time of radicle protrusion. Ascorbic acid was only detected preceding radicle protrusion, whereas its oxidized form was present during the whole germination period. In general, therefore, antioxidant enzymes, such as SOD and CAT, increased significantly prior to radicle

protrusion, while oxidized concentrations diminished during further germination of these seeds. Previously, Cu/Zn SOD from the *Radix lethospermi* seed that is used as a medicinal material was purified and characterized by ammonium sulfate fractionation and successive column chromatographic procedures including DEAE-52, Sephadex G-200 and DEAE-52 again. The purified enzyme had a specific activity of 4843 U/mg, and was purified 267.2 fold with a yield of 23.55%.

2.3.3 SOD in flowers and fruits

SOD activities during flower budding and fruit development of apple have also been studied (Abassi *et al*., 1998). In the dormant flower buds of this plant, SOD and catalase activities were very low, but these then increased 2 - 5 fold during bud swelling. SOD activity fell 5 - 8 fold, however, with the onset of the bud break and during further fruit development. In fact, SOD activity was only detected in the peel, but not in the cortex and seed tissue, of immature and mature apple fruit. Similarly, when pepper and cucumber fruits were studied, SOD activity was mainly detected in the pepper's pericarp and in the skin and peeled pericarp of the cucumber (Rabinowitch and Sklan, 1981). It was also found that SOD activities were high in immature-green fruits of both species, but these decreased to a minimum during the mature green stages. SOD levels went up again, however, until the peppers turned orange in colour and the cucumbers became yellow, and then only declined again after this ripening. It has been found that free radicals have a significant role in fruit ripening, and that superoxide dismutase and catalase are the most important antioxidant enzymes influencing fruit ripening patterns

2.4 Other antioxidants in plants

2.4.1 Catalase

Catalase is an unusual enzyme since, although hydrogen peroxide is its only substrate, it follows a ping-pong mechanism. Here, its cofactor is oxidised by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate. Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase "acatalasemia" or mice genetically engineered to lack catalase completely, suffer few ill effects. Hydrogen peroxide causes the formation of $^{\bullet}OH$ radicals, and thus it damages living organisms. Compared with other plant cells, photosynthesizing plant

cells show especially high rates of H_2O_2 production. Furthermore, low concentrations of H_2O_2 can rapidly inactivate photosynthesis because of the way they inhibit CO_2 fixation. It is thought that H_2O_2 is especially harmful, even though H_2O_2 is a less reactive oxidant when compared to other ROS, because it is comparatively stable and may therefore diffuse within cells. As a result, H_2O_2 can generate more ROS, which greatly increases its toxicity, meaning that the effective scavenging of H_2O_2 is very important for the survival of plants. This indicates, in turn, that the fact that hydrogen peroxide can be converted to oxygen and water by the enzyme catalase means that this enzyme plays an important role in plants. Generally catalases are small subunit monofunctional enzymes, or tetrameric proteins with a molecular weight of around 240 kDa. The typical inhibitors of these plant catalases are cyanide, azide and 2-aminotriazole. In addition, micromolar concentrations of nitrate and millimolar concentrations of sulfite can also inhibit plant catalases (Streb *et al*., 1993).

2.4.2 Glutathione and glutathione reductase

Glutathione and glutathione reductase, as well as cytochrome c function in the scavenging of H_2O_2. Oxygenase, or more specific as peroxygenase, in pea microsome catalyzes the hydroxylation of indoles, by which oxygen from H_2O_2 is incorporated to form hydroxylated products. Glutathione also contributes to ascorbate regeneration by way of dehydro-ascorbate reductase, and it is also formed by glutathione reductase (GR) in a NADPH-dependent reaction.

2.4.3 Ascorbic acid

Ascorbate is both the most abundant small molecular weight antioxidant and the most important antioxidant in plants. Its basic role is the elimination of hydrogen peroxide. Ascorbic acid is redox catalyst which can reduce, and thereby neutralize, reactive oxygen species such as hydrogen peroxide. In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the redox enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants. Ascorbic acid is present at high levels in all parts of plants and can reach concentrations of 20 millimolar in chloroplasts. High concentrations of ascorbate are commonly found in fruits, but ascorbate concentrations in fruit are, in fact, not always higher than those found in leaves and may even be lower in some species (Davey *et al*., 2000). There are two successive steps in the oxidation of this ascorbate: firstly, mono-dehydro-ascorbate is generated, and secondly, the mono-dehydroascorbate is

converted disproportionately to ascorbate, or dehydro-ascorbate if it is not swiftly re-reduced to ascorbate (Foyer, 1994).

2.4.4 Ascorbate peroxidase

Ascorbate peroxidase, participating in the removal of H_2O_2, is found in almost every compartment of the plant cell. Its activity has been frequently reported in the chloroplasts and cytosol, but some recent research has shown that it is also active in mitochondria (Anderson *et al*., 1995).

2.4.5 Carotenoids

Carotenoids can scavenge $^{\bullet}OH$, $O_2^{\bullet-}$ and peroxyl radicals, and can also prevent the oxidation of vitamin A. Additional functions of carotenoids are to quench singlet oxygen and to shield cells by absorbing excess excitation energy from chlorophyll (Arora *et al*., 2002).

2.4.6 Thioredoxin

The thioredoxin system contains the 12 kDa protein thioredoxin and its companion thioredoxin reductase. Proteins related to thioredoxin are present in all sequenced organisms with plants, such as Arabidopsis thaliana, having a particularly great diversity of isoforms. The active site of thioredoxin consists of two neighboring cysteines, as part of a highly conserved CXXC motif, that can cycle between an active dithiol form (reduced) and an oxidized disulfide form. In its active state, thioredoxin acts as an efficient reducing agent, scavenging reactive oxygen species and maintaining other proteins in their reduced state. After being oxidized, the active thioredoxin is regenerated by the action of thioredoxin reductase, using NADPH as an electron donor.

2.4.7 Uric acid

The antioxidant in highest concentration in human blood is uric acid, which provides about half of the total antioxidant capacity of human serum. Uric acid is an oxypurine produced from xanthine by the enzyme xanthine oxidase, and is a waste product of purine metabolism in primates, birds, and reptiles. An overabundance of this chemical in the body causes gout. The effects of uric acid in conditions such as stroke and heart attacks are still not well understood, with some studies linking higher levels of uric acid with increased mortality. This apparent effect might either be due to uric acid being activated as a defense mechanism against oxidative stress, or uric acid acting as a pro-oxidant and contributing to the damage caused in these diseases.

Uric acid is released from tissues that are short of oxygen and elevated uric acid levels may be an important part of acclimatisation to high altitude.

2.4.8 Melatonin

Melatonin is a powerful antioxidant and, unlike conventional antioxidants such as vitamins C and E and glutathione, it is both produced in the human body and is acquired in the diet (fruits, vegetables, cereals and herbs etc., contain melatonin). Melatonin easily crosses cell membranes and the blood-brain barrier. Unlike other antioxidants, melatonin does not undergo redox cycling, which is the ability of a molecule to undergo repeated reduction and oxidation. Redox cycling may allow other antioxidants (such as vitamin C) to act as pro-oxidants and promote free radical formation. Melatonin, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant.

2.5 Applications of SOD

Anti-oxidative stress mechanisms, including SOD, in the human body are limited to the inside of cells and to tissues where ROS occur. If the quantity of ROS exceeds the ability of the defence mechanisms of the body to respond, severe diseases, such as cancer and arteriosclerosis, may develop (Ukeda, 2004). Moreover, cell damage as a result of excessive ROS is also considered to be one of the principal causes of aging and aging-related diseases. Therefore, there has been much interest in the use of ROS trapping agents for their potential use as anti-aging agents and as a possible cure for many diseases. To this end, SODs are often included in nutritional supplements, and as coenzymes in cosmetic products, in order to combat anti-oxidative stress and to prevent aging. More specifically, SOD is included in a variety of cosmetic products to decrease free radical damage to skin, because SOD is found naturally in both the dermis and epidermis and is crucial for the production of healthy fibroblasts. SOD is also used in decreasing fibrosis following radiation for breast cancer. As a result of all of these interests, SOD can nowadays be applied in a variety of forms, including injections, sublingual oral supplements, enteric-coated pills, and topical creams. In its topical form, for example, it is thought that SOD will help to decrease scar tissue, heal wounds and protect against harmful UV rays (Paramonov *et al*., 2005).

SODs are also applied as treatments for some diseases because of their function as anti-inflammatory and autoimmune agents. In these cases, SODs are applied to compensate for a lack of natural SOD, and to help neutralize free radicals, in patients with Crohn's disease (Phylactos *et al*., 2001), prostate problems, corneal ulcers, inflammatory diseases and rheumatoid arthritis (Eugenia *et al*., 2005). Furthermore, the recent significant developments in SOD therapy in two new areas, namely ischaemia-reperfusion and the practice of grafting and transplantation (Domanski *et al*., 2006), suggest further uses for the SODs. Determination of SOD activity could also be employed as a means of indicating and diagnosing certain health conditions, as well as for investigating the mechanisms that cause disease, because the correlation between some diseases and SOD activity appears to be direct and strong. For instance, the SOD activity level in diabetes patients is very low, as SOD activity is dramatically reduced by the *in vivo* Maillard reaction, and, moreover, continuous decreases in SOD level points towards the development of diabetes complications (Ukeda, 2004). In addition, the detection of increased levels of SOD-1 in amniotic fluid is now used as a screening test for Down's syndrome in the foetus. Higher levels of SOD-1 can also be detected in the blood, sera, and other extra-cellular body fluids of a mother carrying foetal Down syndrome (Netto *et al.* 2004). As interest has increased in the anti-oxidative qualities of plants, the study of SOD has come to play an important role in the genetic engineering of crops. There have, for example, been a number of studies into the genetic variability of plants that contain SOD including research that found that the gel pattern of SOD isozymes was affected by both the iron supply and the age of plants. This research into the effects on SOD isozyme production of various environmental alterations has, in turn, predicted the possibility of research into the sophisticated genetic control of many plants.

Recently, for instance, genetic engineering for stress tolerance in crop and forest plants has been a high research priority in plant biotechnology, and a number of transgenic plants with controlled SOD levels have been introduced. An example of this was the creation of transgenic alfafa plants that overexpress either Mn SOD or Fe SOD cDNA (McKersie *et al*., 1997). These transgenic plants showed 25% higher freezing tolerance than non-transgenic plants. Another study found that Mn SOD, overexpressed in *Arabidopsis thaliana*, had a significant role in protecting cells against ROS due to salt stress, and, as a consequence, this research resulted in enhanced salt-tolerance in transgenic plants (Wang *et al*., 2004). More recently,

transgenic rice plants, where a Mn SOD gene from peas was introduced into chloroplasts of rice using *Agrobacterium*-mediated transformation, have presented enhanced drought tolerance (Wang *et al*., 2005). This method had also been used in an earlier study, in which Cu/Zn SOD cDNA (mSOD1) from cassava was inserted into cucumber fruits with *Agrobacterium*-mediated transformation, utilizing an ascorbate oxidase promoter, in order to create transgenic cucumber fruits containing high levels of SOD for an anti-aging cosmetic material (Lee *et al*., 2003). In this research, the transgenic fruits had levels of specific SOD activity roughly three times higher than those in nontransgenic plants.

2.6 Literature reviews of Zingeberaceae family

The plants members of Zingiberaceae family are notice characterized by their tuberous rhizomes its have strong aromatic and medicinal properties. It is usually found as ginger, and exists in about 50 genera and 1,300 species worldwide, distributed mainly in South and Southeast Asia. Species of the Zingiberaceae are the ground plants of the tropical forests. They mostly grow in damp and humid shady places. They are also found infrequently in secondary forest. Some species can fully expose to the sun, and grow on high elevation. Zingiberaceae plants including terpenes, alcohols, ketones, flavonoids, carotenoids and phytoestrogens. The Zingiberaceae have been reported for their biological activities in antifungal, antioxidant, insecticidal, and anti-inflammatory activities. Plants in this family are abundant in Asia including Thailand and there is still lacking of report concerned with superoxide dismutase in these herbs, thus it is interested to search among these herbs for the activity in order to apply in pharmacological area in future.

Habsah *et al*. (2000) have been reported of dichloromethane and methanol extracts of 13 Zingiberaceae species from the *Alpinia*, *Costus* and *Zingiber* genera were screened for antimicrobial and antioxidant activities. The antimicrobial activity of most of the extracts was antibacterial with only the methanol extract of *Costus discolor* showing very potent antifungal activity against only *Aspergillus ochraceos* and all the extracts showed strong antioxidant activity comparable with or higher that of a-tocopherol. Wilson *et al*. (2005) have been reported about the biological activity of extracts extracts of *Curcuma zedoaria* and *C. malabarica* tubers. The biological activity of this study is antibacterial and antifungal activity. They found that the extract of petroleum ether, hexane, chloroform, acetone and ethanol extracts exhibited

antibacterial as well as antifungal activity. This study is the first report of the antimicrobial properties of *C. malabarica*. The findings also support the use of *C. zedoaria* tubers in traditional medicine for the treatment of bacterial and fungal infections. Yao *et al*. (2008) have been reported of β-elemene, a natural plant drug extracted from *C. wenyujin*, has been used as an antitumor drug for different tumors, including glioblastoma. They had a report that anti-proliferation of glioblastoma cells induced by β-elemene was dependent on p38 MAPK activation. Treatment of glioblastoma cell lines with β-elemene, led to phosphorylation of p38 MAPK, cell-cycle arrest in G0/G1 phase and inhibition of proliferation of these cells.

CHAPTER llI

EXPERIMENTAL

3. Material and methods

3.1. Plant materials

The fresh rhizomes of 15 plants in Zingiberaceae family were periodically (April 2010 - May 2010) purchased from Chatuchak park market in Bangkok, Thailand.

3.2. Chemical and biological materials

Nitroblue tetrazolium (NBT), riboflavin and bovine serum albumin (BSA), were purchased from Sigma-Aldrich (USA). The reagents used in polyacrylamide gel electrophoresis (PAGE) were obtained from Plusone Pharmacia Biotech (Sweden), except the low molecular weight calibration kit, used as standard molecular weight marker proteins, which was purchased from Amersham Pharmacia Biotech (UK). All other biochemical reagents and general chemicals used in the investigation were of analytical grade.

3.3. Preparation of the Zingiberaceae rhizomes extract

The rhizomes (1.5 kg wet weight) of the 15 selected plant species from within the Zingiberaceae family were peeled, cut into small pieces (~ 10 × 10 × 10 mm) and then homogenized in 5 L of PBS (20 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl) using a blender and then left with stirring overnight at 4 ^{o}C. The suspension was then clarified by filtration through double-layered cheesecloth followed by centrifugation at 15,000 × g for 30 min. The clarified supernatant ("crude homogenate") was harvested and ammonium sulfate added, with stirring, to 80% saturation and then left with stirring overnight at 4 ^{o}C. The precipitate was collected from the suspension by centrifugation at 15,000 × g for 30 min with discarding of the supernatant. The pelleted materials were then dissolved in PBS, dialyzed (3,500 MWCO) against 3 changes of 5 L of water at 4 ^{o}C and then freeze dried. This is referred to as the "ammonium sulphate cut fraction".

3.4. Measurement of superoxide dismutase (SOD) activity

To determine the SOD activity, the riboflavin-NBT assay was adapted from Lai (Lai *et al*., 2008). The test sample (0.1 ml) at different protein concentrations were

first mixed with 2.9 ml of 20 mM phosphate buffer (pH 7.2) containing 0.2 ml of 0.1 M EDTA and 0.1 ml of 1.5 mM NBT. After incubation at 37 °C for 10 min, 0.05 ml of 1.2 mM riboflavin was then added. The reaction mixture was illuminated with a 25 W light tube for 15 min in a foil-lined box. The microtiter plate reader was used to measure the absorbance at 560 nm. Deionized water instead of the protein sample was used as a control along with a sample blank which was evaluated by adding water instead of the riboflavin solution. The concentration of protein that provides 50% inhibition of the riboflavin-mediated reduction of NBT, taken as SOD activity, was calculated. The SOD in the sample competes for superoxide, inhibiting the reaction rate of superoxide with NBT. The percentage of this inhibition is the basis on which the amount of activity is calculated as below:

$$\% \text{ inhibition} = \frac{\text{absorbance (reaction blank)} - \text{absorbance (sample)}}{\text{absorbance (reaction blank)}} \times 100$$

One unit of SOD was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%.

3.5. DEAE-cellulose ion exchange chromatography

DEAE-cellulose ion exchange chromatography was performed with a 1.6 cm × 15 cm column using an automatic liquid chromatography system (AKTA prime, Amersham Pharmacia Biotech, Sweden). The column was equilibrated with 5 column-volumes of 20 mM Tris-HCl (pH 7.0). Thereafter, 5 ml samples (400 mg protein) of the ammonium sulfate cut fraction were injected into the column and eluted with the same buffer at a flow rate of 1.0 ml/min, collecting 10 ml fractions before a linear 0 - 1.0 M NaCl gradient in the same buffer was applied over the next 30 fractions. The eluted fractions were monitored for protein content with a UV detector at 280 nm and for SOD activity as described in section 3.4. The fractions containing SOD activity from the column were pooled, dialyzed against 3 changes of 5 L of distilled water and concentrated, and is referred to as the “post-DEAE-cellulose SOD fraction”.

3.6. Superdex-75 gel filtration chromatography

The post-DEAE-cellulose SOD fraction was then further enriched by preparative Superdex-75 column (1.6 cm × 60 cm) chromatography. The column was equilibrated with two column-volumes of 100 mM NaCl / 20 mM Tris-HCl (pH 7.0), and then 2 ml of the post-DEAE-cellulose SOD fraction solution (50 mg protein) was

injected and eluted in the same buffer at a flow rate of 0.5 ml/min and collecting 5 ml fractions. Fractions were monitored for protein with a UV detector at 280 nm and for lipase activity as described in section 2.3. The SOD active fractions were pooled, dialyzed against 3 changes of 5 L of distilled water and concentrated, and is referred to as the "enriched SOD fraction".

3.7. Protein content determination

For evaluation of protein levels in the column chromatography, the elution peak profiles of the proteins were determined by measuring the absorbance at 280 nm. For all other samples the protein contents were determined by Bradford's procedure (Bradford, 1976), using BSA as the standard with four different concentrations (5, 10, 15 and 20 µg/ml) to construct the calibration curve. For each serial 2-fold dilution of the sample in deionized water, 50 µl aliquots were transferred into each of three wells of a microtiter plate and 50 µl of Bradford's reagent added to each well, shaken for 5 min and then left for 10 min before reading the absorbance at 595 nm with an ELISA plate reader. The obtained absorbance was converted to protein concentration using the linear equation computed from the standard curve.

3.8. Determination of enzyme purity by native-PAGE and SOD activity staining

The enzyme from each step of purification was analyzed by its native protein pattern and its purity according to the method of Bollag *et al*. (1996). Electrophoresis conditions, protein and activity staining are described below.

3.8.1. Non-denaturating gel electrophoresis

Native PAGE was performed with 12.5% and 5% (w/v) acrylamide separating and stacking gels, respectively, with 100 mM Tris-glycine (pH 8.3) as the electrode buffer. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Hoefer model miniVE, Pharmacia Biotech, UK). After electrophoresis, proteins in the gel were visualized by Coomassie blue R-250 (Sigma) staining and activity staining.

3.8.2. Coomassie blue staining

Native (section 3.8.1.) and reducing SDS-PAGE (section 3.9) gels were stained by immersion in 0.1% (w/v) Coomassie blue R-250 in1 0% (v/v) acetic acid / 45% (v/v) methanol for 45 min. Destaining was performed by immersing the gel in 10% (v/v) acetic acid / 45% (v/v) methanol, with several changes of this destaining solution until the background was clear.

3.8.3 SOD activity staining on non-denaturing PAGE

Protein samples were resolved by non-denaturing -12.5% (w/v) PAGE (8 × 10 cm gel size) without boiling. Following electrophoresis, the gel was stained for SOD activity by the method of Chopra (Chopra and Sabarinath, 2004), except with some modifications as outlined below. Gels were first soaked in 50 ml riboflavin-NBT solution (5 mg riboflavin, 12.5 mg NBT) for 15 min at room temperature in the dark. After incubation, the riboflavin-NBT solution was removed and 50 ml of 0.1% (v/v) TEMED was added. Gels were incubated again in the dark for 15 min. The solution was then removed and superoxide synthesis was induced by exposure to a 25 W light tube for 15 min in foil-lined box. The gel color turns into blue-purple and the SOD bands are white. To identify the prosthetic group of SOD, two inhibitors (KCN and H_2O_2) were used. For hydrogen peroxide treatment, the gel was first soaked in 8 mM H_2O_2 for 30 min, followed by the SOD activity staining as detailed above. For KCN treatment, 8 mM KCN was included in the riboflavin-NBT solution.

3.9. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and size estimation

Discontinuous reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared with 0.1% (w/v) SDS in 12.5% and 5% (w/v) acrylamide separating and stacking gels, respectively, with Tris-glycine buffer (pH 8.3) containing 0.1% (w/v) SDS as the electrode buffer, according to the procedure of Laemmli (1970). Samples to be analyzed were treated with reducing sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were co-resolved in the gel alongside the samples to determine the subunit molecular weight of the purified protein(s). After electrophoresis, the proteins in the gel were visualized by standard Coomassie blue R-250 (section 3.8).

3.10. Effect of temperature on the SOD activity and thermostability

The effect of temperature on the SOD activity was determined by incubating SOD samples in 20 mM phosphate buffer pH 7.2 at various temperatures (-20 - 90 ^{o}C at 10 ^{o}C intervals) for 30 min. The thermostability of the SOD was investigated by incubating SOD samples at 40 - 80 ^{o}C in 10^{o}C intervals in the same buffer for the indicated fixed time intervals (10 - 120 min), cooling to 4 ^{o}C and then assaying the residual SOD activity as described above.

3.11. The pH-dependence of the SOD activity

Incubating the SOD in buffers of broadly similar salinity levels but varying in pH from 2 - 14 was used to assess the pH stability and the pH optima of the SOD. The buffers used were 20 mM glycine-HCl (pH 2-4), 20 mM sodium acetate (pH 4-6), 20 mM potassium phosphate (pH 6-8), 20 mM Tris-HCl (pH 8-10), and 20 mM glycine-NaOH (pH 10-12). The purified SOD was mixed in each of the different buffer-pH compositions (control was just 20 mM phosphate buffer pH 7.2) and left for 1 hr at room temperature. Then the samples were adjusted back to PBS and assayed for SOD activity as above were compared with the control which was set as 100%.

3.12. Effect of metal ions on the SOD activity

The effect of different divalent metal cations on the SOD activity was evaluated. The enriched SOD fraction (1 mg / ml) was incubated with one of the divalent cation salts of Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} (all as chlorides), Cu^{2+} or Zn^{2+} (as sulphates) at 0 - 100 mM for 30 min with continuous shaking. After that, the SOD activity was determined as described (section 3.4) using at least three replicates for each assay.

3.13. Estimation of kinetic parameters

The rate of inhibition of NBT and riboflavin by SOD over an NBT concentration range of 0.1 – 0.25 mM and riboflavin concentration range of 0.05 – 0.1 mM in the presence or absence of various concentrations of the enriched post-Superdex-75 SOD fraction was measured. Then, the data from enzyme assays was subjected to double-reciprocal (Lineweaver-Burk) plot analysis to determine the Michaelis-Menten constant (K_m), maximum velocity (V_{max}) of the enriched enriched post-Superdex-75 SOD fraction.

3.14. Statistical analysis

All determinations were done in triplicate, and the results are reported as the mean $\pm$ 1 standard error of the mean (SEM). Regression analyses and calculation of IC_{50} values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc.).

CHAPTER lV

RESULT AND DISCUSSION

4.1. Screening for SOD in plant samples

In this study we screened the crude homogenates and ammonium sulphate cut fractions from the rhizomes of 15 Zingiberaceae plant species for SOD activity using the NBT assay (section 3.4). The IC_{50} values were calculated from the regression equation obtained from evaluation of different concentrations of each test extract (Table 4.1). Among all of the plant species screened, *Curcuma aeruginosa* Roxb. showed the highest specific activity for SOD enzyme. According to these results several candidates have been selected for further studies.

Table 4.1. The SOD activity in the crude homogenates and ammonium sulphate cut fractions of 15 Thai species from within the Zingiberaceae family[a].

Scientific name	IC_{50} value (µg/ml)[a]	
	crude homogenates [b]	ammonium sulphate cut fractions[c]
Alpinia galanga (Linn.) Swartz.	0.0262 ± 0.002	2.0733 ± 0.005
Boesenbergia pandurata Roxb.	0.0497 ± 0.003	0.9672 ± 0.004
Curcuma aeruginosa Roxb.	0.0537 ± 0.005	0.1780 ± 0.004
Curcuma amarissima Roscoe.	0.2277 ± 0.002	ND
Curcuma aromatica.	0.0255 ± 0.002	1.2327 ± 0.009
Curcuma longa Linn.	0.0179 ± 0.004	0.7927 ± 0.004
Curcuma sp. (Kan-ta-ma-la)	0.2189 ± 0.002	0.6438 ± 0.004
Curcuma xanthorrhiza Roxb.	ND	ND
Curcuma zedoaria (Berg) Roscoe.	0.0703 ± 0.003	0.2592 ± 0.006
Hedychium coronarium.	0.2071 ± 0.003	0.7292 ± 0.008
Kaempferia galanga Linn.	0.0526 ± 0.002	0.6807 ± 0.003
Zingiber cassumunar	0.3301 ± 0.002	0.7269 ± 0.004
Zingiber officinale Roscoe.	0.2246 ± 0.003	0.4315 ± 0.003
Zingiber ottensii Valeton.	0.2099 ± 0.003	ND
Zingiber zerumbet Smith.	ND	ND

[a]Data are shown as the mean ± 1 SEM and are derived from 3 replicate enrichments.
[b]Crude extract and [c]crude protein represent the crude homogenate and ammonium sulphate cut fraction, respectively.
ND = Not detected

4.2. Purification of SOD with conventional chromatography

Ammonium sulphate cut fractions from *C. aeruginosa* was sequentially purified by two successive chromatographies using DEAE-cellulose and Superdex 75.

The major active peak was un-adsorbed onto the DEAE-cellulose column, whereas the minor active peak was adsorbed on the resin (Figure 1A). Active fractions were pooled, concentrated, and injected onto a Superdex 75 Fast Flow column. Some inactive proteinaceous material was observed, while SOD activity was eluted (Figure 1B). The SOD was purified 4.36 fold with 2.51% retention of total extracellular activity. The specific activity of the purified enzyme was 812.20 U/mg of protein (Table 4.2). Several workers have reported the purification of SOD from different plant species using techniques of ammonium sulphate fractionation, anion exchange chromatography and gel filtration. Sevilla *et al*. (1980) purified SOD from *Pisum sativum* with 2.3% yield and Duke and Salin (1985) from *Ginkgo biloba* with 8% yield. Four isoenzymes of SOD have been observed in groundnut (*Arachis hypogea*) seedlings which were purified with 20.12, 5.06, 6.8 and 5.3% recovery, respectively (Sulochana and Venkaiah, 1990).

Table 4.2. Enrichment summary for the SOD from *C. aeruginosa*

Purification step	**Total protein (mg)**	**Total activity (U)**	**Specific activity (U/mg)**	**Yield (%)**	**Purification (fold)**
Culture filtrate	950.24	176983.24	186.25	100	1
80% $(NH_4)_2SO_4$	780.80	43865.17	56.18	24.78	0.30
DEAE-cellulose	20.06	6512.50	324.50	3.56	1.74
Sephadex-75	5.64	4582.70	812.20	2.51	4.36

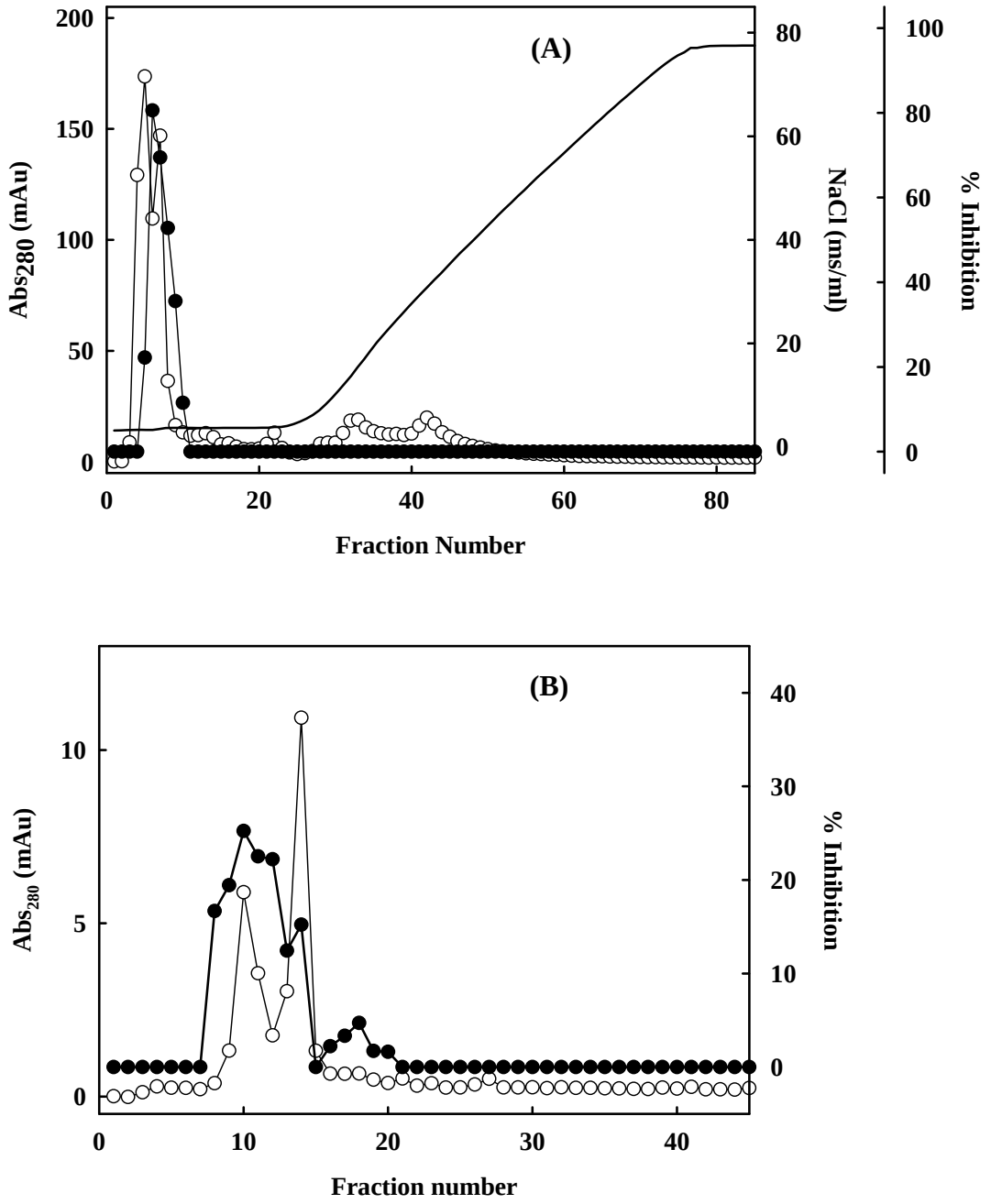

Figure 4.1. Profile of the enrichment of the SOD from *C. aeruginosa* by; (A) DEAE-cellulose ion-exchange chromatography of the ammonium sulfate cut fraction (400 mg protein) eluted in 20 mM Tris-HCl (pH 7.0) with a 0 - 1 M NaCl linear gradient; and (B) Superdex-75 gel chromatography of the post-DEAE-cellulose SOD fraction (50 mg) eluted in 100 mM NaCl / 20 mM Tris-HCl (pH 7.0). For both panels A and B; absorbance at 280 nm (○), SOD activity (●).

4.3. Determination of enzyme purity on native-PAGE, zymography, and reducing SDS-PAGE

The SOD from each step of enrichment was analyzed for purity and protein pattern by native-PAGE, with protein and enzyme activity staining. Whilst the post-DEAE-cellulose SOD fraction still showed multiple components, the enriched SOD fraction (post-Superdex-75 SOD fraction) showed a single protein band on native-PAGE, suggesting a high degree of purity (Figure 4.6A). Prosthetic groups of SOD were determined by native-PAGE followed by SOD activity staining in the presence of sodium cyanide or hydrogen peroxide (KCN or H_2O_2). As shown in Figure 4.6B, the enzyme is insensitive to KCN or hydrogen peroxide inhibitor, suggesting that *C. aeruginosa* SOD belongs to the MnSOD category. Mn SOD is usually localized in the matrix of mitochondria and in prokaryotes, but a membrane-related Mn SOD has also been isolated from chloroplast thylakoids. In pea leaves, Mn SOD is localized in mitochondria as well as peroxisome (Rio *et al*., 2003). In higher plants, Mn SODs are mainly present in mitochondria from different plants (Rio *et al*., 1992, Halliwell and Gutteridge, 2000). On the other hand, in the fruit and leaves of most plants, Mn SOD has been shown to be only 3-5% of the total SOD activity (Fridovich, 1986).

The implication that the enriched post-Superdex-75 SOD fraction was a relatively homogenous protein preparation was supported by the presence of a single band after reducing SDS-PAGE analysis, and gave an estimated size of about 31.5 kDa (Figure 4.6C). Several workers have reported the molecular weight of SOD between 31-39 kDa (Misra and Fridovich, 1972; Beauchamp and Fridovich, 1973; Lumsden and Hall, 1974; Rabinowitch and Sklan, 1981 and Shivaprakasam *et al.*, 2004). Federico *et al*. (1985) isolated and purified SOD from *Lens esculenta* cotyledons. Gel filtration gave a molecular weight of 33 kDa. Kumar *et al*. (2004) predicted molecular weight of 36.3 kDa for SOD in tomato. These reports are in fair agreement with our results.

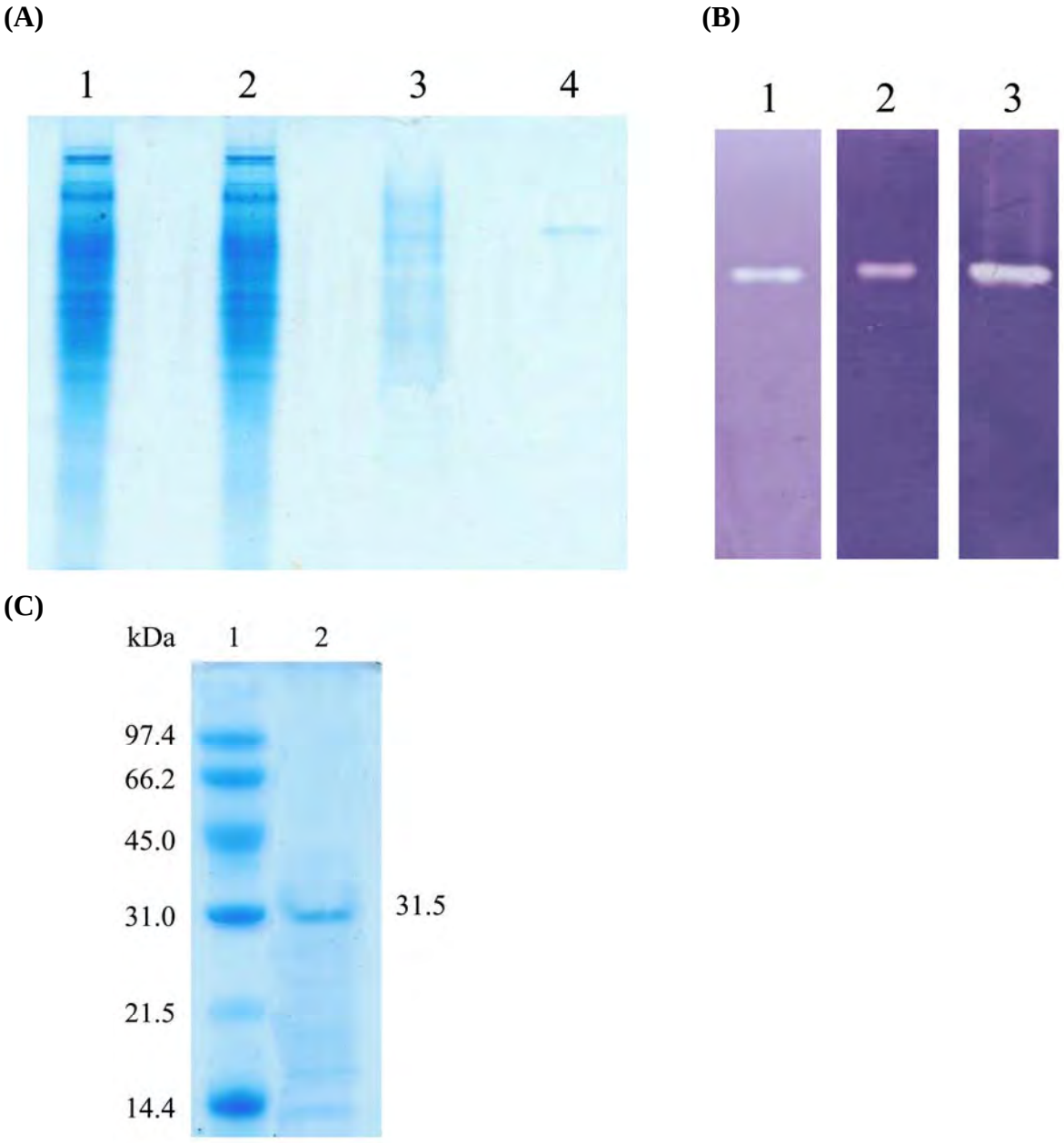

Figure 4.2. (A) Coomassie blue stained non-denaturing PAGE of the from *C. aeruginosa* rhizome protein from each step of enrichment. Lanes 1 - 4 of total protein from (1) 20 µg of the crude homogenate, (2) 20 µg the 80% saturation ammonium sulphate cut fraction and (3) 10 µg the post-DEAE-cellulose unbound fraction and (4) 5 µg post-Superdex-75 SOD fraction. (B) Native PAGE, stained for 10 µg of SOD activities active staining of gels after pre-incubation with KCN or H_2O_2. Lane (1) control, (2) KCN, and (3) H_2O_2. (C) Reducing SDS-PAGE analysis of the enriched post-Superdex-75 SOD fraction. Lane 1, molecular weight standards; Lane 2, post-post-Superdex-75 SOD fraction.

4.4. Effect of temperature on the SOD activity and stability

The effect of temperature in the range of -20 – 90 ^{o}C on the SOD activity was examined. Within the temperature range of -20 – 45 ^{o}C, the purified enzyme is highly active, with >50% of relative activity. The activity was dropped significantly as the temperature >50 – 90 ^{o}C (Figure 4.3A). This result is comparable to the result reported for SOD from pearl millet seedlings, for which the temperature optimum occurred at 28 ^{o}C, but actually had broad temperature optima with not much activity change over 20 - 35 ^{o}C (Babitha *et al*. 2002). Figure 4.3B shows the temperature stability. It was found that the purified SOD was thermally stable after holding at 40 – 50 ^{o}C for 50 min. The residual SOD activity was about 50-60%. Nevertheless, as the temperature was raised to 60 ^{o}C, the SOD activity declined.

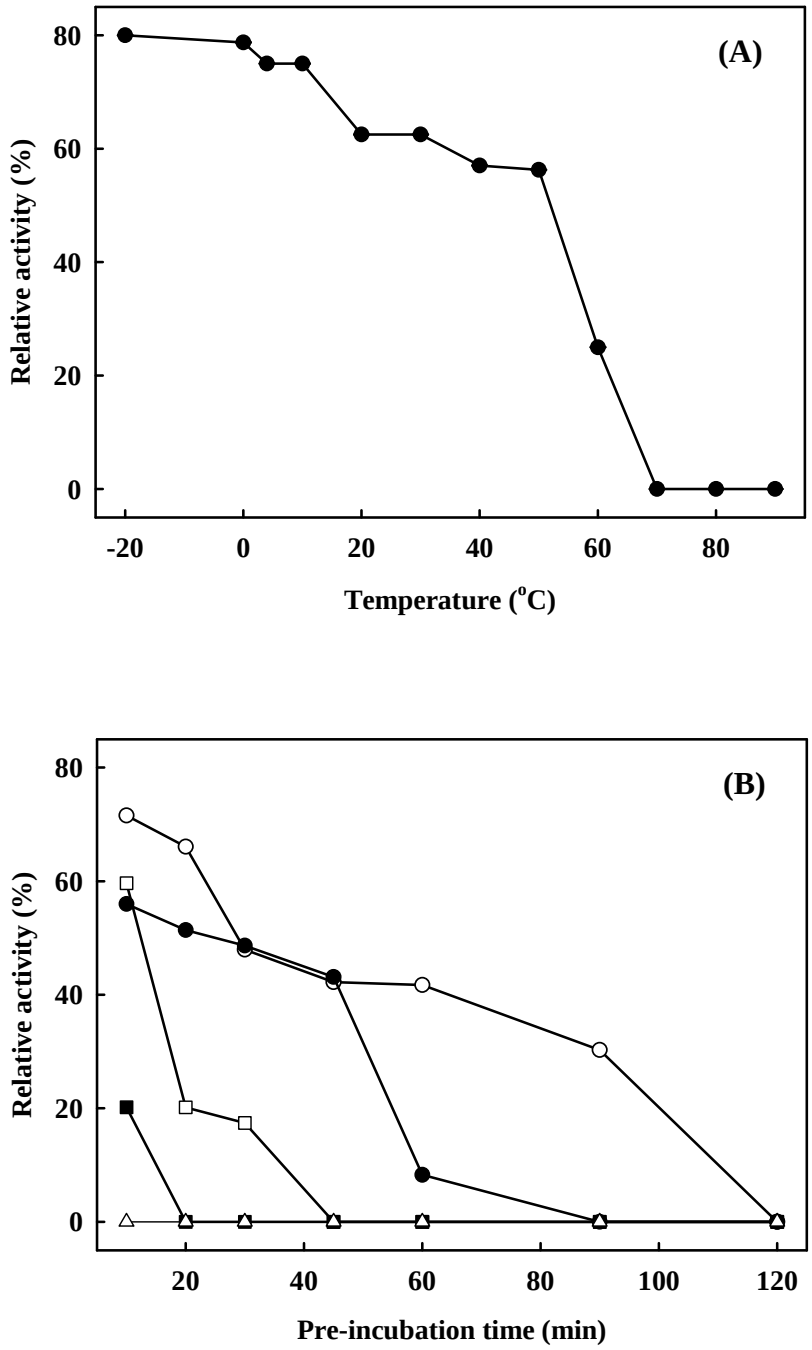

Figure 4.3. (A) Effect of temperature on the NBT inhibition of the purified *C. aeruginosa* rhizome SOD. (B) Thermostability of the same purified SOD at: (○) 40, (●) 50, (□) 60, (■) 70, and (△) 80°C. For both panels the data are shown as the mean±SD and are derived from 3 repeats.

4.5. Effect of pH on the SOD activity and stability

The effect of pH in the range of 2 - 12 on the activity of SOD was examined. It was found that within the pH range of 4 - 6, the crude enzyme solution was highly active. The pH optimum purified SOD occurred at pH 4 (Figure 4.4A). The pH stability of SOD was further investigated over the pH range of 4 - 6 (Figure 4.4B). Correspondingly, the crude SOD extract of wheat seedlings was found to be stable after being held at pH 4 - 6 for 30 min. The residual SOD activity was >80%. (Lai *et al*., 2008).

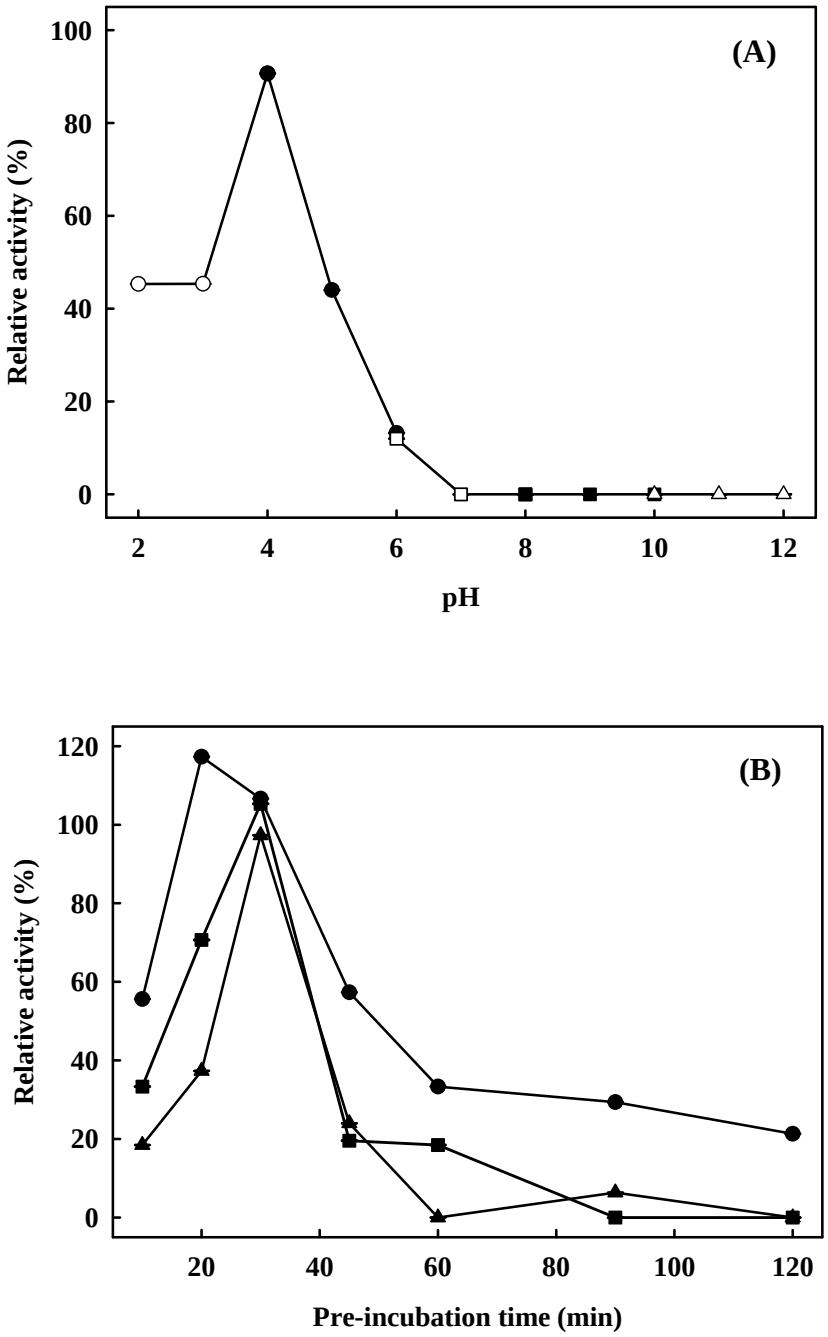

Figure 4.4. (A) Effect of pH on the NBT inhibition of the purified *C. aeruginosa* rhizome SOD. Buffer systems were used: (○) 20 mM glycine-HCl (pH 2-4), (●) 20 mM sodium acetate (pH 4-6), (□) 20 mM potassium phosphate (pH 6-8), (■) 20 mM Tris-HCl (pH 8-10), and (△) 20 mM glycine-NaOH (pH 10-12). (B) pH stability of the same purified SOD on 20 sodium acetate at: (●) pH 4.0, (■) pH 5.0, and (▲) pH 6.0. For both panels the data are shown as the mean±SD and are derived from 3 repeats.

4.6. Effect of divalent metal ions on the activity of SOD

Effect of bivalent metal cations on the enzyme activity was investigated (Table 4.3). The enzyme was pre-incubated with various bivalent metal cations at concentrations given in Table 4.3 for 30 min during enzyme assay. $Mn^{2+}$and Fe^{2+} ions stimulated SOD inhibition activity when increase metal ions concentrations. It is also interesting that a low concentration of Mn^{2+} (0.1 mM) can enhance the SOD activity. However, the high concentration of Mn^{2+} (100.0 mM) inhibited SOD activity significantly. Such results suggest that an appropriate amount of Mn^{2+} could enhance the affinity between SOD and substrates, possibly by changing the electron density of SOD. However, excess Mn^{2+} may induce the denaturation or deassociation of SOD, resulting in significant reduction of enzyme activity.

Table 4.3. Effect of bivalent metal cations on the SOD activity on the enriched post-Superdex-75 SOD fraction from *C. aeruginosa* rhizomes[a].

Concentration (mM)	Relative activity (%)					
	Ca^{2+}	Cu^{2+}	Fe^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}
0.1	63.2±0.003	84.8±0.005	78.9±0.007	112.4±0.002	81.6±0.02	100.8±0.03
0.5	99.2±0.002	97.6±0.003	95.2±0.009	117.6±0.001	89.5±0.10	100.0±0.01
1	98.4±0.005	104.8±0.001	98.4±0.001	107.2±0.001	91.5±0.03	103.2±0.01
5	111.6±0.001	108.8±0.001	106.4±0.003	92.0±0.001	94.4±0.02	118.4±0.03
10	0	83.3±0.001	107.2±0.003	12.3±0.004	112.8±0.02	0
50	0	7.9±0.001	121.1±0.006	4.4±0.009	114.9±0.02	0
100	0	0	141.2±0.006	0	123.7±0.01	0

[a]Data are shown as the mean $\pm$ 1 SEM and are derived from 3 replicate enrichments

4.7. Determination of kinetic parameters

Different concentration of NBT ranging from 0.1 – 0.25 mM was included in the assay mixture to study their effect on the enzyme activity. Maximum activity was observed to 333.7 µmol/min/mg NBT. From double reciprocal plot (Figure 4.5A), the Km of enzyme for NBT was found to be 57.31 M. The enzyme activity was also determined in the presence of different concentration of riboflavin (0.05 – 0.1 mM).

Maximum SOD activity was observed at 254.1 µmol/min/mg riboflavin and Km for riboflavin observed to be 1.51 M as showed in Figure 4.5B Pruthi (2002) reported higher Km values of 310×10^{-6} M and 41×10^{-6} M for its substrates NBT and riboflavin, respectively for SOD purified form *Brassica juncea* (cv. Varuna).

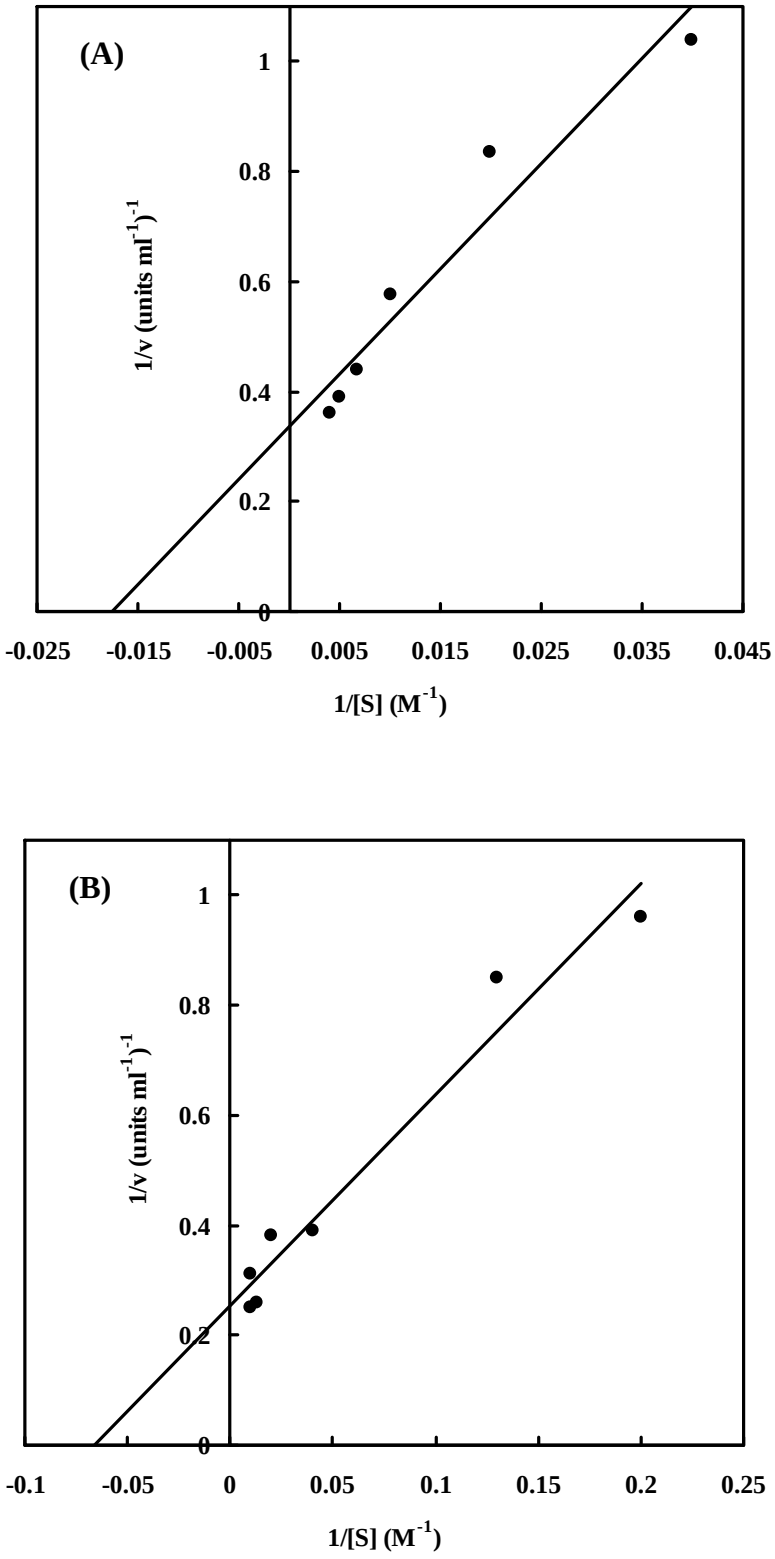

Figure 4.5. Lineweaver-Burk plots showing K_m value for SOD function of (A) NBT and (B) riboflavin concentration.

CHAPTER V

CONCLUSION

SOD have been found aboundantly in many organisms from microorganims to plants and animal. Plants are important producer of biomolecules. These molecules play a role in protection against predators or hostile environment and also support the plant's survival. SOD has long been application in medicinal treatment, cosmetic and other chemical industries. Zingiberaceae, family is used to alleviate female problems such as irregular, painful or excessive menstruation and for uterine pain or dysfunction. However, to the best of our knowledge, despite the widespread use of rhizomes from *C. aeruginosa* in dietary-based medical applications, there are no reports on the purification SOD from *C. aeruginosa*. Consequently, the aims of this study were to purify and characterize SOD from the rhizomes of *C. aeruginosa*.

SOD was purified by ammonium sulphate saturation, DEAE-cellulose chromatography and Superdex ^{TM} 75 chromatography. The SOD was purified 4.36 fold with 2.51% retention of total extracellular activity. The specific activity of the purified enzyme was 812.20 U/mg of protein. SOD was checked the subunit and molecular weight by SDS-PAGE appear that one subunit and 31.5 kDa. SOD characterization was tested the optimum temperature, the thermal stability, the optimum pH, the effect of various metal ions and the kinetic analysis. It was found that the purified SOD was thermally stable after holding at 40 – 50 ^{o}C for 50 min. The residual SOD activity was about 50-60%. Nevertheless, as the temperature was raised to 60 ^{o}C, the SOD activity declined. It was found that within the pH range of 4 - 6, the crude enzyme solution was highly active. Effect of bivalent metal cations on the enzyme activity. $Mn^{2+}$and Fe^{2+} ions stimulated SOD inhibition activity when increase metal ions concentrations. Maximum activity was observed to 333.7 µmol/min/mg NBT, the Km of enzyme for NBT was found to be 57.31 M. The enzyme activity was also determined in the presence of different concentration of riboflavin (0.05 – 0.1 mM). Maximum SOD activity was observed at 254.1 µmol/min/mg riboflavin and

Km for riboflavin observed to be 1.51 M. This is the first report for SOD from *C. aeruginosa*, a traditionally used Thai medicinal plant.

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APPENDICES

APPENDIX A

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solutions

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10% SDS (w/v)

Sodium dodecyl sulfate (SDS) 10 g

50% Glycerol (w/v)

100% Glycerol 50 ml

Added 50 ml of distilled water

1% Bromophenol blue (w/v)

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide	29.2 g
N,N,-methylene-bis-acrylamide	0.8 g
Adjust volume to 100 ml with distilled water	

Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)

2 M Tris-HCl (pH 8.8)	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5 M Tris-HCl pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50 ml
10% SDS	4 ml
Distilled water	46 ml

10% Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)

Tris (hydroxymethyl)-aminomethane	3 g
Glycine	14.4 g
SDS	1 g
Dissolved in distilled water to 1 litre without pH adjustment	
(final pH should be 8.3)	

5x sample buffer

(60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol)

1 M Tris-HCl (pH 6.8)	0.6	ml
Glycerol	5	ml
10% SDS	2	ml
1% Bromophenol blue	1	ml
2-mercaptoethanol	0.5	ml
Distilled water	0.9	ml

3. SDS-PAGE

15% Separating gel

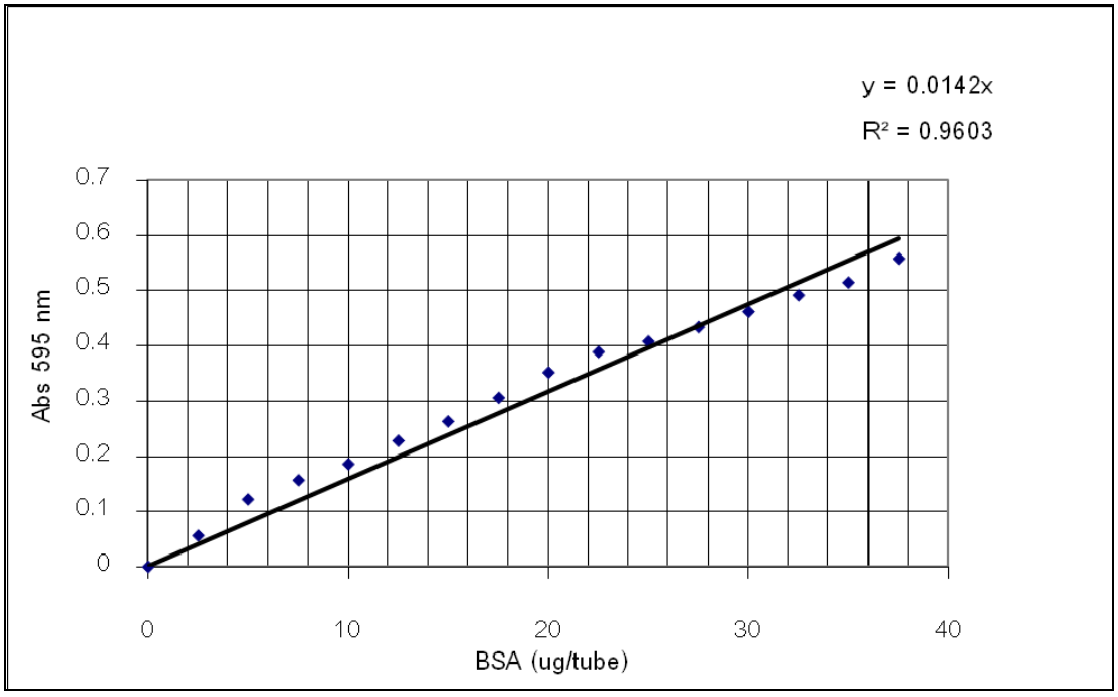
Solution A	10.0	ml
Solution B	5.0	ml
Distilled water	5.0	ml
10% Ammonium persulfate	100	µl
TEMED	10	µl

5.0% Stacking gel

Solution A	0.67	ml
Solution B	1.0	ml
Distilled water	2.3	ml
10% Ammonium persulfate	30	µl
TEMED	5.0	µl

APPENDIX B

Calibration curve for protein determination by Bradford method



APPENDIX C

Amino acid abbreviations

Amino acid	Three-letter	One-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic-acid	Asp	D
(Asn + Asp)	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
(Gln + Glu)	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

BIOGRAPHY

Miss Wanwisa Moon-ai was born on May 22, 1986 in Lampang, Thailand. She graduated with a Bachelor Degree of Science from Department of Biotechnology, Faculty of Science, Thammasat University in 2009. She had been studies for a Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2010.

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