CHAPTER IV

A superoxide dismutase purified from the root of *Stemona tuberosa* Lour

4.1 INTRODUCTION

Superoxide dismutase (SOD, EC 1.15.1.1) is a well-known antioxidant enzyme that plays an important role in the removal of harmful superoxide radicals, which are generated by metabolic processes in oxygen-utilizing organisms and that cause many diseases, by their reduction to hydrogen peroxide (H_2O_2) and molecular oxygen.[48, 52] SOD isozymes can be classified into four main groups depending on the metal cofactor in their active sites and their sensitivity to cyanide and H_2O_2: (1) CuZn-SOD and (2) Ni-SOD are sensitive to inhibition by both cyanide and H_2O_2, (3) Mn-SOD is insensitive to both cyanide and H_2O_2 and (4) Fe-SOD is inhibited by only H_2O_2.[70] In general, SOD is found in microorganisms, animals and plants,[71] but most SOD preparations for use as an antioxidant agent in medicinal treatment, cosmetics and the chemical and food industries are extracted from animal plasma or plant cells.[55] In addition to the antioxidant activity, SOD isozymes have also been reported to display other biological properties, such as anti-inflammatory, anti-cancer and anti-aging activities.[72, 73] Furthermore, SOD is used as an indicator in the diagnosis of some disorders, such as Alzheimer's, Prion and Behcet's diseases.[74]

In higher plants, protection against predators or pathogens and environmental changes involve in the generation of free radicals, including the superoxide radical.[75] Although the superoxide radical is important in plants for the elimination of

predators or pathogens and to aid in the plant's responses to and so existence in a hostile environment, including chilling, hypersalinity, heavy metal ion exposure and ultraviolet light irradiation,[76-81] a high superoxide radical level can damage the living cells. Thus, the appearance of SOD has an important role in supporting the plant's survival from different stress conditions. SOD is the most abundant in plants such as *Radix lethospermi* seeds,[53] *Curcuma comosa*,[64] *Oryza sativa*,[84] lemon,[70] tobacco leave,[85] and sweet potato.[86]

Stemona tuberosa (Stemonaceae family), called wild asparagus in English and Non-Tai Yak in Thailand, is found in Asia, including central China, Indochina, Taiwan, India and Thailand, tropical Australia and North America. The dried roots of *S. tuberosa* (and related plants) have a long history of use in folklore medicine. It is often used as an anti-tussive drug for treating respiratory disorders, such as tuberculosis, bronchitis and pertussis, as well as an anti-helmintic agent.[87-90] Indeed, fairly diverse biological properties have been ascribed to the extracts from roots of *S. tuberosa*, including anti-bacterial,[4] anti-fungal,[90] anti-tussive,[5, 6] antioxidant,[9] insecticidal,[91] demulcent and anticancer[8, 92] activities. Most of this research has focused upon the low polar contents of the *S. tuberosa* roots, especially the alkaloids, stilbenoides and tocopherols, where a high degree of chemical diversity has been reported.[93, 94] Recently, potential antioxidant agents, such as dehydrotocopherols and phenolic compounds, from the roots of *S. tuberose* have been reported,[1] but there are no reports on SOD as an antioxidant protein from *S. tuberose*. However, *S. tuberosa* is potentially an alternative plant source for SOD and consequently the extraction, purification and characterizations of purified SOD from the root of *S. tuberosa* are reported.

4.2 Materials and methods

4.2.1 Plant material

Three kilograms of fresh roots of *S. tuberosa* were purchased from the Chatujak market, Bangkok, Thailand, in June 2009. The samples were quickly taken to laboratory and kept in dark 4 °C room until used.

4.2.2 Chemicals

N-(1-naphthyl) ethylene-diamine dihydrocholide (NED), 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). Low molecular weight calibration kit used as standard molecular weight marker proteins was purchased from Amersham Pharmacia Biotech (UK). All other biochemical reagents and general chemicals used in the investigation were analytical grade.

4.2.3 Isolation and extraction of SOD from *S. tuberosa*

The roots of *S. tuberosa* were peeled, cut into small cubes (~ 10 x 10 x 10 mm) and homogenized in 5 L of 0.1 M NaCl / 20 mM phosphate buffer pH 7.2 at 4°C overnight. The suspension-solution mixture was clarified by centrifugation (15,000 x g for 30 min) and the supernatant was harvested. Then, ammonium sulfate was added into the supernatant to 90% saturation and left overnight at 4 °C. The precipitated crude protein extracted was collected by centrifugation (15,000 x g for 30 min), dissolved in de-ionized water and dialyzed against 3 changes of 5 L of 20 mM

phosphate buffer pH 7.2 over 24 h. The crude protein extract solution was then freeze-dried.

4.2.4 Purification of SOD from *S. tuberosa*

The dried crude protein extract was dissolved in distilled water to 1 mg/ml and then 10 ml of solution was injected into a DEAE cellulose anion exchange column. The unbound protein was eluted from the column with 20 mM phosphate buffer pH 7.2 (buffer A) at 1 ml/min collecting 5 ml fractions. Then the bound fractions were eluted as above but with the addition of a stepwise gradient of 0.25, 0.50, 0.75 and 1 NaCl in the same phosphate buffer. The collected fractions were dialyzed against water and analyzed for protein concentration using the Bradford assay and for SOD activity.

4.2.5 SOD assay activity

To determine the SOD activity, the riboflavin-NBT assay was adapted from Lai *et al.*[82] The test sample (0.1 ml) at different protein concentrations in distilled water was 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 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 (blank solvent control), was used as a control along with a sample blank, evaluated by adding water instead of the riboflavin solution. The concentration of protein that provided 50 % inhibition of the riboflavin-mediated reduction of NBT, taken as SOD

activity, was calculated and reported as the IC 50 value (Appendix B). 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:

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

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

4.2.6 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,[95] using BSA as the standard with four different concentrations (5, 10, 15 and 20 µg/ml) to construct the calibration curve. For each serial two-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 (Appendix C).

4.2.7 Determination of the SOD enzyme purity on Native-PAGE

The likely metal ion group of the SOD enzyme was determined by native-PAGE resolution of the protein followed by SOD activity staining (zymogram) using the modified method of Lia *et al*[82] and Boonmee *et al.*[64] The purified SOD solution was mixed with 1/10 volume of 0.01% (w/v) bromophenol blue and 50% (v/v) glycerol in a 1:3 (v/v) ratio, respectively, and the electrophoresis was conducted at 280 V (7 x 7 cm gel). After electrophoresis, the gel was moved to a foil-lined box and illuminated with 25 W light tube for 15 min (15 cm light to gel distance; 20-200 the umol photons m^2 s^{-1}). SOD activity was determined as the presence of a white unstained zone against a purple background. Sensitivity of the SOD activity to H_2O_2 was determined by preincubation of the resolved gel in 8 mM H_2O_2 for 15 min prior to performing the SOD activity staining zymogram, whilst sensitivity to KCN was evaluated by the co-addition of 8 mM KCN into the riboflavin solution for the SOD activity staining.

4.2.8 SOD characterization by tandem mass spectrometry

Tryptic peptides were prepared from the excised single protein band (ST-1) from the native-PAGE following the reported method.[96] The tryptic peptides were then mixed with 0.1% (v/v) formic acid before being analyzed by nano liquid chromatography electrospray ionization coupled with tandem mass spectrometry (nano-LC-ESI/MS-MS). The tandem mass spectra of protein were interpreted and searched against the Mascot MS/MS Ions search data base (http://www.matrixscience.com).

4.2.9 Determination of molecular weight by SDS-PAGE

The molecular weight of the enriched SOD subunits was analyzed by comparison to the standard protein markers according to the method of Boonmee *et al*[64] on reducing SDS-PAGE gels with a 12.5% (w/v) polyacrylamide resolving gel and 0.1% (w/v) Coomassie brilliant blue G-250 staining of the resolved gel.

4.2.10 Determination of optimum pH and pH stability

To determine the optimum pH for SOD enzyme activity, the SOD solution was incubated at a 1:3 (v/v) ratio with the respective 20 mM universal buffer; glycine-HCl (pH 2-4), sodium acetate (pH 4-6), potassium phosphate (pH 6-8), Tris-HCl (pH 8-10) and glycine-NaOH (pH 10-12), at different pH values for 30 min and then the SOD activity was determined using the riboflavin-NBT assay.

To evaluate the pH stability, the SOD solution was mixed with the appropriate 20 mM buffers above at the optimum pH values for 10-120 min and then the SOD activity was measured using the riboflavin-NBT assay.

4.2.11 Determination of optimum temperature and thermal stability

To determine the optimum temperature for enzyme activity the SOD solution was incubated at the selected temperature (0, 4, 10, 25, 30, 40, 50, 60, 70, 80 and 90 °C) for 30 min before evaluating the SOD activity level using the riboflavin-NBT assay.

To evaluate the thermal stability, the SOD solution was incubated at the optimum temperature for 10-120 min before then measuring the SOD activity with the riboflavin-NBT assay.

4.2.12 Effect of bivalent metal ions and SDS on the SOD activity

The purified SOD solution was incubated with 0.1, 0.5, 1 and 5 mM of $ZnSO_4$, $CuSO_4$, $CaCl_2$, $MnCl_2$, $FeCl_2$, $MgCl_2$, $HgCl_2$ and SDS for 30 min and then the SOD activity was determined using the riboflavin-NBT assay.

4.2.13 Effect of riboflavin and NBT on SOD activity

The effect of varying the riboflavin concentration (0.1, 0.075, 0.05, 0.025, 0.0075 and 0.005 mM) or the NBT concentration (0.25, 0.2, 0.15, 0.1, 0.05, 0.025 and 0.01 mM) upon the SOD activity was evaluated using the riboflavin-NBT assay.[82]

4.2.14 Partial nucleotide sequences of SOD from *S. tuberosa*

100 mg of the fresh root of *S. tuberosa* was ground to a fine powder before RNA extraction using Plant Total RNA Mini Kit (FavorPrep). The total RNA was continually converted to double strand cDNA and library of adaptor ligated double strand cDNA, respectively by using Marathon™ cDNA Amplification Kit (Clontech). Finally, six primers were designed and used to determine the partial nucleotide sequences of SOD from *S. tuberosa* as the following.

3-pine (5'- TAGCAGTGAGGGTGTTAA-3')

5-solo (5'- AGGCCAATAATACCACAAGCAA-3')

F1 (5'- CCTGTGTGAGATTGTTATCCGTCAC-3')

R1 (5'- GAATTGTGACGGATAACAATCTCAC-3')

F2 (5'- CCTAATAAGAATCACTATAGGGTCC-3')

R2 (5'- GGACCCTATAGTGATTCTTATTAGG-3')

The PCR condition (hot lid thermal cycler) started with using 94°C for 30 cycles then, 94 °C, 5 sec and 86 C° for 4 min. Finally the partial nucleotide sequence of SOD from *S.tuberosa* was analyzed by using A plasmid Editor (ApE) program version 8.5.2.0.

4.3 Results and discussions

4.3.1 Isolation and purification of SOD enzyme from *S. tuberosa*

From three kilograms (wet weight) of fresh S. *tuberosa* roots, extracted in 20 mM phosphate buffer (pH 7.2) 430 mg of crude protein was obtained (0.014% of the starting mass), which contained just under 12 kU SOD activity (Table 4.1). Following 90% saturation ammonium sulfate precipitation to remove small chemical compounds from the extract, a dark brown crude protein extract was obtained which represented a 1.16-fold enrichment of the SOD specific activity for a 1.15-fold loss of the total enzyme activity yield. Further fractionation by DEAE-cellulose anion exchange column chromatography resulted in one main protein peak in the unbound fractions (ST-1) and two minor peaks (ST-2 and ST-3) in the bound fractions that were eluted with 0.25 and 0.50 M NaCl, respectively (Figure 4.1). The major peak from the unbound fraction (ST-1) had the highest SOD activity (~75% of the total ammonium sulfate precipitate activity) and was comprised of 34.5% of the loaded protein level, or 25.8% of the total crude root extract protein level compared to ST-2 and ST-3(Table 4.1). In addition ST-1 also has the most potent for SOD activity compared to other 10 plants (Table 4.2). In contrast, the two minor bound fractions (ST-2 and ST-3) had a much lower SOD activity as well as a lower protein yield. Thus,

fraction ST-1 was selected for further characterization and represents a three-fold enrichment of the SOD specific activity for a yield of 77.4% total activity.

The native-PAGE resolution of ST-1 (Figure 4.2) revealed the presence of only a single protein band, suggesting enrichment to apparent homogeneity, whilst this band was revealed to have SOD activity in terms of the single white unstained band seen against the purple background in the SOD zymogram (Figure 4.3). Furthermore, ST-1 was also classified as Mn-SOD because the activity of enzyme was not significantly inhibited by either H_2O_2 or KCN (Figure 4.3).

Table 4. 1 Purification procedures of SOD from *S. tuberosa*.

Sample	Total protein (mg)	Total SOD activity (U)	Specific activity (U/mg)	Protein yield (%)	Enrichment (Fold)
Crude extract (5 L)	430.0	11,944	27.8	100.0	1.00
90% $(NH_4)_2SO_4$ cut	320.8	10,347	32.3	74.6	1.16
ST-1 (unbound)	110.9	9,240	83.3	25.8	3.00

Table 4. 2 IC_{50} value of SOD from *S. tuberosa* compared to other plants

Plants	IC_{50} value (μg/mL)	Reference
Wheat germ	250	Chen *et al*[97]
Alfalfa leaf	700	Xie *et al*[98]
Sphenostylis stenocarpa	> 1000	Ajibola *et al*[99]
Pea (F2)	1000	Pownall *et al*[100]
Gnetum gnemon (Gg-AOPI)	40	Siswoyo *et al*[101]
Melon	800	Carillon *et al*[102]
Hedychium coronarium	0.729 ± 0.008	Moon-ai *et al*[103]
Kaempferia alangal Linn.	0.681 ± 0.003	Moon-ai *et al*[103]
Zingiber officinale Roscoe.	0.432 ± 0.003	Moon-ai *et al*[103]
Curcuma zedoaria Roscoe.	0.259 ± 0.006	Moon-ai *et al*[103]
Stemona tuberosa	**0.039 ± 0.006**	Appendix B

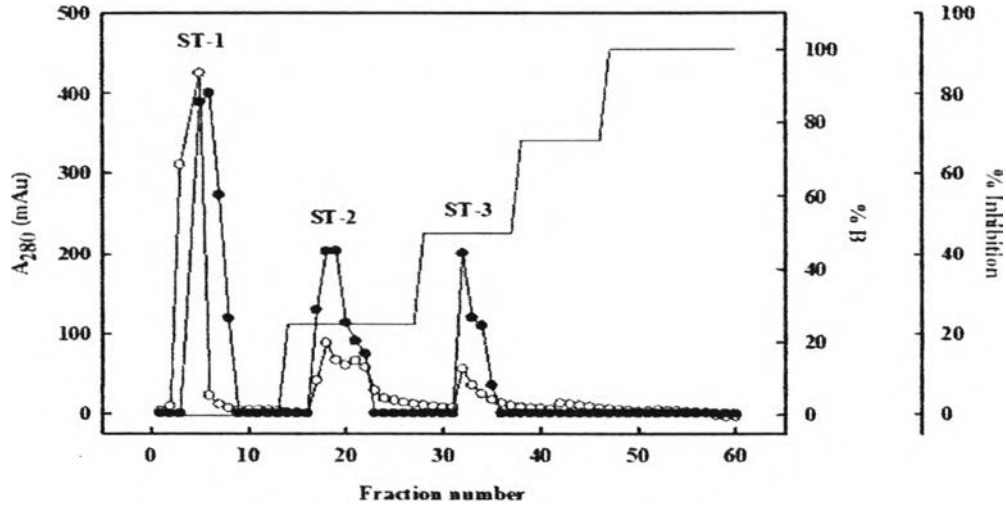

Figure 4. 1 DEAE-cellulose anion exchange column chromatogram showing the separation profile of the crude protein preparation from S. tuberosa roots, yielding the unbound fraction (ST-1) and bound fractions (ST-2 and ST-3). ST-1 was eluted in 20 mM phosphate buffer pH 7.2 while ST-2 and ST-3 were eluted in the same buffer but supplemented with a stepwise gradient of 0.25 and 0.50 M NaCl, respectively. All fractions were assayed for SOD activity (●) and absorbance at 280 nm (○).

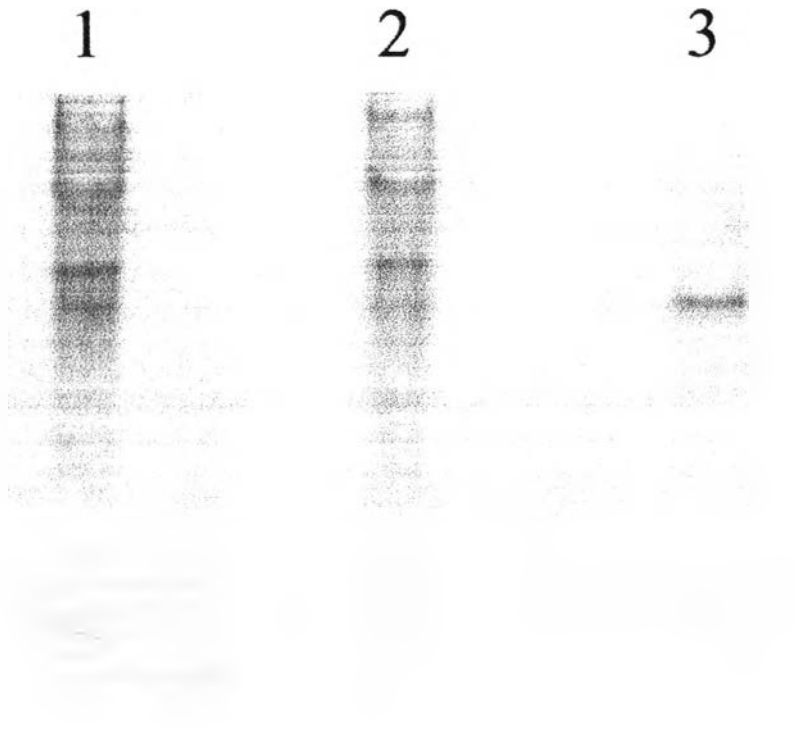

Figure 4. 2 Native-PAGE analysis of the enriched SOD from *S. tuberosa* roots. Lane 1:10 µg of the root crude extract. Lane 2: 10 µg of the post-ammonium sulfate cut protein fraction. Lane 3: 5 µg of ST-1 (enriched SOD fraction).

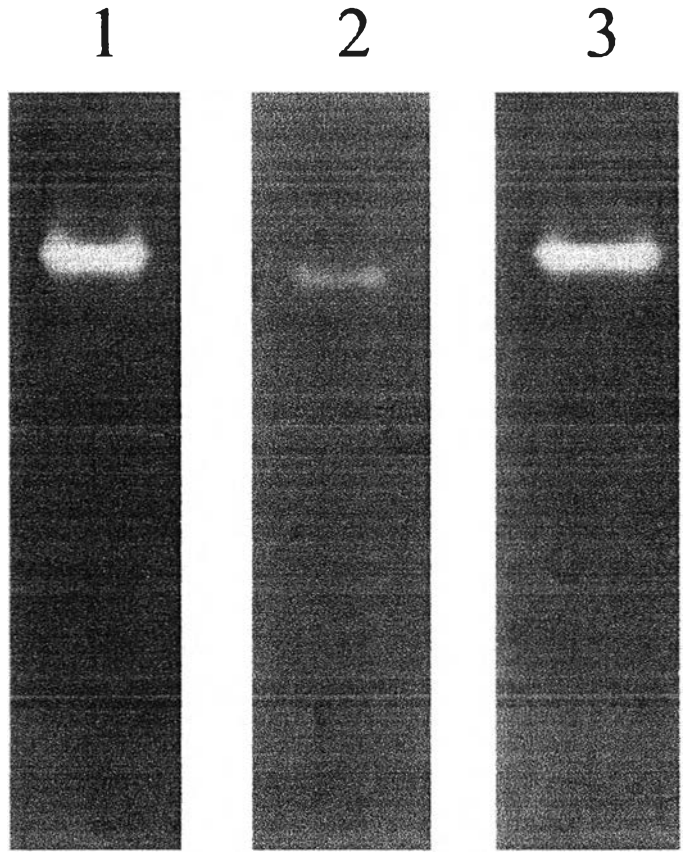

Figure 4. 3 SOD activity zymograms used to identify the type of SOD from *S. tuberosa* roots in terms of its sensitivity to KCN and H_2O_2 inhibition. ST-1 (10 µg / lane) was resolved by native-PAGE and then stained for SOD activity. Lane 1: SOD zymogram without inhibitors. Lane 2: SOD zymogram after pretreatment with 8 mM H_2O_2. Lane 3: SOD zymogram in the presence of 8 mM KCN. Gels shown are representative of 3 independent repeats

4.3.2 SOD characterization by tandem mass spectrometry

The single protein band of the ST-1 fraction resolved by native-PAGE was excised and subjected to tryptic digestion and identification of the amino acid sequences of the tryptic peptides using nano-LC-EIS/MS-MS. MASCOT database obtained tryptic peptide sequences from the nano-LC-EIS/MS-MS analysis revealed three peptides (10, 12 and 13 amino acids) matched to the CuZn-SOD isozymes from *Ananas comosus* and *Solanum lycopersicum* (Figure 4.4). In the case of the SOD from *A. comosus*, two peptides matched identically, with the third (C-terminal matching) peptide having one mismatch, giving 34/35 identical residues from 152 amino acids (23% coverage), whilst for *S. lycopersicum* the N-terminal matching peptide had two mismatches giving 33/35 identical residues over the 23% sequence coverage. The results from Native-PAGE coupled with SOD staining activity compared to the results from mass spectrometry are incompatible because limited data in the plant protein sequence database of plant preventing matches of the tryptic peptides from SOD band from 3 (Figure 4.2). Consequently it should be noted that the enriched SOD from the roots of *S. tuberosa* is Mn-SOD.

4.3.3 Determination of the molecular weight of the SOD enzyme by reducing SDS-PAGE

The molecular weight of SOD was evaluated using reducing SDS-PAGE (Figure 4.5), where it appeared to be comprised of at least two heterologous subunits with a molecular weight of 17.6 and 31.5 kDa, respectively. Most plant SODs are general composed of two or four protein subunits. That are between 18-35 kDa in size, such as those from corn germ,[104] pearl millet seedlings,[48] and soybean.[105]

(A)

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  1 MVKAVAVLGSSEGVKGTIYFTQEGDGPTTVTGSISGLKPGLHGFHVHALG
 51 DTTNGCMSTGPHFNPAGNEHGAPEDETRHAGDLGNVTVGEDGTVNVNIVD
101 SQIPLSGSNSIIGRAVVVHADPDDLGKGGHELSKTTGNAGGRVACGIIGL
151 QG
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(B)

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  1 MVKAVAVLNSSEGVSGTYLFTQVGVAPTTVNGNISGLKPGLHGFHVHALG
 51 DTTNGCMSTGPHYNPAGKEHGAPEDEVRHAGDLGNITVGEDGTASFTITD
101 KQIPLTGPQSIIGRAVVVHADPDDLGKGGHELSKSTGNAGGRIACGIIGL
151 QG
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Figure 4. 4 Three tryptic peptide amino acid sequences from ST-1 (underlined) with their matches (bold, mismatch in italics) against the CuZn-SOD isozyme from (a) *Ananas comosus* and (b) *Solanum lycopersicum*.

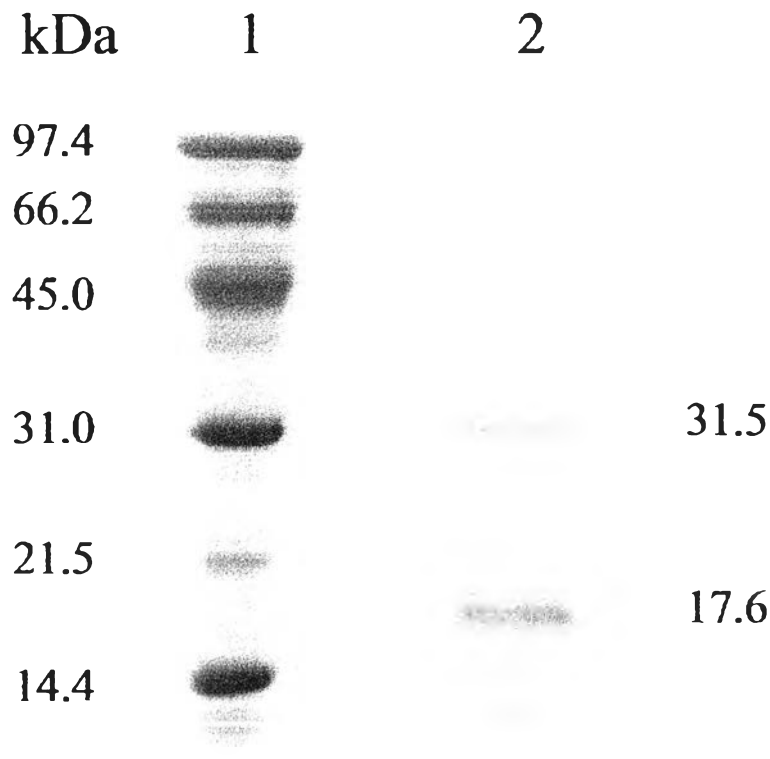

Figure 4. 5 Determination of the molecular weight of the enriched CuZnSOD from *S. tuberosa* roots by reducing SDS-PAGE. Lane 1: protein molecular weight markers. Lane 2: 7.5 µg of the enriched SOD preparation (ST-1), which appears to consist of two subunits with the indicated sizes (17.6 and 31.5 kDa). Gel shown is representative of 3 independent repeats

4.3.4 The optimum pH and pH stability of the Mn-SOD enzyme from *S. tuberosa* roots

The effect of the pH, within the range of pH 1-12, on the SOD activity was evaluated in 20 mM universal buffers (Figure 4.6A) and was found to exhibit a SOD activity level of more than 70% of the maximal level (that at pH 5 and set to be 100%) over a wide pH range (pH 3-10). In addition, SOD activity levels of ~50-60% were still maintained at extreme acidic (pH 1) and alkaline (pH 10-11) conditions. In contrast, the enzyme activity was less than 20% at pH 12. Note, that although some buffer-dependent affects upon the SOD activity were noticed (e.g. pH 4, 8 and 10 but not at pH 6), but these were less marked than the effect of the pH changes within each buffer type. Thus, the pH is the main factor in the changes in the observed SOD enzyme activity levels and so the optimum pH was determined to be pH 5 (this enzyme activity level was nominally set to 100%). In slight contrast, the optimum pH value of most plant SODs are reported to be less or not acidic, being in the range of pH 6-9, such as the SOD isozymes from *Radix lethospermi* seed,[53] pear millet seedlings,[48] wheat seedlings,[82] tea,[49] mung bean,[106] and corn.[104]

Over the pH range of pH 4-10, the Mn-SOD enzyme activity was stable for 120 min and retained the highest activity at pH 5, but still retained some 80% activity at pH 7 and pH 8 for 60 min and at pH 6 for 45 min, before decreasing to 70% and 60% residual activity after 120 min at pH 7-8 and pH 6, respectively (Figure 4.6B). At pH 4, the Mn-SOD enzyme retained 80% of activity for only 10 min and then the activity dropped to lower than 40% at 120 min. The pH stability of the enriched Mn-SOD enzyme from *S. tuberosa* was somewhat broader than that compared to results from the SOD from wheat seedlings,[82] which after 15 min at pH 7-9 displayed only ~60-

80% activity, and less than 40% activity after longer times, compared to a higher activity (78-100%) over a broader pH range (pH 5-9) for longer (up to 45 min) for the purified SOD from *S. tuberosa*. Likewise, the pH stability of the SOD from *R. lethospermi* seeds[53] was lower than that reported here for *S. tuberosa*. For example, at pH 5, the activity of purified SOD from *R. lethospermi* seeds were reduced to 50% while that from *S. tuberosa* was 100%.

4.3.5 The optimum temperature and thermal stability of SOD enzyme

The enriched Mn-SOD enzyme from *S. tuberosa* roots maintained an ~100% enzyme activity over the broad temperature range of 0-50 °C, but this decreased to 50% and 30% at 60 °C and 70 °C, respectively, and was completely inactivated at 80-100 °C (Figure 4.7A). This optimum temperature of 0-50 °C is the same as that reported for the SODs from garlic and wheat seedlings,[82] but wider than that reported from other plant SODs, such as pearl millet seedlings (20-35 °C) and *R. lethospermi* seeds (25-45 °C).[53]

The enriched Mn-SOD from *S. tuberosa* roots was found to be thermostable up to 40 °C with the enzyme activity remaining at ~100% for 90 min at these temperatures, but declining to 90% after 120 min (Figure 4.7B). However, at 50 °C the SOD activity was around 90% for 30 min and then dropped to 80% at 120 min, whilst at 60 °C the SOD activity decreased to 30% after 120 min, and the enzyme was completely inactivated after 90 to 120 min at 70 °C.

The thermal stability of the enriched Mn-SOD from *S. tuberosa* roots observed here (Figure 4.7B) was higher than the purified SOD from wheat seedlings, the latter of which displayed only 20% activity after 60 min at 60 °C compared to

50% activity after 60 min and 30% activity after 120 min for that from *S. tuberosa* roots. After 60 min at 70 ^{o}C the enzyme activity of the enriched SOD from *S. tuberosa* was around 30% compared to less than 10% for the SOD from wheat seedlings.[82]

4.3.6 Determination of the effect of different bivalent metal ions on the SOD activity

The enriched Mn-SOD from *S. tuberosa* roots was pre-incubated with various metal ions at 0.1, 0.5, 1 or 5 mM for 30 min before determining the residual SOD activity of the enzyme using the riboflavin-NBT assay. The prior incubation with Fe^{2+} significantly enhanced the SOD enzyme activity level at all four tested concentrations, but in a dose-independent manner, at least at these tested concentrations, which were outside of the 0-0.1 mM range (Table 4.2). Pre-incubation with Ca^{2+} also significantly increased the enzyme activity level at 0.5 to 5 mM, but only weakly at 0.1 mM. A weak increase in enzyme activity level was seen for Zn^{2+} and Cu^{2+} at low concentrations (0.1 and 0.5 mM), but these two metal ions were either strongly (Zn^{2+}) or weakly (Cu^{2+}) inhibitory at higher concentrations (1.0 and 5.0 mM). In contrast, Hg^{2+}, Mn^{2+} and Mg^{2+} showed a dose-dependent strong inhibition of the SOD enzyme activity, with Hg^{2+} causing complete enzyme inhibition at 5 mM. Finally, the enzyme was completely inactivated by SDS at concentrations of 0.5 mM and above.

The inhibition of SOD enzyme activity by both SDS and Hg^{2+} at low concentrations has been reported for other plant SODs, such as wheat seedlings,[82] and tea.[49] In addition, plant SODs have been reported to use Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} as prosthetic groups in their active site, which mainly aid electron transfer

during the oxidation-reduction reaction,[82] whilst Zn^{2+} has been reported to not only be an electron carrier but also play a role in the stability of the enzyme structure.[53]

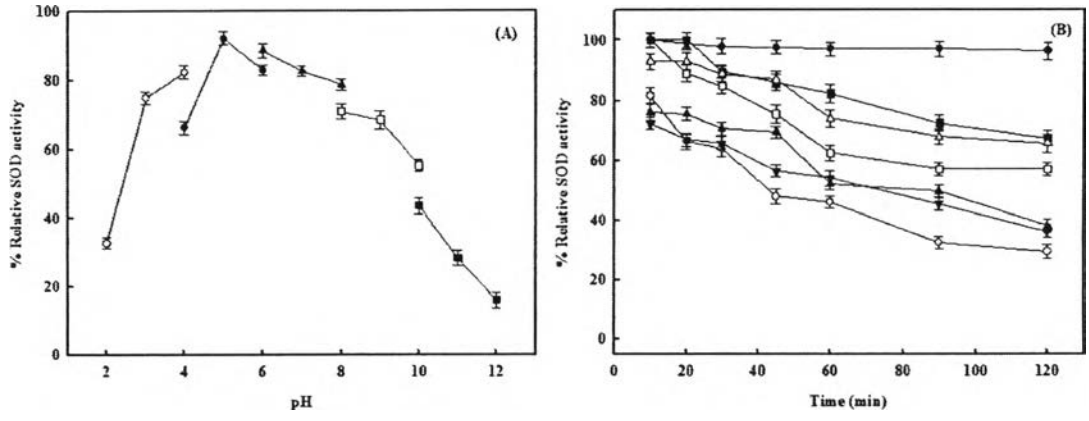

Figure 4. 6 The effect of pH on the enriched MnSOD from *S. tuberosa* roots showing: (A) the relative SOD activity level in different 20 mM buffers and pH values, for (○) glycine-HCl (pH 2-4), (●) sodium acetate (pH 4-6), (▲) potassium phosphate (pH 6-8), (□) Tris-HCl (pH 8-10) and (■) glycine-NaOH (pH 10-12) and (B) the pH stability of the enzyme after 10-120 min preincubation in 20 mM sodium acetate (pH 4; (○), pH 5; (●), pH 6; (□), potassium phosphate ((■); pH7 & (△); pH8) and Tris-HCl ((▲); pH 9 & (▼); pH10). (B) The pH stability of the enzyme for 10-120min. In all cases the data, reported as the relative activity where the highest seen was set at 100%, are shown as the mean ± 1 SD and are derived from 3 independent repeats.

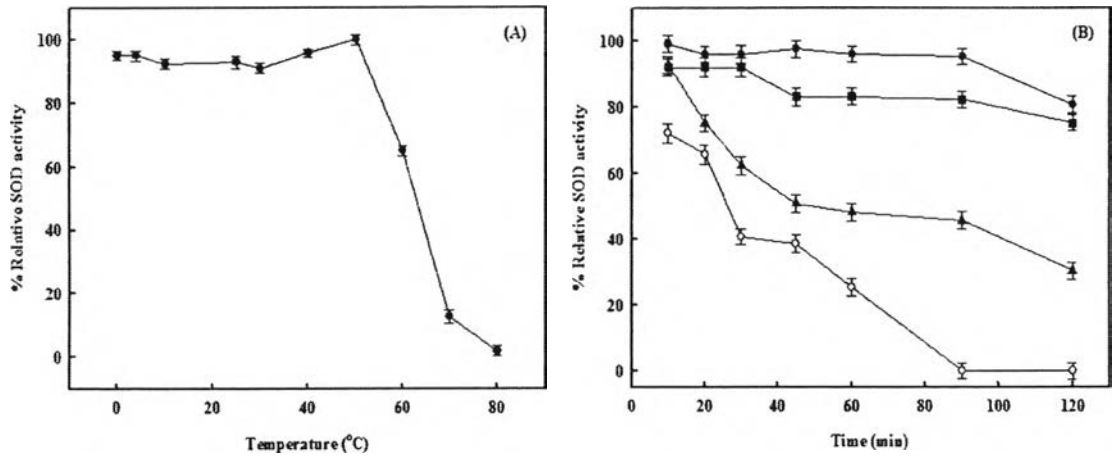

Figure 4. 7 The effect of temperature on the enriched MnSOD from *S. tuberosa* roots showing:- (A) the relative SOD activity level at different temperatures, and (B) the thermal stability of the enzyme after 10-120 min pre-incubation at different temperatures. In all cases the data, reported as the relative activity where the highest seen was set at 100%, are shown as the mean ± 1 SD and are derived from 3 independent repeats (B) The effect of appropriate temperature from optimum temperature range at 10-120 min. (●); 40°C, (■); 50°C, (▲); 60°C and (○); 70°

Table 4. 3 The effect of bivalent metal ions or SDS on the enriched MnSOD enzyme activity.

% Relative SOD activity[1]				
Additive	0.1 mM	0.5 mM	1 mM	5 mM
Zn^{2+}	101.2 ± 0.008	103.6 ± 0.003	88.4 ± 0.009	88.4 ± 0.010
Cu^{2+}	102.6 ± 0.004	102.6 ± 0.017	97.2 ± 0.009	97.2 ± 0.004
Ca^{2+}	102.8 ± 0.005	110.0 ± 0.008	110.0 ± 0.002	109.2 ± 0.004
Mn^{2+}	95.6 ± 0.012	82.0 ± 0.008	67.6 ± 0.017	65.2 ± 0.006
Fe^{2+}	110.0 ± 0.007	110.0 ± 0.013	110.0 ± 0.017	110.0 ± 0.006
Mg^{2+}	86.8 ± 0.002	85.2 ± 0.010	80.4 ± 0.001	75.6 ± 0.008
Hg^{2+}	65.2± 0.044	60.5 ± 0.023	56.5 ± 0.012	0
SDS	81.3 ± 0.038	0	0	0

[1]Data, reported as the % relative enzyme activity where that of the control with no addition was set as 100%, are shown as the mean ± standard deviation and are derived from three independent replications. Means within a column or row followed by a different lowercase superscript letter are significantly different ($P<0.05$; ANOVA and Tukey test on log transformed data).

4.3.7 Evaluation of the effect of riboflavin and NBT on SOD activity

Different concentrations of NBT (0.1-0.25 mM) and riboflavin (0.05 - 0.1 mM) were used to determine the kinetics of the SOD activity. Double reciprocal (Lineweaver-Burke) plots of the data revealed that the relationships were linear (R^2 = 0.964 and 0.954 for NBT and riboflavin, respectively), giving derived K_m and V_{max} values of SOD of 62.414 ± 0.015 M and 101.010 ± 0.022 µmol/min/mg protein for NBT and 27.389 ± 0.032 M (Figure 4.8A)and 38.167 ± 0.021 µmol/min/mg protein for riboflavin (Figure 4.8B).

4.3.8 Partial nucleotide sequences of SOD from *S. tuberosa*

To determine the partial nucleotide sequences of SOD from *S. tuberosa* the 5'RACE and 3'RACE PCR were generated by using two primers 3-pine and 5-solo which were designed according to the sequences of pine apple (*Ananas comosus*) and tomato (*Solanum lycopersicum*) SODs respectively. With these two primers and AP1 primer (5'-CCATCCTAATACGACTCACTATAGGGC-3') and the PCR condition as mentioned in section 4.2.14, the approximately 400 bp PCR products was extracted and submitted for DNA sequencing (Macrogen, Korea). Then, 70 bp nucleotide sequences were obtained from the alignment by using ApE program (Appendix D). The primers F1, F2, R1 and R2 were designed from the previous 70 bp for obtaining the longer nucleotide sequences under the same PCR condition. There are 178 bp

(5'GAGGGGATTTGCGGGGGTATAGTTGTCAGGGTCTCCTTATAGGTCTGGT

GAGAATAAAACTAGTGATATTAAGATTAGAAGTAAGAGTAAGGCTCCTAGGATGTCTTTGATTG

TATAGTAGGGGTGAAATGGGATTTTGTCTGAGTCTGATGGCAATTCCTGAGGGTTTGTTCCATC

C-3') of nucleotide sequence of SOD from *S. tuberosa* was obtained from using the

primers R1 and R2. Consequently, this is partial nucleotide sequence of SOD from *S. tuberosa*. Therefore, is not long enough to compare with the database so more primers are needed to be designed from the partial nucleotide sequences (178bp) to obtain a longer nucleotide sequences then compare to the database.

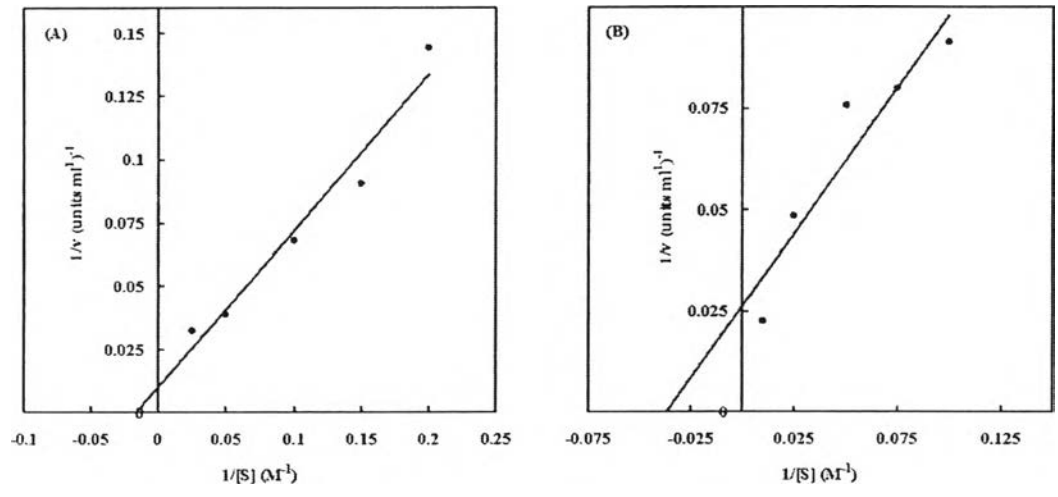

Figure 4. 8 Kinetics, shown as a double reciprocal (Lineweaver-Burk) plots, of the enriched SOD activity from *S. tuberose* with different concentrations of (A) NBT and (B) riboflavin as substrates.

4.4 CONCLUSION

Superoxide dismutases were discovered from the root of *S. tuberosa*. There are at least three different SOD was found from this plant after purified using ion exchange chromatography called ST-1, ST-2 and ST-3. ST-1 from unbound fraction has the strongest SOD activity. ST-1 was characterized as a purified Mn-SOD which normally find in mitochondria. Mn-SOD is usually found in mitochondria for protecting the organelle from free radical damage. Mn is a cofactor of SOD and can scavenge superoxide radical to hydrogen peroxide and oxygen molecule. Because of the mode of action, there are publications reported on biological activities of SOD such as antioxidant, ant-aging and anti-inflammatory properties which can be applied to use in pharmaceutical industry. Consequently ST-1 was characterized its biochemical properties for preparing to use in future pharmaceutical application. In addition, ST-1 is still required to characterize its biological properties that have not been reported in this research, yet.