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Catalytic Characteristics of Peroxidase from Wheat Grass

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The crude enzyme extract of wheat grass was heated at 60 °C for 30 min, followed by ammonium sulfate fractionation and isoelectric chromatofocusing on Polybuffer exchanger (PBE 94) for purification. The purified peroxidase was then characterized for its catalytic characteristics. It was found that $AgNO_3$ at a concentration of 0.25 mM and $MnSO_4$ and EDTA at concentrations of 5 mM significantly inhibited the activity of wheat grass peroxidase. However, KCl, NaCl, $CuCl_2$, $CaCl_2$, $ZnCl_2$, and $MgCl_2$ at concentrations of 5.0 mM and $HgCl_2$ at a concentration of 0.25 mM enhanced enzyme activity. Chemical modification significantly influenced the activity of wheat grass peroxidase. Particularly, *N*-bromosuccinimide (5 mM) inhibited 16% of the enzyme activity, whereas *N*-acetylimidazole (2.5 mM), diethyl pyrocarbonate (2.5 mM), and phenylmethanesulfonyl fluoride (2.5 mM) enhanced by 18−29% of the enzyme activity. Such results implied that tryptophan, histidine, tyrosine, and serine residues are related to enzyme activity. The pH optima for wheat grass peroxidase to catalyze the oxidation of *o*-phenylenediamine (OPD), catechol, pyrogallol, and guaiacol were 5.0, 4.5, 6.5, and 5.0, respectively. The apparent K_m values for OPD, catechol, pyrogallol, and guaiacol were 2.9, 18.2, 2.5, and 3.8 mM, respectively. Under optimal reaction conditions, wheat grass peroxidase catalyzed the oxidation of OPD (an aromatic amine substrate) 3−11 times more rapidly than guaiacol, catechol, and pyrogallol (phenolic substrates containing one to three hydroxy groups in the benzene ring).

KEYWORDS: Wheat grass peroxidase; catalytic characteristics; chemical modification reagents; metal ions; substrate specificity

INTRODUCTION

Wheat grass is the tender small plant grown from wheat seed (*Triticum aestivum* L.). It contains significant amounts of protein, vitamins, minerals, phenolic compounds, and flavonoids (*1*−*3*). Phenolic compounds and flavonoids have been shown to have the capability of scavenging various free radicals such as $^{\bullet}O_2^-$ or $^{\bullet}HO_2$ and can significantly reduce the oxidative stress for living cells (*4*, *5*). Therefore, wheat grass juice has received a lot of interest recently as a vitality drink.

Peroxidase (POD; EC 1.11.1.7) is an oxidoreductase that catalyzes the oxidation of various electron donor substrates (such as phenols and aromatic amines) with H_2O_2 and carries a b-type heme as a prosthetic group (*6*). Peroxidase is widely distributed in fruits and vegetables. It often contributes to deteriorative changes in flavor, color, texture, and mouthfeel in raw and processed fruits and vegetables (*7*−*13*). However, peroxidase is also involved in adjusting and controlling the biosynthesis of plant growth hormone, serving as a blanching indicator due to its thermal stability under limited heat treatment, and is widely employed in microanalysis due to its ability to yield chromogenic products at low concentrations and its relatively good stability (*7*−*13*). In a study of the processing of wheat grass juice, Shih (*14*) reported that significant residual activity of peroxidase was detected in the wheat grass juice extracted from steam-blanched wheat grass. Such results implied the presence of thermally resistant peroxidase. In our previous study (*15*), we have reported that the molecular mass of the predominant peroxidase from wheat grass was about 23 kDa and consisted of two isoforms with isoelectric points of 8.88 and 8.43, respectively (*15*). As OPD was used as the hydrogen-donor substrate, the pH and temperature of optimum activity were 5.0 and 30 °C, respectively. The thermal inactivation reaction of wheat grass peroxidase followed the first-order reaction kinetics, and the temperature dependence of the rate constants was in agreement with the Arrhenius equation with an activation energy value of 3.1×10^4 J/mol (*15*). The objective of this study is to further characterize the catalytic characteristics of the purified predominant peroxidase from wheat grass. Specifically, the effect of metal ions and salts, chemical modification reagents, and buffer pH and concentration, as well as apparent Michaelis constants and substrate specificity for wheat grass peroxidase, would be evaluated.

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Table 1. Corresponding Solvents, Reaction Buffers, and Possible Amino Acid Residues Modified for Various Chemical Modification Reagents

chemical modification reagent[a]	solvent	reaction buffer	possible amino acid residues modified	ref
NBS	water	0.1 M acetate buffer, pH 4.0	Cys, His, Trp, Tyr	Viswanatha and Lanwson (*17*)
EAM	water	0.01 M phosphate buffer, pH 7.0	Lys	Shaw and Chang (*18*)
NAI	water	0.025 M phosphate buffer, pH 7.0	Tyr	Yamasak et al. (*19*)
DNFB	DMSO[b]	0.2 M phosphate buffer, pH 8.0	Lys	Welches and Baldwin (*20*)
PMSF	DMSO[b]	0.2 M phosphate buffer, pH 8.0	Ser	Gold and Fahrney (*21*)
DEPC	absolute alcohol	0.06 M phosphate buffer, pH 8.0	His	Miles (*22*)
WRK	water	0.5 M phosphate buffer, pH 6.0	Asp, Glu	Vangrysperre et al. (*23*)
PHMB	water	0.05 M imidazole buffer, pH 7.0	Cys	Liu and Speer (*24*)

[a] NBS, *N*-bromosuccinimide; EAM, ethyl acetimidate; NAI, *N*-acetylimidazole; DNFB, 2,4-dinitro-1-fluorobenzene; PMSF, phenylmethanesulfonyl fluoride; DEPC, diethyl pyrocarbonate ; WRK, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K); PDMB, *p*-hydroxymercuribenzoic acid. [b] DMSO, dimethyl sulfoxide.

MATERIALS AND METHODS

Wheat Grass. Wheat seeds (*T. aestivum* L. Nungryuan 2) were purchased from a contracted farmer in Taichung, Taiwan. The wheat seeds were then planted to obtain wheat grass according to the method of Wang et al. (*15*). Wheat seeds (200 g) were first soaked in distilled water (24−25 °C, 2000 mL) for 5 h. The steeped seeds were then covered with a moistened cloth to promote the onset of germination for 24 h (120 mL of water was sprayed onto the cloth every 8 h to keep the cloth moistened). The seeds were then planted onto 500 g of cultivation soil (Finnpeat, Code AA, Kekkilä, Tussla, Finland) (with a maximum particle of 65 mm, pH 5.5−6.0, electric conductivity, EC, ≥ 0.3 mS/cm press extract) in an air-conditioned room (23 ± 2 °C), and watered (120 mL) every 12 h to keep the soil moistened. The wheat grass was cut when its height reached about 10−12 cm.

Crude Enzyme Extract. Crude enzyme extract of wheat grass was obtained by using a grass juice extractor (Green Power, KP-E1201, Kempo Co. Ltd., Seoul, Korea), then centrifuged (CR20B2, Hitachi, Tokyo, Japan) at 16300*g* and 4 °C for 15 min, and filtered (Whatman no. 4 filter paper, Whatman International Ltd., Maidstone, U.K.). Soluble solids 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 solids content of the supernatant was adjusted to 5 °Brix by the addition of deionized water.

Measurement of Peroxidase Activity. Peroxidase activity was measured at 30 °C using *o*-phenylenediamine (OPD) as a hydrogen-donor substrate (*15*). The reaction mixture contained 0.8 mL of 10 mM OPD, 0.8 mL of 2.5 mM H_2O_2, and 1 mL of 0.1 M citrate−phosphate buffer (pH 5.0). After 5 min of incubation at 30 °C, 0.15 mL of enzyme solution with appropriate dilution was added; 0.05 mL of 4 N HCl was added to stop the reaction after 10 min of reaction. The absorbance at 445 nm was then determined. The amount of oxidative OPD produced was calculated from the increase in absorbance by using an extinction coefficient (ϵ) of 11.1 mM^{-1} cm^{-1}. One enzyme activity unit was defined as the amount of enzyme required to produce 1 μmol of oxidative OPD per minute.

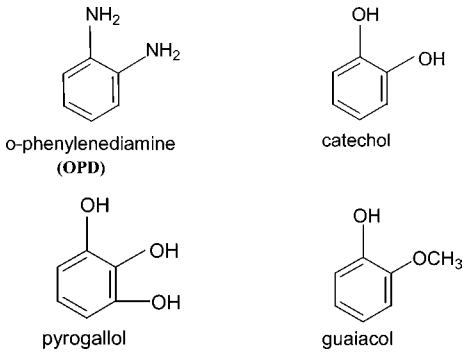
Protein Determination. Protein content was quantified by using bicinchoninic acid (BCA) and the micro BCA protein assay kit (Pierce Biotechnology Inc., Boston, MA) according to the method of Smith et al. (*16*). Bovine serum albumin (BSA) was used as protein standard.

Purification of Peroxidase. The crude enzyme extract was purified according to the method of Wang et al. (*15*). Crude enzyme extract was first heated at 60 °C for 30 min and then centrifuged at 16500*g* and 4 °C for 20 min to remove the precipitate. The supernatant was then added with 50% saturation of ammonium sulfate and centrifuged at 16500*g* (20 min at 4 °C) to remove the precipitate. The supernatant was then added with 70% saturation of ammonium sulfate and centrifuged at 16500*g* (20 min at 4 °C) to collect the precipitate. The precipitate obtained from 50−70% saturation of ammonium sulfate was then dissolved with 0.025 M of imidazole−HCl buffer (pH 7.4) and centrifuged at 16500*g* (20 min at 4 °C). The supernatant was 10 times concentrated by using an Amicon Ultra-15 ultrafiltration unit with a 10 kDa molecular weight cutoff (MWCO) membrane (Millipore Co., Bedford, MA). The enzyme solution was eluted by using isoelectric chromatofocusing on a Polybuffer exchanger (PBE 94) column (1.0 × 20 cm, V_t = 11.87 mL) (Amersham Pharmacia Biotech A, Uppsala, Sweden) in a pH gradient between 7.4 and 4.0. The column was previously equilibrated with 0.025 M imidazole−HCl buffer (pH 7.4) using a flow rate of 30 mL/h. One milliliter of the enzyme solution was applied to the column and eluted with 0.025 M imidazole−HCl buffer (pH 7.4). Samples were collected every 3 mL for 15 fractions, and then a Polybuffer 74−HCl buffer (pH 4.0) was applied to the column to form a pH gradient between 7.4 and 4.0. Samples were collected every 3 mL for another 75 fractions. Fractions containing higher peroxidase activity were pooled and dialyzed against 0.025 M ethanolamine−CH_3COOH buffer (pH 9.6) for 24 h using a snake skin pleated dialysis tubing with a MWCO of 10 kDa (Pierce Biotechnology Inc.). The dialyzed sample was first concentrated by using an Amicon Ultra-15 ultrafiltration unit with a 10 kDa MWCO membrane (Millipore Co.) and then eluted by using isoelectric chromatofocusing on another Polybuffer exchanger (PBE 94) column (1.0 × 20 cm, V_t = 11.87 mL) (Amersham Pharmacia Biotech A) in a pH gradient between 9.6 and 6.0. Similarly, the column was previously equilibrated with 0.025 M ethanolamine−CH_3COOH buffer (pH 9.6) using a flow rate of 30 mL/h. One milliliter of the enzyme solution was applied to the column and eluted with 0.025 M ethanolamine−CH_3COOH buffer (pH 9.6). Samples were collected every 3 mL for 15 fractions, and then a Polybuffer 96−CH_3COOH buffer (pH 6.0) was applied to the column to form a pH gradient between 9.6 and 6.0. Samples were collected every 3 mL for another 75 fractions. Fractions containing peroxidase activity were then pooled. After the consecutive purification steps, about 87 purification fold was achieved (*15*). The purified enzyme contained predominantly two distinct cationic peroxidase isofroms with isoelectric points of 8.88 and 8.43, respectively, and a negligible trace amount of anionic peroxidase isoforms with isoelectric points between 6 and 7, as analyzed by isoelectrofocusing electrophoresis and peroxidase activity staining (*15*). Due to the low yield (4.8%) and difficulty of isoform separation, the purified peroxidase described above without further purification was used for catalytic properties characterization.

Effcet of Metal Ions and Salts. The purified peroxidase (0.05 mL) was incubated with 0.05 mL of 0.5 or 10 mM of various salts ($AgNO_3$, $HgCl_2$, KCl, NaCl, $CaCl_2$, $CuCl_2$, $MgCl_2$, $ZnCl_2$, $MnSO_4$, and EDTA) or deionized water as a control at 25 °C for 30 min. The enzyme activity was then measured. Results were expressed as the relative activity percentage calculated by the ratio of the specific activity of the purified peroxidase with metal ions or salts to that without metal ions and salts.

Effect of Chemical Modification Reagents. The purified peroxidase (0.05 mL) was incubated with 0.1 mL of the corresponding reaction buffer shown in **Table 1** (*17*−*24*) and 0.05 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 (PHMB, 2 mM) at 25 °C for 30 min. The enzyme activity was then measured. Results were expressed as the relative activity percentage calculated by the ratio of the specific activity of the purified peroxidase with chemical modification reagents to that without chemical modification reagents.

Effect of Buffer pH for Different Hydrogen-Donor Substrates. Peroxidase activity was measured at 30 °C. The reaction mixture contained 0.8 mL of various hydrogen-donor substrates (including OPD, catechol, pyrogallol, and guaiacol), 0.8 mL of H_2O_2, and 1 mL of



100 mM of citrate−phosphate buffer at various pH values. After 5 min of incubation at 30 °C, 0.15 mL of enzyme solution with appropriate dilution was added; 0.05 mL of 4 N HCl was added to stop the reaction after 10 min of reaction. The absorbance at 445, 295, 420, and 470 nm was measured for OPD, catechol, pyrogallol, and guaiacol, respectively. Values of extinction coefficient are 11.1, 1.7, 2.64, and 5.2 mM^{-1} cm^{-1} for OPD, catechol, pyrogallol, and guaiacol, respectively. One enzyme activity unit was defined as the amount of enzyme required to produce 1 μmol of oxidizing product per minute under standard conditions.

Effect of Buffer Concentration for Different Hydrogen-Donor Substrates. Peroxidase activity was measured at 30 °C. The reaction mixture contained 0.8 mL of various hydrogen-donor substrates, 0.8 mL of H_2O_2, and 1 mL of various concentrations of citrate−phosphate buffer at the optimum pH for each particular hydrogen-donor substrate determined as in the previous section. Peroxidase activity was measured according the steps mentioned in the previous section.

Determination of Apparent Michaelis Constants and Substrate Specificity. When the apparent Michaelis constants were studied, the peroxidase activity was measured under the optimal buffer pH and concentration determined as in the previous sections. The apparent Michaelis constant (K'_m) and apparent maximal velocity (V'_{max}) of the peroxidation of various hydrogen donors (AH) and H_2O_2 by the purified peroxidase were determined at various substrate concentration ranges. The saturation concentration for each substrate was first determined. The apparent K_m and V_{max} of each hydrogen-donor substrate and H_2O_2 were calculated using the Lineweaver−Burk (*25*) reciprocal plot graphic method for the two-substrate ping-pong mechanism followed by peroxidase (*26*, *27*). A general equation for this mechanism in the forward direction was given by Whitaker (*28*)

$$\frac{1}{V_0} = \frac{1}{V_{max}} + \frac{K_a}{A_0 V_{max}} + \frac{K_b}{B_0 V_{max}} \quad (1)$$

where V_{max} = maximum velocity, $K_a = K_m$ for substrate A, $K_b = K_m$ for substrate B (H-donor), A_0 = concentration of substrate A, and B_0 = concentration of substrate B. Under constant A_0, eq 1 will yield

$$\frac{1}{V_0} = \frac{K_b}{V_{max}}\left(\frac{1}{B_0}\right) + \left(1 + \frac{K_a}{A_0}\right)\frac{1}{V_{max}} \quad (2)$$

Therefore, the plot of $1/V_0$ versus $1/B_0$ will produce a straight line. The *y*-intercept of the line will yield

$$\frac{1}{V_0} = \left(1 + \frac{K_a}{A_0}\right)\left(\frac{1}{V_{max}}\right) = \frac{1}{V'_{max}} \quad (3)$$

Table 2. Effect of Metal Ions and Various Chemical Compounds on the Activity of Wheat Grass Peroxidase

chemical	concn (mM)	rel activity[a] (%)
none		100.0 ± 1.0 f
KCl	5.0	146.0 ± 1.9 c
NaCl	5.0	144.0 ± 0.7 c
$CuCl_2$	5.0	166.0 ± 2.6 a
$CaCl_2$	5.0	164.0 ± 2.8 a
$ZnCl_2$	5.0	110.0 ± 0.7 e
$MgCl_2$	5.0	150.0 ± 0.3 b
$HgCl_2$	0.25	137.0 ± 2.9 d
$AgNO_3$	0.25	69.0 ± 2.4 h
$MnSO_4$	5.0	77.0 ± 2.2 g
EDTA[b]	5.0	41.0 ± 0.7 i

[a] Enzyme activity was determined using OPD as a hydrogen-donor substrate. Data are expressed as the mean ± standard deviation ($n = 3$). Means with different letters differ significantly ($p < 0.05$). [b] EDTA, ethylenediaminetetraacetic acid disodium salt.

where V'_{max} is the apparent maximum velocity of substrate B. The *x*-intercept of the line will yield

$$\frac{1}{B_0} = \frac{-\left(1 + \frac{K_a}{A_0}\right)}{K_b} = \frac{-1}{K'_b} \quad (4)$$

where K'_b is the apparent K_m for substrate B. Similarly, under constant B_0, V'_{max} and K'_a for substrate A can be obtained from the *y*-intercept and *x*-intercept of the plot of $1/V_0$ versus $1/A_0$.

In the study of the substrate specificity, the peroxidase activity was measured under optimal conditions (including buffer pH, buffer concentration, hydrogen-donor concentration, and H_2O_2 concentration) determined for each hydrogen-donor substrate.

Statistical Analysis. All data were expressed as mean ± standard deviation. Analysis of variance was performed by ANOVA procedures. Duncan's new multiple-range test was used to determine the difference of means, and $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of Metal Ions and Salts. As shown in **Table 2**, $AgNO_3$ at a concentration of 0.25 mM and $MnSO_4$ and EDTA at concentrations of 5 mM significantly inhibited the peroxidase activity. In contrast, KCl, NaCl, $CuCl_2$, $CaCl_2$, $ZnCl_2$, and $MgCl_2$ at concentrations of 5.0 mM and $HgCl_2$ at a concentration of 0.25 mM enhanced the peroxidase activity. Many plant peroxidases thus far investigated have all been shown to possess ferriprotoporphyrin III (hematin) as a prosthetic group. They are iron-containing enzymes. Appropriate metal ions or salts could therefore possibly enhance the affinity between peroxidase and substrates and immobilize the reaction group through electron pairs or electrostatic force. For example, turnip peroxidase activity was enhanced by the presence of calcium and magnesium ions but inhibited by the presence of sodium, potassium, or manganese ions or EDTA (*29*, *30*). Peroxidase from buckwheat was enhanced by the presence of ammonium ion (*31*). In peroxidase isozyme PC3 from *Pleargonium grareolense* (*32*), peroxidase activity was enhanced 7-fold by imidazole and 4-fold by adenine when *o*-dianisidine was used as a substrate.

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 peroxidase from wheat grass. Among the chemical modification reagents tested, it was found

Table 3. Effect of Various Chemical Modification Reagents on the Activity of Wheat Grass Peroxidase[a]

chemical modification reagent[b]	concn (mM)	rel activity (%)
none		100.0 ± 1.6 g
NBS	0.5	109.0 ± 0.8 d
NBS	5.0	84.0 ± 1.2 i
EAM	0.25	104.0 ± 0.2 f
NAI	2.5	121.0 ± 1.2 b
DNFB	2.5	110.0 ± 0.2 d
PMSF	2.5	118.0 ± 1.3 c
DEPC	2.5	129.0 ± 1.7 a
WRK	50.0	91.0 ± 0.5 h
PHMB	0.5	106.0 ± 1.0 e

[a] Enzyme activity was determined using OPD as a hydrogen-donor substrate. Data are expressed as the mean ± standard deviation ($n = 3$). Means with different letters differ significantly ($p < 0.05$). [b] See **Table 1** for abbreviations.

Table 4. Effect of Citrate–Phosphate Buffer pH on the Percentage Activity of Wheat Grass Peroxidase for Different Hydrogen-Donor Substrates at 30 °C

buffer pH	rel activity[a] (%)			
	OPD[b]	catechol[c]	pyrogallol[d]	guaiacol[e]
2.6	0.39 ± 0.89 g			0.94 ± 1.63 f
3.0	0.97 ± 0.67 g		1.04 ± 0.90 g	1.89 ± 1.63 f
3.5	4.84 ± 2.35 g		7.04 ± 1.41 f	3.77 ± 1.63 f
4.0	22.63 ± 3.07 f	43.23 ± 4.57 b	6.22 ± 0.01 f	35.85 ± 3.27 d
4.5	71.18 ± 3.20 c	100.00 ± 2.23 a	20.21 ± 0.01 e	83.02 ± 3.27 b
5.0	100.00 ± 1.87 a	47.42 ± 4.84 b	66.67 ± 0.81 c	100.00 ± 3.27 a
5.5	96.32 ± 2.32 a	42.58 ± 1.68 b	67.61 ± 1.41 c	87.74 ± 2.83 b
6.0	80.08 ± 4.06 b	18.06 ± 3.40 c	85.45 ± 0.81 b	77.36 ± 4.32 c
6.5	55.51 ± 2.42 d		100.00 ± 0.01 a	38.68 ± 1.63 d
7.0	39.85 ± 4.12 e		87.79 ± 2.15 b	21.70 ± 4.32 e
7.5	23.40 ± 4.39 f		46.48 ± 4.88 d	5.66 ± 2.83 f

[a] Data are expressed as mean ± standard deviation ($n = 3$). Means with different letters within the same column differ significantly ($p < 0.05$). [b] [OPD] = 3.49 mM, [H_2O_2] = 0.87 mM, [buffer] = 36.36 mM in the reaction mixture. [c] [Catechol] = 25.43 mM, [H_2O_2] = 5.08 mM, [buffer] = 36.36 mM in the reaction mixture. [d] [Pyrogallol] = 5.59 mM, [H_2O_2] = 4.07 mM, [buffer] = 36.36 mM in the reaction mixture. [e] [Guaiacol] = 5.82 mM, [H_2O_2] = 5.82 mM, [buffer] = 36.36 mM in the reaction mixture.

that NBS (5 mM) inhibited the enzyme activity (16%) most significantly, followed by WRK (9%) to a lesser extent (**Table 3**). Whereas NAI (2.5 mM), DEPC (2.5 mM), and PMSF (2.5 mM) enhanced by 18−29% of the enzyme activity, PHMB (0.5 mM) did not inhibit enzyme activity. Because NBS is a protein-oxidizing agent that is particularly specific for tryptophan and the SH group, NAI and DEPC could modify tyrosine and histidine, and PHMB is a mercaptide-forming agent that can react with enzymes containing SH groups and subsequently inhibit their activity, we believed that tryptophan should be related to the activity of peroxidase from wheat grass. However, it should be noted that the activity of peroxidase from wheat grass was not completely inhibited by 5 mM NBS and enhanced by 2.5 mM DEPC, NAI, and PMSF. Therefore, histidine, tyrosine, and serine residues should be also possibly 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.

Effect of Buffer pH and Concentration for Different Hydrogen-Donor Substrate. Peroxidase catalyzes the oxidation of various hydrogen-donor substrates, such as OPD, catechol, pyrogallol, and guaiacol, by hydrogen peroxide. Because the optimum conditions for different hydrogen-donor substrates are not necessarily identical, we first determined the pH optimum for wheat grass peroxidase to catalyze the oxidation of each particular hydrogen donor substrates. As shown in **Table 4**, the pH optima for OPD, catechol, pyrogallol, and guaiacol were 5.0, 4.5, 6.5, and 5.0, respectively. Interestingly, as OPD, pyrogallol, or guaiacol was used as the hydrogen-donor substrate, reasonable peroxidase activity could be obtained within a wider buffer pH range. However, as catechol was used as the hydrogen-donor substrate, peroxidase activity decreased significantly as the buffer pH deviated from 4.5.

Table 5. Effect of Citrate–Phosphate Buffer Concentration on the Percentage Activity of Wheat Grass Peroxidase for Different Hydrogen-Donor Substrates at 30 °C

buffer concn (mM)	rel activity[a] (%)			
	OPD[b]	catechol[c]	pyrogallol[d]	guaiacol[e]
3.64	100.00 ± 1.15 a	31.11 ± 3.47 i	100.00 ± 2.45 a	100.00 ± 4.03 a
7.27	95.23 ± 4.15 ab	39.44 ± 4.19 h	96.26 ± 1.60 a	76.74 ± 0.01 b
10.91	91.21 ± 3.77 bc	52.78 ± 3.47 g	81.82 ± 4.81 bcd	74.42 ± 4.03 bc
14.55	89.95 ± 1.15 bcd	75.00 ± 1.67 ef	78.61 ± 3.21 cd	65.12 ± 4.03 de
18.18	89.70 ± 3.02 bcde	83.89 ± 3.85 cd	84.49 ± 1.85 b	67.44 ± 4.03 de
21.82	89.70 ± 3.45 bcde	100.00 ± 4.41 a	82.89 ± 1.85 bc	69.77 ± 0.01 cd
25.45	85.93 ± 2.72 cde	70.00 ± 3.33 f	79.68 ± 2.45 bcd	67.44 ± 4.03 de
29.09	88.69 ± 1.90 cde	78.89 ± 1.92 de	77.54 ± 3.70 d	65.12 ± 4.03 de
32.73	84.42 ± 3.99 de	91.67 ± 2.89 b	72.73 ± 0.93 e	62.79 ± 0.01 ef
36.36	83.92 ± 3.79 e	89.44 ± 2.55 bc	70.59 ± 1.60 e	58.14 ± 4.03 f

[a] Data are represented as mean ± standard deviation ($n = 3$). Means with different letters within the same column differ significantly ($p < 0.05$). [b] [OPD] = 3.49 mM, [H_2O_2] = 0.87 mM, buffer pH = 5.0 in the reaction mixture. [c] [Catechol] = 25.43 mM, [H_2O_2] = 5.08 mM, buffer pH = 4.5 in the reaction mixture. [d] [Pyrogallol] = 5.59 mM, [H_2O_2] = 4.07 mM, buffer pH = 6.5 in the reaction mixture. [e] [Guaiacol] = 5.82 mM, [H_2O_2] = 5.82 mM, buffer pH = 5.0 in the reaction mixture.

Table 6. Saturation Concentrations and Apparent K_m for Several Hydrogen-Donor Substrates and Hydrogen Peroxide

hydrogen donor	hydrogen donor[a]		H_2O_2[b]	
	saturation concn (mM)	apparent K_m (mM)	saturation concn (mM)	apparent K_m (mM)
OPD	3.6	2.9	4.4	0.93
catechol	23.7	18.2	3.5	0.31
pyrogallol	8.7	2.5	2.3	0.12
guaiacol	8.7	3.8	2.9	2.36

[a] As OPD was the hydrogen donor, [H_2O_2] = 2.9 mM in the reaction mixture. For the other three types of hydrogen donors, [H_2O_2] = 5.8 mM in the reaction mixture. [b] Corresponding hydrogen-donor concentrations in the reaction mixture were [OPD] = 3.6 mM, [catechol] = 23.7 mM, [pyrogallol] = 8.7 mM, and [guaiacol] = 8.7 mM, respectively.

As the peroxidase activity for some anionic peroxidases depends on the buffer concentration (*11*, *12*), we also determined the concentration optimum for wheat grass peroxidase to catalyze the oxidation of each particular hydrogen donor substrate under optimum pH determined previously. As shown in **Table 5**, the buffer concentration optima for OPD, catechol, pyrogallol, and guaiacol were 3.64, 21.82, 3.64, and 3.64 mM, respectively. It should be noted that the sharpest dependence was again observed with catechol, followed by guaiacol, pyrogallol, and OPD, respectively. Such results implied that these substrates would bind to different parts of the active site of wheat grass peroxidase and confirmed a need to determine the optimal reaction conditions for each.

Apparent Michaelis Constants and Substrate Specificity. Hydrogen peroxide is a suicide substrate for peroxidases. The hydrogen-donor substrate may protect the active site of peroxi-

Table 7. Optimum Conditions and Specific Activities of Wheat Grass Peroxidase for Several Hydrogen-Donor Substrates

			conditions for max activity					
hydrogen donor [AH]	λ (nm)	ϵ (mM^{-1} cm^{-1})	[H_2O_2] (mM)	[AH] (mM)	citrate–phosphate buffer pH	citrate–phosphate buffer concn (mM)	specific activity (units/mg)	rel activity (%)
OPD	445	11.1	4.4	3.6	5.0	3.6	221.2	100.0
catechol	295	1.7	3.5	23.7	4.5	21.8	60.6	27.4
pyrogallol	420	2.64	2.3	8.7	6.5	3.6	68.9	31.1
guaiacol	470	5.2	2.9	8.7	5.0	3.6	20.2	9.1

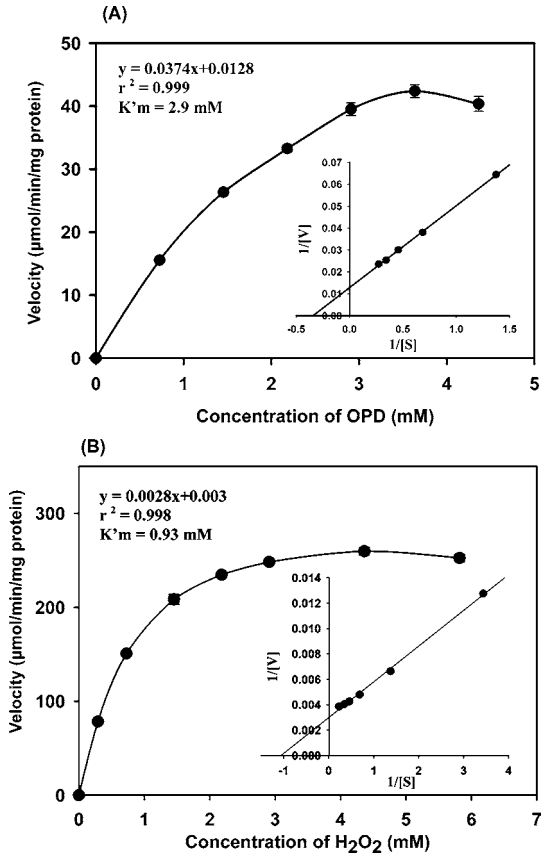


Figure 1. Effect of OPD hydrogen donor and hydrogen peroxide concentration on the activity of wheat grass peroxidase: (**A**) H_2O_2 concentration = 2.9 mM; (**B**) OPD concentration = 3.6 mM.

dase from the inactivation by a high concentration of hydrogen peroxide (*12*). Hence, we have first determined the saturation concentration for each hydrogen-donor substrate studied to obtain the maximum catalytic activity. A similar approach was then taken to determine the saturation concentration for hydrogen peroxide for each hydrogen-donor substrate. As shown in **Figure 1** as an example, the saturation concentration was 3.6 mM for OPD and was 4.4 mM for hydrogen peroxide when OPD was used as the hydrogen-donor substrate. The apparent K_m values of each hydrogen-donor substrate and hydrogen peroxide were then calculated using the Lineweaver−Burk (*25*) reciprocal plot graphic method for the two-substrate ping-pong mechanism followed by peroxidase (*26*, *27*). It was found that the K'_m value for H_2O_2 calculated by Lineweaver−Burk reciprocal plot was identical to that estimated from the substrate saturation curve (**Figure 1B**). However, the K'_m value for OPD under constant [H_2O_2] (2.9 mM) calculated by Lineweaver−Burk reciprocal plot was higher than that estimated from the substrate saturation curve (**Figure 1A**). This is possibly due to the fact that the oxidized intermediates (compounds **I** and **II**) of peroxidase could react with H_2O_2 at lower hydrogen donor concentration, producing an inactive form of the enzyme (*12*, *27*). As shown in **Table 6**, the hydrogen-donor saturation concentration was highest for catechol, followed by pyrogallol and guaiacol and then OPD. The apparent K_m was also highest for catechol, followed by guaiacol, OPD, and pyrogallol, respectively. On the other hand, the hydrogen peroxide saturation concentration was highest for OPD, followed by catechol, guaiacol, and pyrogallol, respectively. The apparent K_m was highest for guaiacol, followed by OPD, catechol, and pyrogallol, respectively.

The activity of peroxidase from wheat grass was then measured under the optimal reaction conditions (including buffer pH, buffer concentration, hydrogen-donor concentration, and H_2O_2 concentration) determined for each hydrogen-donor substrate. As shown in **Table 7**, the relative activity of peroxidase were highest as OPD was the hydrogen-donor substrate, followed by pyrogallol, catechol, and guaiacol, respectively, in decreasing order. As compared to the specific activity values for each hydrogen-donor substrate, it was interesting to note that under the optimal reaction conditions, wheat grass peroxidase catalyzed the oxidation of OPD (an aromatic amine substrate) 3−11 times more rapidly than guaiacol, catechol, and pyrogallol (phenolic substrates containing one to three hydroxy groups in the benzene ring). Peroxidases from sweet potato (*11*) and royal palm tree (*12*) were reported to oxidize 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and ferulic acid more effectively than *o*-dianisidine, OPD, and guaiacol. Therefore, the efficiency of plant peroxidase catalysis depends strongly on the chemical nature of the reducing substrates and the catalytic properties of the enzyme.

Wheat grass peroxidase, like royal palm and sweet potato peroxidases, had an optimal buffer pH and concentration for different reducing substrates. At optimal reaction conditions, it oxidized aromatic amine substrate (OPD) more efficiency than phenolic substrates. Further characterization of the catalytic properties of this enzyme, such as catalytic efficiency for other reducing substrates, other activity effectors, and catalytic reactions involving amino acid residues, remains to be investigated.

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