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Isolation and Characterization of Superoxide Dismutase from Wheat Seedlings

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Two major superoxide dismutases (SODs; SODs I and II) were found in the crude enzyme extract of wheat seedlings after heat treatment, ammonium sulfate fractionation, anionic exchange chromatography, and gel permeation chromatography. The purification fold for SODs I and II were 154 and 98, and the yields were 11 and 2.4%, respectively. SOD I was further characterized. It was found that SOD I from wheat seedlings is a homodimer, with a subunit molecular mass of 23 kDa. Isoelectric focusing electrophoresis (IEF) and zymogram staining results indicated that the isoelectric point of SOD I is 3.95. It belongs to the MnSOD category due to the fact that it was insensitive to KCN or hydrogen peroxide inhibitor. This MnSOD from wheat seedlings was found to be stable over pH 7−9, with an optimum pH of 8, but was sensitive to extreme pH, particularly to acidic pH. It was stable over a wide range of temperatures (5−50 °C). Thermal inactivation of wheat seedling MnSOD followed first-order reaction kinetics, and the temperature dependence of rate constants was in agreement with the Arrhenius equation. The activation energy for thermal inactivation of wheat seedling MnSOD in the temperature range of 50−70 °C was found to be 150 kJ/mol. $HgCl_2$ and SDS at a concentration of 1.0 mM significantly inhibited enzyme activity. Chemical modification agents, including diethyl pyrocarbonate (2.5 mM) and Woodward's reagent K (50 mM), significantly inhibited the activity of wheat seedling SOD, implying that imidazole groups from histidine and carboxyl groups from aspartic acid and glutamic acid are probably located at or near the active site of the enzyme.

KEYWORDS: pH and temperature optimum; thermal inactivation kinetics; metal ions; chemical modification reagents

INTRODUCTION

Superoxide dismutase (SOD, EC 1.15.1.1) is one of the important antioxidant enzymes found in living organisms, including microorganisms and plants (*1*–*3*). It catalyzes the dismutation of the superoxide anion radicals ($O_2^{\bullet -}$ to O_2 and H_2O_2. This enzyme is therefore able to prevent redox reactions directly involving superoxide anion radicals and reactions by more reactive radicals generated by superoxide anion radicals, such as hydroxyl radicals ($OH^{\bullet}$). SODs isolated from different sources belong to three classes containing Fe, Mn, or Cu plus Zn as prosthetic groups (metal cofactor requirement). Cu/ZnSOD located both in cytosol and in chloroplasts is the most abundant form of SOD in higher plants. On the other hand, FeSOD and MnSOD are primarily located in plastids and mitochondria of higher plants, respectively (*3*).

Wheat seedling (or commonly called "wheat grass" in Taiwan due to its grass-like appearance) is the tender small plants (or the vegetative tissues) grown from wheat seed (*Triticum aestivum* L.). It is thought to have the capability of reducing the oxidative stress for living cells and has received much interest recently as one of the functional foods (*4, 5*). The water and ethanol extracts of wheat seedlings have been shown to be capable of scavenging various free radicals such as superoxide anion radicals and DPPH radicals, chelating Fe^{2+}, and reducing Fe^{3+} as well as inhibiting the autoxidation of linoleic acid (*6, 7*). In a study on the effect of dehydration on the antioxidative activity of wheat seedling powder, Lin et al. (*6*) reported that the water extract of freeze-dried wheat seedling powder showed the strongest superoxide radical scavenging activities, followed by vacuum dehydration and air convective dehydration in decreasing order. Furthermore, Hong and Lai (*7*) found that steam blanching pretreatment of wheat seedlings caused the water extract of freeze-dried powders of wheat seedlings to lose the superoxide radical scavenging capability. Such results implied that the superoxide radical scavenging activities of wheat seedlings could possibly result from SOD activity in addition to the SOD-like components in wheat

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seedlings. However, no efforts have been reported on the characterization of SODs from wheat seedlings.

During food processing, which may include mechanical disruption, freezing, heat treatment, and drying, etc., the SOD isozymes may be wholly or partially inactivated, which may result in an increase in the susceptibility of food ingredients to autoxidation and enzyme-catalyzed oxidation reactions. Characterization of the SODs from wheat seedlings would be helpful not only for developing a suitable processing condition to preserve the SOD activity for wheat seedling juice or powder but also for assessing the application potential of wheat seedling SOD as a natural food antioxidant or functional ingredient. Therefore, this study aimed at testing the hypothesis that the nutritional benefits of wheat seedlings are associated in part with their SOD activity by isolation of the SOD isoforms in the extracts of wheat seedlings. Furthermore, the biochemical characteristics of wheat seedling SOD, including molecular mass, isoelectric point, prosthetic group, and pH/temperature optimum and stability, were analyzed. Effects of metal ions and chemical modification agents on the activity of wheat seedling SOD are reported as well.

MATERIALS AND METHODS

Wheat Seedlings. Wheat seeds (*T. aestivum* L. Nungyuan 2) were purchased from a contracted farmer in Taichung, Taiwan. The wheat seeds were then planted to obtain wheat seedlings according to the method of Lai et al. (*8*). The vegetative tissues of wheat seedlings were cut when the height of wheat seedlings was about 10−12 cm.

Crude Enzyme Extract. Crude enzyme extract of wheat seedlings was obtained by using a grass juice extractor (Green Power, KP-E1201, Kempo Co. Ltd., Seoul, Korea), then centrifuged (CR20B2, Hitachi, Tokyo, Japan) at 15000*g* and 4 °C for 15 min, and filtered (Whatman no. 4 filter paper, Whatman International Ltd., Maidstone, U.K.). Soluble solid content of the supernatant was measured using a refractometer (Pallette PR-101, Atago Co. Ltd., Tokyo, Japan). To standardize the crude enzyme extract from each batch, the soluble solid content of the supernatant was adjusted to 5 °Brix by the addition of deionized water.

Measurement of SOD Activity. A riboflavin−nitroblue tetrazolium (NBT) assay was adapted from Babitha et al. (*9*) with modification. One tenth of a milliliter of enzyme solution with appropriate dilution, 0.2 mL of 0.1 M EDTA, 0.1 mL of 1.5 mM NBT, and 2.9 mL of 67 mM sodium phosphate buffer (pH 7.8) were first mixed and incubated at 37 °C for 5 min, after which 0.05 mL of 1.2 mM riboflavin was added. The entire reaction mixture was moved to a foil-lined box and illuminated with a 13 W light tube from a distance of approximately 12 cm at room temperature for 14 min. To provide homogeneous illumination, a digital lux meter (Lutron Lx-101, Taichung, Taiwan) was used to control the illumination in the range of 6500−7500 lx. The inhibition of NBT reduction was measured at 560 nm in a Hitachi spectrophotometer (model 2001) equipped with a thermostated cell compartment at 25 °C. One unit of SOD activity was defined as the required amount of enzyme to provide 50% inhibition of riboflavin-mediated reduction of NBT.

Measurement of Protease Activity. Protease activity was measured by using casein hydrolysis as an index according to the method of Anson (*10*) with modification. Five milliliters of 0.6% casein in 0.1 M sodium phosphate buffer (pH 8.0) was first incubated at 37 °C for 5 min, followed by the addition of 0.1 mL of enzyme solution with appropriate dilution. After 10 min of incubation at 37 °C, 5 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid was added to terminate the reaction. The reaction mixture was then thoroughly mixed, filtered, and centrifuged. The absorbance of the supernatant at 280 nm was determined. The protease activity was then calculated from the calibration curve, which was performed with 0−0.16 mg/mL tyrosine under the same reaction condition. One unit of protease activity was defined as the amount of enzyme that gave an increase in absorbance at 280 nm equivalent to 1 μg of tyrosine per minute at 37 °C.

Protein Determination. Protein content was quantified by using the BCA (bicinchoninic acid) and micro BCA protein assay kit (Pierce Biotechnology Inc., Boston, MA) according to the method of Smith et al. (*11*). Bovine serum albumin (BSA) was used as the protein standard.

Purification of SOD. *Heat Treatment.* Crude enzyme extract was first heated at 40 °C for 15 min and then centrifuged at 15000*g* and 4 °C for 15 min to remove the precipitate. An aliquot of the supernatant was analyzed for SOD activity and protein content. The remainder was saved for further purification.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was slowly added to the stirred solution of the above supernatant to give 30% saturation of ammonium sulfate and equilibrated for 30 min at room temperature. The mixture was centrifuged at 15000*g* (20 min at 4 °C) to separate the precipitate (P_1) and supernatant (S_1). The collected precipitate was redissolved in a 67 mM sodium phosphate buffer (pH 7.8) and analyzed for SOD activity and protein content. A further addition of ammonium sulfate was made to the supernatant (S_1) to give a 40% saturation of ammonium sulfate and equilibrated for 30 min at room temperature followed by centrifugation at 15000*g* (20 min at 4 °C) to separate the precipitate (P_2) and supernatant (S_2). The precipitate was redissolved in a 67 mM sodium phosphate buffer (pH 7.8) and analyzed for SOD activity and protein content. Similar steps were performed until 90% saturation of ammonium sulfate was achieved. SOD activity and protein content versus saturation of ammonium sulfate were plotted to determine the optimum saturation of ammonium sulfate fractionation. The precipitate obtained from optimum saturation of ammonium sulfate fractionation was dissolved in 67 mM of sodium phosphate buffer (pH 7.8) and dialyzed against a 50-fold volume of 67 mM sodium phosphate buffer (pH 7.8) at 4 °C for 24 h by using a snake skin pleated dialysis tubing with a molecular weight cutoff (MWCO) of 10 kDa (Pierce Biotechnology Inc.) and centrifuged at 15000*g* (10 min at 4 °C). The dialyzed sample was filtered through a 0.45 μm membrane (0.45 μm HV, Millipore Co., Bedford, MA) and concentrated by using an Amicon Ultra-15 ultrafiltration unit with a membrane having a MWCO of 10 kDa (Millipore Co.).

DEAE-Sephacel Anion Exchange Chromatography. The DEAE-Sephacel column (2.6 × 40 cm) was equilibrated with 67 mM sodium phosphate buffer (pH 7.8) using a flow rate of 30 mL/h. One milliliter of enzyme solution obtained from the ammonium sulfate fractionation step was applied to the column and eluted with a stepwise NaCl gradient (0.05−0.5 M) in 67 mM sodium phosphate buffer (pH 7.8). Each of the collected fractions (2 mL) was analyzed for SOD activity and protein content (absorbance at 280 nm). Fractions containing SOD activity were pooled, dialyzed, and concentrated as described above.

Sephacryl S-100 HR Gel Permeation Chromatography. The Sephacryl S-100 HR column (1.6 × 70 cm) was equilibrated with 67 mM sodium phosphate buffer (pH 7.8) using a flow rate of 30 mL/h. One milliliter of enzyme solution obtained from the ion-exchange step was applied to the column and eluted with the equilibrium buffer. Each of the collected fractions (1 mL) was analyzed for SOD activity and protein content (absorbance at 280 nm).

Determination of Native Molecular Mass. The native molecular mass of the purified SOD was determined by gel filtration. A Superose 12 Hiprep 16/70 column (1.6 × 70 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweeden) was equilibrated with 5 mM sodium phosphate buffer (pH 7.8) with 0.15 M NaCl using a flow rate of 12 mL/h. Half a milliliter of enzyme solution was applied to the column and eluted with the equilibrium buffer. Each of the collected fractions (0.5 mL) was analyzed for SOD activity and protein content (absorbance at 280 nm). Calibration was made with blue dextran 2000 and purified protein standards (including ribonuclease A, molecular mass = 13.7 kDa; chymotrypsinogen A, molecular mass = 25 kDa; ovalbumin, molecular mass = 43 kDa, and bovine serum albumin, molecular mass = 67 kDa). The elution volume of the protein standard was converted to the apparent distribution coefficient (K_{av}) using the equation

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

where V_e = the elution volume of sample, V_0 = the void volume of the column, and V_t = the resin bed volume of the column. Calibration curve was obtained by plotting the apparent distribution coefficient (K_{av}) versus logarithmic molecular mass. The native molecular mass of SOD was calculated from the calibration curve.

SDS−Polyacrylamide Gel Electrophoresis (SDS-PAGE). The molecular mass of the monomer of SOD isozymes was determined by using a vertical SDS-PAGE according to the method of Laemmli (*12*) with modification. Before loading onto the miniformat gel, protein standards including phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), chicken egg albumin (45.0 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa) (Pharmacia low molecular weight calibration kit vial) were treated with equal volumes of sample buffer that contained 4% SDS and 10% 2-mercaptoethanol followed by heating at 90 °C for 5 min. The enzyme solution was treated with an equal volume of sample buffer that contained 4% SDS only (without 2-mercaptoethanol) followed by heating at 90 °C for 5 min. Electrophoresis (Hoefer Mighty Small Gel Electrophoresis SE260, Amersham Pharmacia Biotech AB) was conducted in a 12.5% acrylamide gel slab (10 × 7 × 0.75 mm) placed in 50 mM Tris−glycine−SDS buffer of pH 8.3. Fifteen microliters of each sample was applied and electrophoresed at 80 V through stacking gel for 15 min and at 130 V through separating gel for 60 min. After electrophoresis, each gel was divided into two parts, one for protein staining by Coomassie brilliant blue R (CBR) and the other for SOD activity staining according to the method of Chen et al. (*13*) with modification. Briefly, the gel was washed with 1% Triton X-100 in 0.1 M sodium phosphate buffer (pH 7.0) for at least 90 min, followed by 0.1 M sodium phosphate buffer (pH 7.0) without 1% Triton X-100 several times and deionized water several times. The gel was soaked in 1.225 mM NBT solution for 15 min, washed with water, and soaked in 100 mM sodium phosphate buffer (pH 7.0) containing 28 mM *N*,*N*,*N*′,*N*′- tetramethylethylenediamine (TEMED) and 0.028 mM riboflavin for another 15 min. After the gel was washed again, it was illuminated for 15 min to initiate the photochemical reaction. All of the procedures were carried out at room temperature, and the two soaking steps were performed with shaking at 50 rpm. SOD isozymes were observed as white unstained zones against a blue background. The molecular mass of the monomer of SOD was calculated by comparing the relative mobility (R_f) of SOD with that of the molecular markers developed in the same gel.

Isoelectric Focusing Electrophoresis (IEF). The p*I* values of wheat seedling SOD were determined by IEF using PhastGel IEF 3−9 gel (Amersham Pharmacia Biotech AB). The detailed procedure is outlined in the Pharmacia PhastSystem Separation Technique File No. 110. After electrophoresis, each gel was divided into two parts, one for protein staining by CBR, and the other for SOD activity staining as described before. An aliquot of the standard protein mixture, including trypsinogen (p*I* = 9.3), lentil lectin-basic band (p*I* = 8.65), lentil lectin-middle band (p*I* = 8.4), lentil lectin-acidic band (p*I* = 8.15), myoglobin-basic band (p*I* = 7.35), myoglobin-acidic band (p*I* = 6.85), human carbonic anhydrase B (p*I* = 6.55), bovine carbonic anhydrase B (p*I* = 5.85), *β*-lactoglobin A (p*I* = 5.2), soybean trypsin inhibitor (p*I* = 4.55), and amyloglucosidase (p*I* = 3.55) (Pharmacia p*I* calibration kit), was run alongside the SOD isozyme samples, and the R_f values directly measured from the gel were used to construct the p*I* calibration curve. The R_f values of wheat seedling SOD isozymes were used to estimate the p*I* values of wheat seedling SOD from the p*I* calibration curve.

Identification of Types of SOD Isozyme. Prosthetic groups of wheat seedling SOD were determined by native-PAGE followed by SOD activity staining using the method of Liu and Pan (*14*), which was modified from that of Beauchamp and Fridovich (*15*). Each enzyme solution was mixed with a $^1/_{10}$ volume of protein tracking dye solution (0.01% bromophenol blue and 50% glycerol) and an equal volume of loading buffer (1.5 M Tris, pH 8.3) in the absence of 2-mercaptoethanol and SDS and loaded directly onto the gel without heating. Fifteen microliters of each sample was applied, and electrophoresis was conducted at 100 V. After electrophoresis, SOD activity staining was performed as described. SOD isozymes were observed as white unstained zones against a blue background. Two types of inhibitors, including 8 mM sodium cyanide (KCN) and 8 mM hydrogen peroxide, were used to identify the prosthetic type of SOD isozymes. Briefly, 8 mM KCN was added into riboflavin solution for SOD activity staining, or the gels were first soaked in 8 mM hydrogen peroxide in 100 mM potassium phosphate buffer at pH 7.0 for 30 min, followed by SOD activity staining as described.

Determination of Optimum pH and pH Stability. The apparent pH optimum of the crude extract and purified SOD from wheat seedlings was determined by performing the NBT reduction in universal buffers at different pH values (2−12) (*16*). Reaction controls were run for each pH value. Results were expressed as the relative activity percentage calculated by the ratio of the specific activity at different pH values to that of maximum activity within the pH range studied. To determine the pH stability of wheat seedling SOD, the enzyme solutions were first equilibrated with universal buffers at appropriate pH values (according to the results from pH optimum test) for 0−60 min at 37 °C. The NBT reduction test was then performed to determine the SOD activity as described. Measurements were carried out in triplicate for each pH/time combination. The SOD activity of the untreated extracts represented the initial (100%) activity from which the proportion (percent) of the remaining activity after pH/time treatment was calculated.

Determination of Optimum Temperature and Temperature Stability. The temperature optimum of the crude and purified SOD from wheat seedlings was determined by performing the NBT reduction in 67 mM sodium phosphate buffer (pH 7.8) after incubation of the reaction mixture at different temperatures (5−80 °C) for 5 min. Reaction controls were run for each temperature. Results were expressed as the relative activity percentage calculated by the ratio of the specific activity of the SOD from wheat seedlings at different temperatures to that of maximum activity within the temperature range studied. To determine the temperature stability of wheat seedling SOD, the enzyme solutions were pipetted into several thin-walled glass tubes pre-equilibrated at the appropriate temperature (according to the results from optimum temperature test) for 0−60 min (*17*). After heat treatment, the contents were cooled quickly in ice-cold water and analyzed for SOD activity. Measurements were carried out in triplicate for each temperature/time combination. The SOD activity of the unheated extracts represented the initial (100%) activity from which the proportion (percent) of the remaining activity after heating was calculated.

Effect of Metal Ions and Various Compounds. The purified SOD (0.05 mL) was incubated with 0.05 mL of 0.2 or 2 mM of various compounds (including KCN, H_2O_2, $CaCl_2$, $NaNO_2$, $ZnCl_2$, $MgCl_2$, $HgCl_2$, SDS, and EDTA-2Na) or deionized water as the control at 25 °C for 30 min. The enzyme activity was measured and expressed as the relative activity percentage calculated by the ratio of the specific activity of SOD with metal ions or other compounds to that without metal ions and other compounds.

Effect of Chemical Modification Reagents. The purified SOD (0.05 mL) was incubated with 0.025 mL of corresponding reaction buffer for each chemical modification reagent (*18*–*25*) and 0.025 mL of *N*-bromosuccinimide (NBS, 2 and 20 mM), ethyl acetimidate (EAM, 1 mM), *N*-acetylimidazole (NAI, 10 mM), 2,4-dinitro-1-fluorobenzene (DNFB, 10 mM), phenylmethanesulfonyl fluoride (PMSF, 10 mM), diethyl pyrocarbonate (DEPC, 10 mM), *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K, WRK, 200 mM), and *p*-hydroxymercuribenzoic acid (*p*HMB, 2 and 4 mM) or 0.05 mL of deionized water as the control at 25 °C for 30 min. The enzyme activity was measured and expressed as the relative activity percentage calculated by the ratio of the specific activity of SOD with chemical modification reagents to that without chemical modification reagents.

Statistical Analysis. All data are expressed as mean ± standard deviation. Analysis of variance was performed by ANOVA procedures of SAS program (version 9.1, Statistical Analysis System Inc., Cary, NC; 2006). Duncan's new multiple-range test was used to determine the difference of means, and $p < 0.05$ was considered to be statistically significant.

Table 1. Purification Scheme of Superoxide Dismutase from Wheat Seedlings

procedure	total vol (mL)	total activity (units)	total protein (mg)	specific activity (units/mg)	purifn (fold)	yield (%)
crude extract	61	1454.84	1252.94	1.16	1.0	100
heat treatment (40 °C; 15 min)	56	1177.05	957.60	1.23	1.1	80.9
ammonium sulfate fractionation (0−60% saturation)	8	915.45	299.60	3.06	2.6	62.9
DEAE-Sephacel ion exchange						
peak I (0.2−0.3 M NaCl)	33	659.28	3.96	166.48	143.4	45.3
peak II (0.4 M NaCl)	18	205.20	2.88	71.25	61.4	14.1
Sephacryl S-100 HR gel filtration						
peak I	15	161.07	0.90	178.96	154.1	11.1
peak II	24	35.5	0.31	113.77	98.0	2.4

RESULTS AND DISCUSSION

Isolation of Wheat Seedling SODs. Like many extracts of plant tissues, extracts of wheat seedlings contained a high concentration of pigments. However, after mild heat treatment and ammonium sulfate precipitation, the specific activity of SOD was increased 3-fold accompanied by simultaneous removal of some colored compounds from the extracts, although the solution remained a deep brown color. Precipitates from 0 to 60% saturation of ammonium sulfate showed significantly higher SOD specific activity and were pooled for DEAE-Sephacel purification. Under the DEAE-Sephacel purification conditions applied, all colored compounds were bound by the DEAE-Sephacel, whereas the SOD was eluted by the gradient. DEAE-Sephacel anion exchange chromatogram resolved the wheat seedling enzyme into a major and a minor peak. The major one was eluted from fractions 24−40 (designated peak I or SOD I hereafter), and the minor one was eluted from fractions 41−49 (designated peak II or SOD II hereafter). Sequential Sephacryl S-100 chromatography resulted in a single peak for both SOD isozymes with specific activities of 179 and 114 units/mg protein for SODs I and II, respectively. **Table 1** summarizes the levels of purification of wheat seedling SOD from each purification scheme. Although purification through gel permeation chromatography increased the purification fold to 154 and 98 for SODs I and II, respectively, the yields dropped to 11 and 2.4% for SODs I and II, respectively, which were approximately one-fourth to one-sixth of the yields from DEAE-Sephacel anion exchange. This is possibly due to the removal of some inert protein or stabilizing substances during gel permeation chromatography, which are necessary to protect the enzyme from inactivation after this step. Because our purification strategy gave priority to the yield and overall production cost over the purification fold, and subsequent heating, ammonium sulfate fractionation, and anionic exchange chromatography already gave a 143-fold purification of wheat seedling SOD with good recovery (45%) and stability, only DEAE-Sephacel anion exchange purified SOD I (the predominant SOD in wheat seedlings) was further characterized in this study.

Identification of SOD from Wheat Seedlings. The molecular mass of the DEAE-Sephacel anion exchange purified SOD I from wheat seedlings was about 46 kDa as estimated by Superose 12 HR gel filtration (**Figure 1**). The molecular mass of the monomer of SOD isozymes was found to be 23 kDa as determined by comparing the relative mobility (R_f) of SOD with that of the molecular markers developed in the same SDS-PAGE gel (**Figure 1**). Such results indicated that the purified wheat seedling SOD I is a homodimer. Most plant SODs such as those

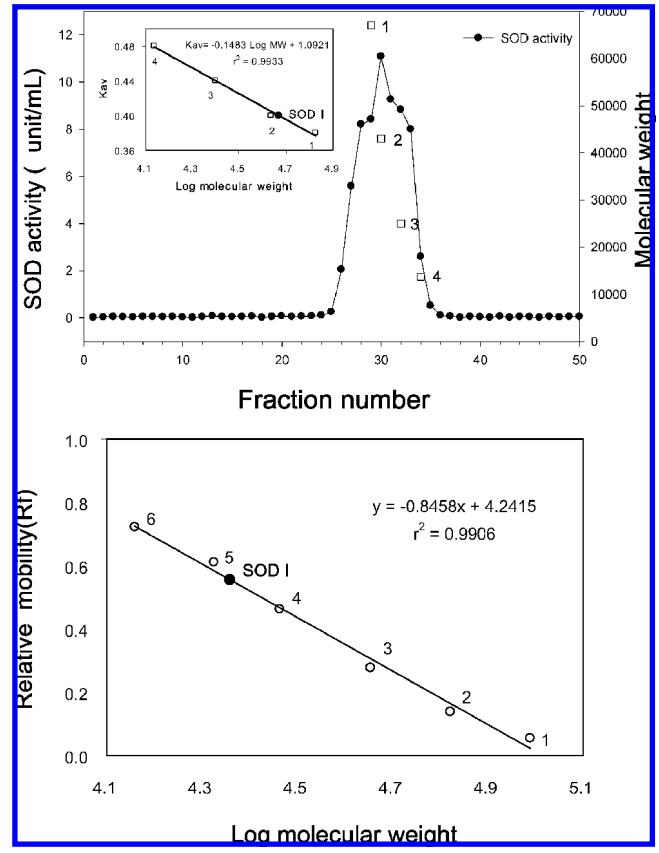

Figure 1. Determination of the molecular mass of SOD I from wheat seedlings by Superose 12 gel filtration (top) and SDS-PAGE (bottom). Top: 1−4 indicate protein standards with known molecular masses. Bottom: 1−6 indicate protein standards from a low molecular weight calibration kit.

from wheat kernel, pearl millet seedlings, maize, and corn germ, were reported to be a dimer or tetramer with a subunit molecular mass in the range of 18−35 kDa (*9, 14, 16, 17, 26–28*). For example, the MnSODs from wheat kernel and Norway spruce (*Picea abies* L. Karst) were tetramers with subunit molecular masses of 20 and 22 kDa, respectively (*17, 28*). The Cu/Zn SODs from corn germ and soybean were dimers with subunit molecular masses of 18−19 kDa (*26, 27*).

As shown in **Figure 2**, the CBR staining of the IEF gel showed several bands, indicating the enzyme preparation up to DEAE-Sephacel anion exchange step contained other proteins in addition to SOD. However, after activity staining of the IEF gel with substrate for SOD, only a single white unstained band against the blue background was observed (**Figure 2**). By comparing the relative mobility (R_f) of SOD with that of the p*I* markers developed in the same gel, the isoelectric point of the DEAE-Sephacel anion exchange purified SOD I from wheat seedlings was found to be 3.95, indicating it is an acidic SOD (**Figure 2**). This result is comparable to the results in the literature, where most of the p*I* values reported for plant SODs were in the range of 4.3−6.5 (*9, 14, 17, 26–30*).

Prosthetic groups of wheat seedling SOD were determined by native-PAGE followed by SOD activity staining in the presence of sodium cyanide or hydrogen peroxide (*15*). As shown in **Figure 3**, the crude enzyme extract and enzyme solutions from each purification steps (including heating, ammonium sulfate fractionation, and DEAE anionic exchange chromatography) are insensitive to KCN or hydrogen peroxide inhibitor, suggesting that both SODs I and II from wheat seedlings belong to the MnSOD category.

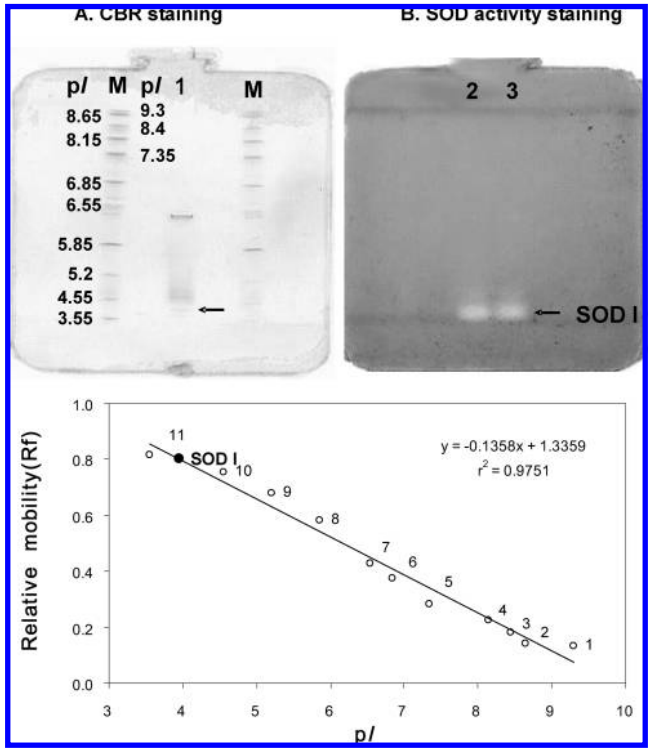

Figure 2. Determination of p*I* of SOD I from wheat seedlings by isoelectrofocusing electrophoresis. Top: Lanes M and 1 indicate Coomassie brilliant blue R (CBR) staining of p*I* marker proteins and purified SOD I. Lanes 2 and 3 indicate activity staining of purified SOD I. Bottom: 1−11 indicate standard proteins from a p*I* calibration kit.

MnSODs purified and characterized so far have been shown to have many similarities to one another. For example, the purified MnSOD from seedlings of pearl millet has a molecular mass of 35 kDa on SDS-PAGE and an isoelectric point of 4.3 (*9*). The MnSODs from wheat kernel (cv. Tonic) showed two closely related tetrameric isoenzymes with p*I* values of 6.0 and 6.1 and a molecular mass of 80 kDa (*17*). Similarly, the predominant MnSOD present in *Flammulina velutipes* was a tetramer with a monomer molecular mass of about 20 kDa. However, the p*I* values revealed three isoforms with p*I* values of 4.8, 5.0, and 5.3 (*14*). MnSOD from maize was a tetramer with a molecular mass of 90 kDa (*29*). The MnSOD from the seed of gymnosperm (*Pinus sylvestris* L.) was also a tetramer with a subunit molecular mass of 23 kDa and a p*I* of 6.5 (*30*).

Optimum pH and pH Stability. The effect of pH in the range of 2−12 on the activity of wheat seedling SOD I was examined (**Figure 4A**). It was found that within the pH range of 5−11, the crude enzyme solution was highly active, with >80% of relative activity remaining. In contrast, the purified SOD I is only highly active within a narrower pH range (pH 5−9). As shown in **Figure 4A**, both the crude extract and purified SOD I were rapidly inactivated at extreme pH, particularly for extreme acidic pH (pH 2 and 3), and to a lesser extent for extreme alkali pH (pH 12). The enzyme retained about 65−70% relative activity at pH 4 (**Figure 4A**). The pH optimum for both crude and purified wheat seedling SOD I occurred at pH 8. This result is comparable to the results in the literature, where the optimum pH values reported for the MnSOD from *Pisum sativum* leaf, pearl millet seedlings, mung bean, and corn were 8.6, 8, 7.8, and 7.8, respectively (*2, 9, 31, 32*).

The pH stability of wheat seedling SOD I was further investigated over the pH range of 4−11 (**Figure 4B**). The crude SOD extract of wheat seedlings was found to be stable after being held at pH 7−10 for 60 min. The residual SOD activity was >80%. Nevertheless, the residual SOD activity dropped to 55% after being held at pH 11 for 60 min and to 30−40% after being held at pH 5−6 for 60 min. SOD activity dropped to about 9% after being held at pH 4 for 15 min. As expected, the pH stability of purified SOD I was poorer than that of the crude enzyme. Only about 50−80% enzyme activity was retained after being held at pH 7−9 for 15 min. The enzyme activity decreased significantly after being held at pH conditions other than 7−9 for >15 min. The SOD residual activity was <40%. This finding is also comparable to those reported in the literature, where the MnSOD from pearl millet seedlings was reported to be stable over a pH range of 7−9 (*9*). The higher pH stability for SOD activity in the crude extract is possibly related to the presence of more pH stable isoforms or enzyme-stabilizing substances.

Optimum Temperature and Temperature Stability. The effect of temperature in the range of 5−80 °C on the activity of wheat seedling SOD I was examined (**Figure 5A**). Within the temperature range of 5−50 °C, both the crude and purified enzymes are highly active, with >90% of relative activity. The activity of wheat seedling SOD I dropped significantly as the temperature was >60−70 °C and was nearly fully inactivated at 80 °C (**Figure 5A**). The apparent temperature optimum for both crude and purified wheat seedling SOD I occurred at 5−50 °C. This result is comparable to the result reported for MnSOD from pearl millet seedlings, for which the temperature optimum

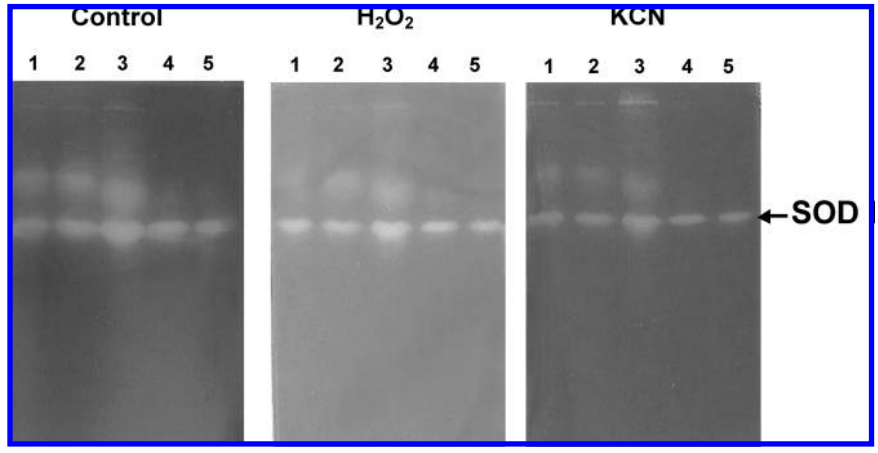

Figure 3. Identification of the prosthetic groups of SOD isozymes from wheat seedlings. Electrophoresis was performed in 12.5% native-PAGE gels. Lane 1 indicates crude enzyme; lane 2 indicates heat-treated SOD; lane 3 indicates ammonium sulfate fractionation purified SOD; lanes 4 and 5 indicate DEAE-Sephacel ion exchange purified SOD I. Control represents without inhibitor; H_2O_2 represents with addition of 8 mM H_2O_2, and KCN represents with the addition of 8 mM KCN.

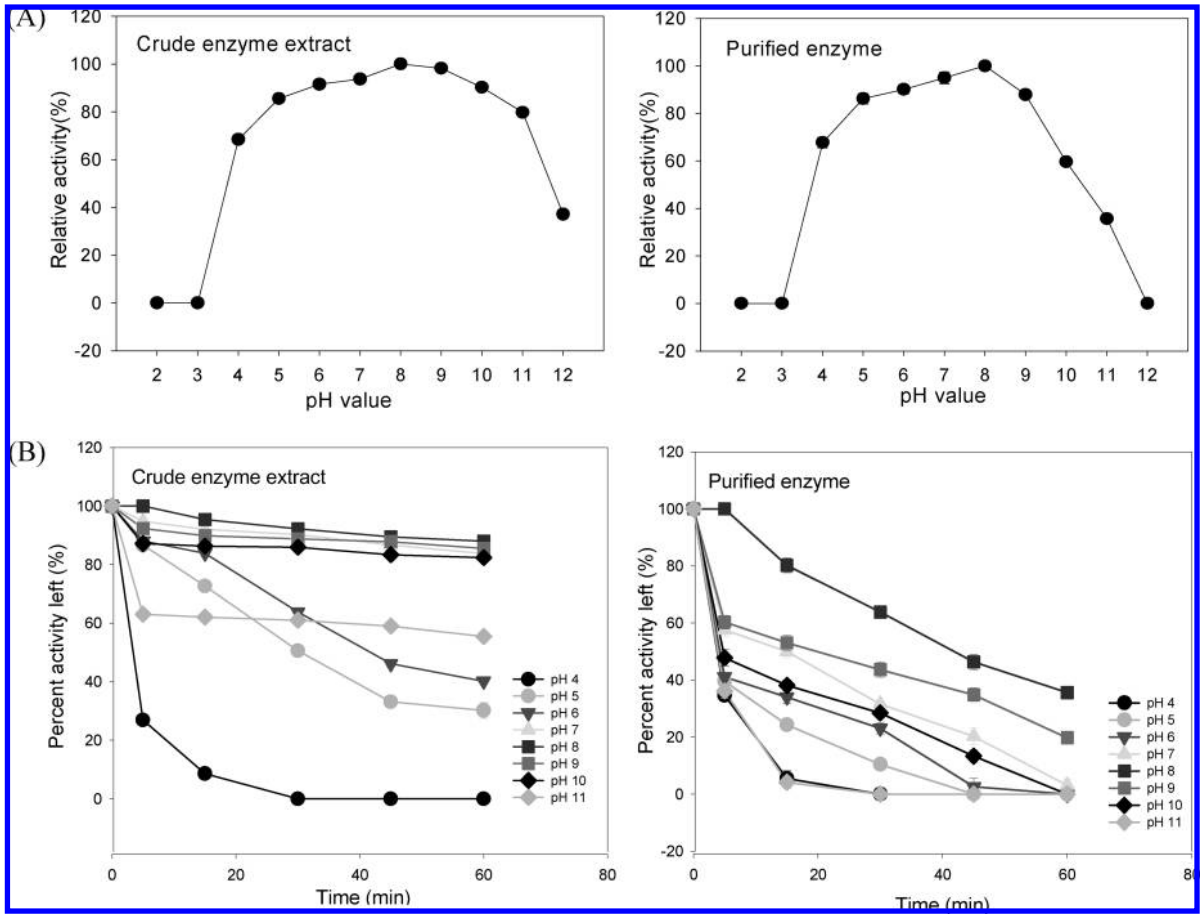

Figure 4. Effect of pH (**A**) and pH stability (**B**) of MnSOD from wheat seedlings.

occurred at 28 °C, but actually had broad temperature optima with not much activity change over 20−35 °C (*9*).

Figure 5B shows the temperature stability. It was found that the crude extract of wheat seedling SODs was thermally stable after holding at 20−40 °C for 60 min. The residual SOD activity was about 75−95%. Nevertheless, as the temperature was raised to 50 °C, the SOD activity declined. Holding times of 30 and 60 min resulted in residual SOD activities of 79 and 47%, respectively. At temperatures >60 °C, even a short holding time (5 min) resulted in a significant loss in enzyme activity. Similarly, the purified SOD I from wheat seedlings was thermally stable at 20−50 °C for 60 min. Approximately 69−82% enzyme activity was retained. However, as the temperature exceeded 60 °C, the enzyme activity decreased significantly, but the purified enzyme seemed to be more thermally stable than crude enzyme. For example, for a short holding (5 min) at 60 °C, the residual SOD activity in crude enzyme solution was <45%, in contrast to >80% for purified enzyme solution. Similarly, after 5 min at 70 °C, the residual SOD activity in crude enzyme solution dropped to about 30%, in contrast to >50% for purified enzyme solution. The stronger thermal stability for purified SOD I at mesothermal conditions is possibly related to the differences in the residual protease activity between the purified and crude enzyme solutions. The protease activity in the crude enzyme solution was found to be 23.43 units/mL, whereas that in the purified enzyme solution was negligible (1.09 units/mL). Therefore, under mesothermal conditions, SOD enzyme in crude enzyme solution was possibly degraded more by protease as compared to the purified enzyme solution, resulting in poorer thermal stability. The temperature stability reported for most plant MnSODs, such as wheat, *P. sativum* leaf, was also below 50−60 °C (*2, 9, 31, 32*).

The initial heat inactivation reaction of the crude and purified SOD isozymes was further simulated by the first-order kinetics as

$$A = A_0 \times e^{-kt} \quad (2)$$

$$t_{1/2} = \frac{-\ln 0.5}{k} \quad (3)$$

where A is the SOD activity after t (min) of heating at a given temperature, A_0 is the initial SOD activity, k is the reaction rate constant of first-order kinetic, and $t_{1/2}$ is the half-life of thermal inactivation of SOD. The reaction rate constant can be obtained from a plot of the natural log of activity versus time plot. The kinetic parameters of thermal inactivation of SOD I during heating are shown in **Table 2**. Results in **Table 2** imply that the thermal inactivation reaction of SOD I during initial thermostat stage followed first-order reaction kinetics, as the determination of correlation coefficients at various temperatures are all >0.92. The $t_{1/2}$ values varied from 12.84 to 2.14 h for crude enzyme extract at 20−40 °C and from 0.89 to 0.04 h at 50−70 °C. The $t_{1/2}$ values varied from 3.50 to 2.57 h for purified enzyme at 20−40 °C and but from 1.99 to 0.07 h at 50−70 °C, which was slightly higher than those for crude enzyme extract.

The temperature dependence of rate constants was further simulated with the Arrhenius equation as

$$k = k_0 \times e^{-E_a/RT} \quad (4)$$

where k_0 is the frequency factor (per minute), E_a is the activation energy (kJ/mol), R is the universal gas constant (8.314 J/mol·K),

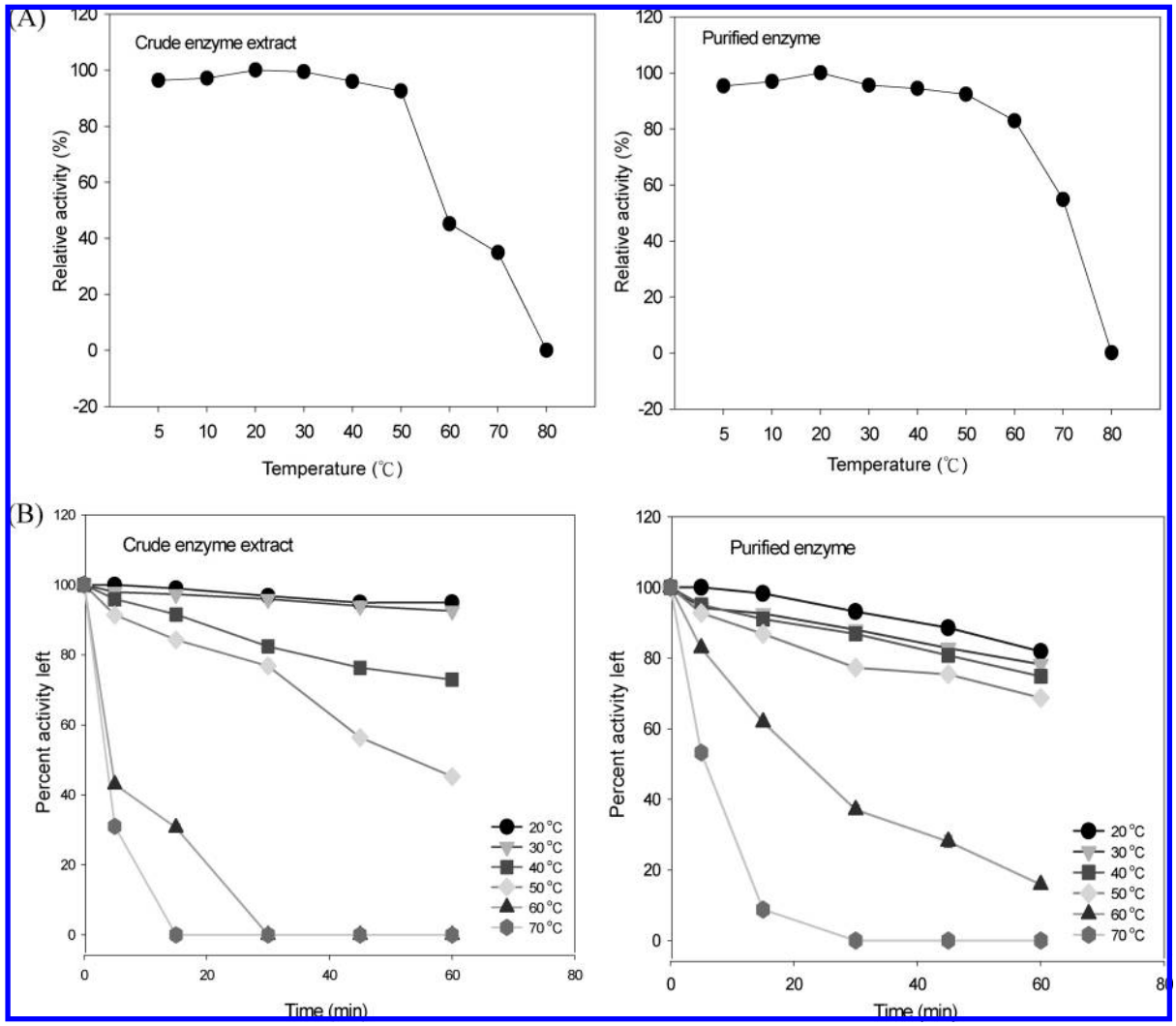

Figure 5. Effect of temperature (**A**) and heating time (**B**) on the activity of MnSOD from wheat seedlings.

Table 2. Rate Constants and Half-Lives of Thermal Inactivation Reaction of Wheat Seedlings MnSOD under Various Temperatures

	crude enzyme extract			purified enzyme		
temp (°C)	k (min^{-1})	$t_{1/2}$ (h)	r^2	k (min^{-1})	$t_{1/2}$ (h)	r^2
20	9.0×10^{-4}	12.84	0.9640	3.3×10^{-3}	3.50	0.9661
30	1.2×10^{-3}	9.63	0.9725	3.8×10^{-3}	3.04	0.9795
40	5.4×10^{-3}	2.14	0.9853	4.5×10^{-3}	2.57	0.9887
50	1.3×10^{-2}	0.89	0.9682	5.8×10^{-3}	1.99	0.9574
60	1.5×10^{-1}	0.08	0.9217	3.0×10^{-2}	0.39	0.9934
70	3.1×10^{-1}	0.04	0.9926	1.6×10^{-1}	0.07	0.9975

and T is the absolute temperature (K). As shown in **Figure 6**, the Arrhenius plot for both crude and purified enzymes showed two distinct regions. At higher temperature range (50−70 °C), activation energy (E_a) determined from the slope of Arrhenius plots for thermal inactivation of SOD I was 150 kJ/mol for both crude and purified SOD I (**Figure 6**). However, at lower temperature range (20−50 °C), the heat activation energy (E_a) for enzyme inactivation was 74 and 15 kJ/mol for crude and purified SOD I, respectively (**Figure 6**). These results are consistent with the behavior of wheat kernel MnSOD, which also showed lower activation energy after purification (*17*). The higher activation energy for SOD inactivation in the crude extract at lower temperature range indicated the presence of more thermostable isoenzymes or enzyme-stabilizing substances. Robinson et al. (*17*) further analyzed the thermal inactivation of wheat kernel MnSOD by using the Eyring equation

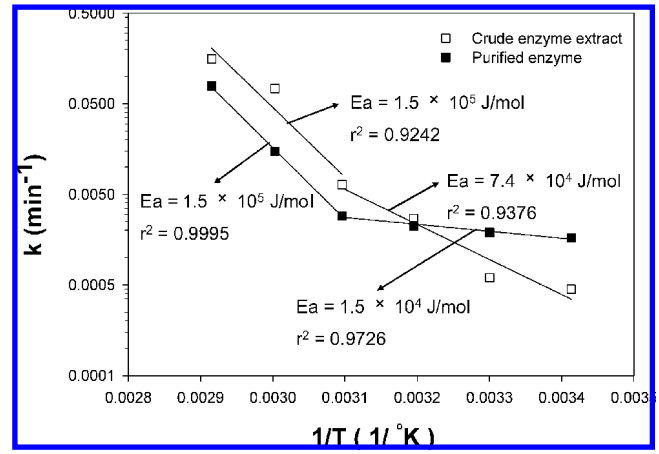

Figure 6. Arrhenius plots for thermal inactivation of MnSOD from wheat seedlings.

and concluded that the lower enthalpy and negative entropy of purified SOD indicated that a simple heat-induced chemical reaction such as release of an essential metal may be involved for inactivation of enzymes and that aggregation of molecules may occur (*17*). Therefore, we believe that the lower activation energy in the lower temperature region may indicate that a simple chemical release of the bound metal may have been responsible for the observed inactivation. However, as the temperature is raised, protein unfolding is probably the rate-limiting step for thermal inactivation,

Table 3. Effect of Metal Ions and Various Compounds on the Activity of Wheat Seedling MnSOD

reagent	relative activity[a] (%) 0.1 mM	1.0 mM
blank	100	100
KCN	122.0 ± 2.7D	95.0 ± 1.9D
H_2O_2	143.0 ± 2.8B	100.0 ± 1.2C
$CaCl_2$	112.0 ± 2.8E	108.0 ± 1.2B
$NaNO_2$	130.0 ± 2.7C	116.0 ± 1.9A
$ZnCl_2$	100.0 ± 1.6F	77.0 ± 2.5F
$MgCl_2$	112.0 ± 2.7E	91.0 ± 1.2E
$HgCl_2$	87.0 ± 2.3G	27.0 ± 1.2G
SDS	148.0 ± 2.1A	5.0 ± 1.2H
EDTA-2Na	128.0 ± 1.3C	78.0 ± 1.9F

[a] Data are presented as mean ± standard deviation ($n = 3$). Means with different letters within the same column differ significantly ($p < 0.05$).

Table 4. Effect of Various Chemical Modification Reagents on the Activity of Wheat Seedling MnSOD

chemical modification reagent[a]	concn (mM)	rel activity[b] (%)
blank		100
NBS	0.5	89.0 ± 1.5B
NBS	5.0	51.0 ± 2.4E
EAM	0.25	82.0 ± 2.3C
NAI	2.5	72.0 ± 1.8D
DNFB	2.5	51.0 ± 1.3E
PMSF	2.5	97.0 ± 1.3A
DEPC	2.5	12.0 ± 0.8F
WRK	50	5.0 ± 2.6G
*p*HMB	0.5	90.0 ± 1.0B
*p*HMB	1.0	73.0 ± 1.1D

[a] NBS, *N*-bromosuccinimide; EAM, ethyl acetimidate; NAI, *N*-acetylimidazole; DNFB, 2,4-dinitro-1-fluorobenzene; PMSF, phenylmethanesulfonyl fluoride; DEPC, diethyl pyrocarbonate; WRK (Woodward's reagent K), *N*-ethyl-5-phenylisoxazolium-3′-sulfonate; *p*-HMB, *p*-hydroxymercuribenzoic acid. [b] Data are presented as mean ± standard deviation ($n = 3$). Means with different letters within the same column differ significantly ($p < 0.05$).

which would require higher energy. Such a conclusion depends on the validity of the assumed straight-line relationship in the Arrhenius plot (**Figure 6**).

Effect of Metal Ions and Various Compounds. As shown in **Table 3**, KCN, H_2O_2, $CaCl_2$, $NaNO_2$, $MgCl_2$, SDS, and EDTA-2Na at concentrations of 0.1 mM enhanced wheat seedling SOD I activity. In contrast, $HgCl_2$ and SDS at a concentration of 1.0 mM inhibited wheat seedling SOD I activity significantly. Many plant SODs thus far investigated have been shown to possess Cu, Zn, Fe, or Mn as a prosthetic group (metal cofactor requirement), which would mainly facilitate the electron transfer during the oxidation−reduction reaction. The presence of other metal ions or compounds could possibly modify the affinity between SOD and substrates by influencing the conformation of SOD through electron pairs and electrostatic force, which result in change in immobilization of the reaction groups. It is also interesting that a low concentration of SDS (0.1 mM) can enhance the SOD I activity from wheat seedlings. However, the high concentration of SDS (1.0 mM) inhibited SOD I activity significantly. Such results suggest that an appropriate amount of SDS could enhance the affinity between SOD I and substrates, possibly by changing the electron density of SOD. However, excess SDS may induce the denaturation or deassociation of SOD I, resulting in significant reduction of enzyme activity.

Effect of Chemical Modification Reagents. It is generally believed that if the amino acid side chain involved in the catalytic activity is chemically modified, the enzyme will be inhibited or inactivated. In this study, we used a number of reagents to modify the purified SOD I from wheat seedlings (**Table 4**). Among the chemical modification reagents tested, diethyl pyrocarbonate (2.5 mM) and Woodward's reagent K (50 mM) significantly inhibited the activity of the enzyme. Therefore, imidazole (histidine) and carboxyl (aspartic and glutamic acid) groups are probably located at or near the active site of the enzyme. However, it should be noted that the activity of SOD I from wheat seedlings was also inhibited 49% by 5 mM NBS and 2.5 mM DNFB. Therefore, lysine, tyrosine, tryptophan, and cysteine residues may also be related to the enzyme activity. These residues are possibly not essential for catalytic reaction, but instead involved only in maintenance of the conformation of the enzyme.

Results from this study imply that during development of functional foods containing wheat seedlings as a natural food antioxidant or functional ingredient, low-temperature processing and low-acid environment should be applied to preserve the SOD activity. In addition, the yield of another SOD in wheat seedlings (SOD II) is about one-third that of SOD I after DEAE purification. It is also a MnSOD. To have a profound understanding of the application potential of wheat seedlings as functional food supplements (*33*) or as a source of therapeutic agent for inflammation (*34, 35*), it would be interesting to determine the differences in biochemical characteristics between these two MnSODs.

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