Purification and Characterization of Windmill Palm Tree (*Trachycarpus fortunei*) Peroxidase

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High peroxidase activity was demonstrated to be present in the leaf of several species of cold-resistant palms. Histochemical studies of the leaf of windmill palm tree (*Trachycarpus fortunei*) showed the peroxidase activity to be localized in hypoderma, epidermis, cell walls, and conducting bundles. However, chlorophyll-containing mesophyll cells had no peroxidase at all. The leaf windmill palm tree peroxidase (WPTP) was purified to homogeneity and had a specific activity of 6230 units/mg, RZ = 3.0, a molecular mass of 50 kDa, and an isoelectric point of pI 3.5. The electronic spectrum of WPTP with a Soret band at 403 nm was typical of plant peroxidases. The N-terminal amino acid sequence of WPTP was determined. The substrate specificity of WPTP was distinct from that of other palm peroxidases, and the best substrate for WPTP was 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid). The palm peroxidase showed an unusually high stability at elevated temperatures and high concentrations of guanidine.

KEYWORDS: Peroxidase; palm; localization; purification; substrate specificity; stability

INTRODUCTION

Peroxidases (EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase) are widely spread in the plant kingdom, and in vivo they are one of the key enzymes controlling plant growth, differentiation, and development. This enzyme is widely employed in practical enzyme immunoassay, for the construction of biosensors, and in organic synthesis (*1*−*3*).

Secretory plant peroxidases have a high degree of polymorphism (*4*). According to their isoelectric point (pI), peroxidases are subdivided into cationic and anionic isozymes. The most studied peroxidase isozyme *c* from horseradish roots (*Armoracia rusticana*) (HRP-C) has a pI of 8.5 (*5*) and belongs to a group of cationic isoperoxidases. Anionic peroxidases (pI < 7.0) were purified from a number of agricultural plants, such as soy, tobacco, tea, potato, sweet potato, and broccoli (*6*−*11*).

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Recently, novel anionic peroxidases have been isolated from the leaves of some palm trees (*Elaeis guineensis*, *Roystonea regia*, and *Metroxylon sagu*) (*12*−*15*). These enzymes showed an extremely high stability at elevated temperatures, at acidic pH values, and also in the presence of hydrogen peroxide and guanidinium chloride (*16*, *17*). A combination of the above properties allowed us to use palm tree peroxidases to construct H_2O_2-sensitive biosensors with improved characteristics (*18*) and to develop the enzymatic synthesis of conducting polyaniline under acidic conditions (*19*).

Palm trees, mentioned above, are cultivated in tropical regions of Asia, Latin America, and Africa and are of economic interest for the food industry and for ornamental use. However, some species of palms grow in more northern regions (up to 56° latitude north), where natural climatic fluctuations are more distinct over the year. The climate effect on biosynthesis and properties of palm peroxidases has not been studied yet. In the present work, we reveal some species of palms spread along the northern coast of the Black Sea having leaves that show a high peroxidase activity. Histochemical localization of the peroxidase activity in the leaf of cold-resistant windmill palm tree (*Trachycarpus fortunei*) was studied. The anionic windmill palm tree peroxidase (WPTP) was purified to homogeneity, and its properties were characterized.

Windmill Palm Tree Peroxidase

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MATERIALS AND METHODS

Materials. Green palm tree leaves harvested in the territory of the Nikita Botanic Garden (Crimea, Ukraine) were used in the work.

Hydrogen peroxide (30%) and $(NH_4)_2SO_4$ were purchased from Merck (Darmstadt, Germany), *N,N′*-Methylenebis(acrylamide), *o*-phenylenediamine, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), *o*-dianisidine, 3,3′-diaminebenzidine (DAB), guaiacol, guanidine−HCl, phloroglucin, $(NH_4)_2S_2O_8$, acrylamide, and TEMED were from Sigma (St. Louis, MO); ferulic acid was from Fluka (Buchs, Switzerland); DEAE-Toyopearl was from Toya Soda (Japan); Sephacryl S200, phenyl-Sepharose, and molecular weight protein and p*I* protein markers were from Pharmacia Biotech (Uppsala, Sweden); ampholines Bio-Lyte 2.5/5 and Tris were from Bio-Rad (Hercules, CA); and polyethylene glycol (MW 10000) was from Aldrich (Milwaukee, WI).

Preparation of Palm Leaf Extract. Palm tree leaves (1 g) were milled and homogenized in 10 mL of 10 mM phosphate buffer (pH 7.0) in accordance with a previously published protocol (*12*). The homogenate was incubated for 1 h at ambient temperature and then centrifuged at 5000*g* for 15 min at 20 °C. The peroxidase activity in the supernatants was measured with guaiacol as substrate. The activity in the extracts of three palms of each species was measured.

Enzyme Assay. Peroxidase activity was determined spectrophotometrically using guaiacol or ABTS as substrate (*13*). WPTP substrate specificity was studied under optimal conditions (pH, buffer concentration) determined earlier for other palm peroxidases (*20*). By varying the concentrations of substrates (AH_2 in the range of 0.001−2.0 mM and H_2O_2 in the range of 0.1−20 mM) their optimal values were determined. The H_2O_2 concentration was determined by monitoring absorbance using $\epsilon_{240} = 43.6$ M^{-1} cm^{-1}. The required dilutions of H_2O_2 were prepared daily.

One unit of activity was defined as the amount of peroxidase oxidizing 1 μmol of substrate per minute under standard conditions. Specific activity was expressed as units of activity per milligram of protein.

Histochemical Studies. Sections of fresh palm leaf (10−30 μm) were prepared using a microtome and soaked in a water drop on a glass slide. For the peroxidase activity localization the leaf tissues were washed three times with water and then incubated in a freshly prepared 50 mM phosphate buffer (pH 7.0) containing hydrogen peroxide (0.006%) and DAB (0.1 mg/mL) (*21*). After 10 min of incubation, the tissues were washed three times with water. In the control experiments H_2O_2 was excluded from the substrate solution. To stain lignin, palm leaf sections were incubated for 5−10 min with 0.5% phloroglucin soluble in ethanol followed by treatment with HCl vapor to obtain a pink color (*22*). After a final washing with water, all palm leaf sections were mounted in 10 μL of glycerol. To localize phenolics, the fluorescence at 360 nm was detected (*23*). Photographs were taken with a fluorescence phase microscope (Zeiss) and an AxioCam camera.

Enzyme Purification. The preparative purification of WPTP was carried out using a modified procedure (*12*). The windmill palm leaves (2.5 kg) were milled and then incubated with periodic agitation in 10 L of distilled water for 15 h at ambient temperature. The tissue debris was removed by filtration, and the second portion (2.5 kg) of milled leaves was added to the obtained extract. After 15 h of incubation, debris was filtered, and then the filtrate was centrifugated (10000*g*, 20 min, 20 °C). To the resulting supernatant were subsequently added solid polyethylene glycol and solid $(NH_4)_2SO_4$ in portions to get 14 and 10% (w/v) concentrations, respectively. Three phases (the top polymer phase, the bottom aqueous phase, and the precipitate at the interface) were formed after the addition of $(NH_4)_2SO_4$. Because the top deep brown phase and the precipitate had no peroxidase activity, they were discarded. Solid $(NH_4)_2SO_4$ was added to the aqueous phase containing peroxidase activity up to 1.7 M. The resultant solution was applied to a phenyl-Sepharose column (1.5 × 30 cm) equilibrated with 100 mM phosphate buffer (pH 6.5) containing 1.7 M $(NH_4)_2SO_4$. The enzyme was eluted by decreasing the $(NH_4)_2SO_4$ concentration in the eluting solution to 0.3 M. Fractions with enzyme activity were collected and concentrated using a YM-10 membrane (Millipore/Amicon, cutoff = 10000). The obtained solution was applied to a Sephacryl S200 column (2.5 × 50 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.5). Elution

Table 1. Peroxidase Activity in Palm Trees Leaves[a]

NN	palm species	peroxidase activity, units/g of leaves
1	*Washingtonia filifera*	88
2	*Trachycarpus fortunei*	680
3	*Jubaea spectabilis*	285
4	*Butia capitata*	415
5	*Sabal minor*	650
6	*Trachycarpus excelsa*	700
7	*Phoenix canariensis*	0.5
8	*Arundo donax*	300

[a] The screening was carried out in July 2004.

was carried out with the same buffer. Fractions with enzyme activity were collected and applied to a DEAE-Toyopearl 650M column (1.5 × 20 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.5). The peroxidase was eluted with a linear 0−50 mM NaCl gradient. The peroxidase preparation was stored at −18 °C.

Stability. The thermal stability of WPTP was studied as follows: after heating of 495 μL of buffer solution [10 mM Tris-HCl buffer (pH 7.5) or 10 mM phosphate buffer (pH 3.0)] to the desired temperature (61−80 °C), 5 μL of WPTP sample ([E] = 6.8×10^{-6} M) was added, and the solution obtained was incubated at the same temperature. At different intervals, aliquots (10 μL) were taken off, and the residual activity was determined.

The guanidine-induced inactivation of peroxidase was carried out as follows: 5 μL of WPTP sample ([E] = 6.8×10^{-6} M) was added to 495 μL of 10 mM Tris-HCl buffer (pH 7.0) containing different concentrations of guanidine−HCl (up to 6 M) and incubated at 25 °C for 20 h. Finally, the peroxidase activity was measured using *o*-dianisidine and ABTS as substrates.

Analytical Methods. The purity and molecular mass value of WPTP were determined by SDS-PAGE (12.5%) electrophoresis in the presence of 1% 2-mercaptoethanol and 5 mM EDTA. Isoelectrofocusing was performed as described previously (*21*) using a pH gradient in the range of 2.5−5.0. Protein concentration was measured using the bicinchonic acid method with purified bovine serum albumin as standard (*24*). The concentration of WPTP was estimated spectrophotometrically using ϵ_{403} = 114 mM^{-1} cm^{-1} (*12*). Electronic spectra of WPTP were recorded using a Shimadzu UV-2401 PC spectrophotometer. The calcium content was determined by atomic absorption spectroscopy (*25*).

RESULTS AND DISCUSSION

Screening. Upon screening of palms cultivated in the Nikita Botanic Garden (Black Sea, 46° latitute north), we found some species having leaves that had a high peroxidase activity (**Table 1**). The highest peroxidase activity was detected in leaves of the palms *Trachycarpus fortunei*, *Trachycarpus excelsa*, and *Sabal minor*. The specific activity values were close to the best ones reported for tropical palm peroxidases (*13*). The peroxidase activity in windmill palm (*T. fortunei*) leaves varied over the year; in summer the enzyme activity was approximately 3-fold as compared to that measured in the winter. This contrasts with the results obtained for royal palm (*Roystonea regia*) cultivated in tropical areas, the activity of which remained the same over the whole year (*13*).

However, not all studied palms showed the high peroxidase activity. The peroxidase activity in the leaves of *Phoenix canariensis* was only 0.5 unit/g of tissue (**Table 1**). Other palm species showed intermediate values. Thus, on the basis of the obtained results, the windmill palm tree was selected for further work.

Localization. A cellular localization of the peroxidase activity was detected using H_2O_2/DAB reagent. **Figure 1C,D** shows the peroxidase activity (brown staining) to be detected in epidermis, hypoderma, conducting bundles, and cell walls, whereas the
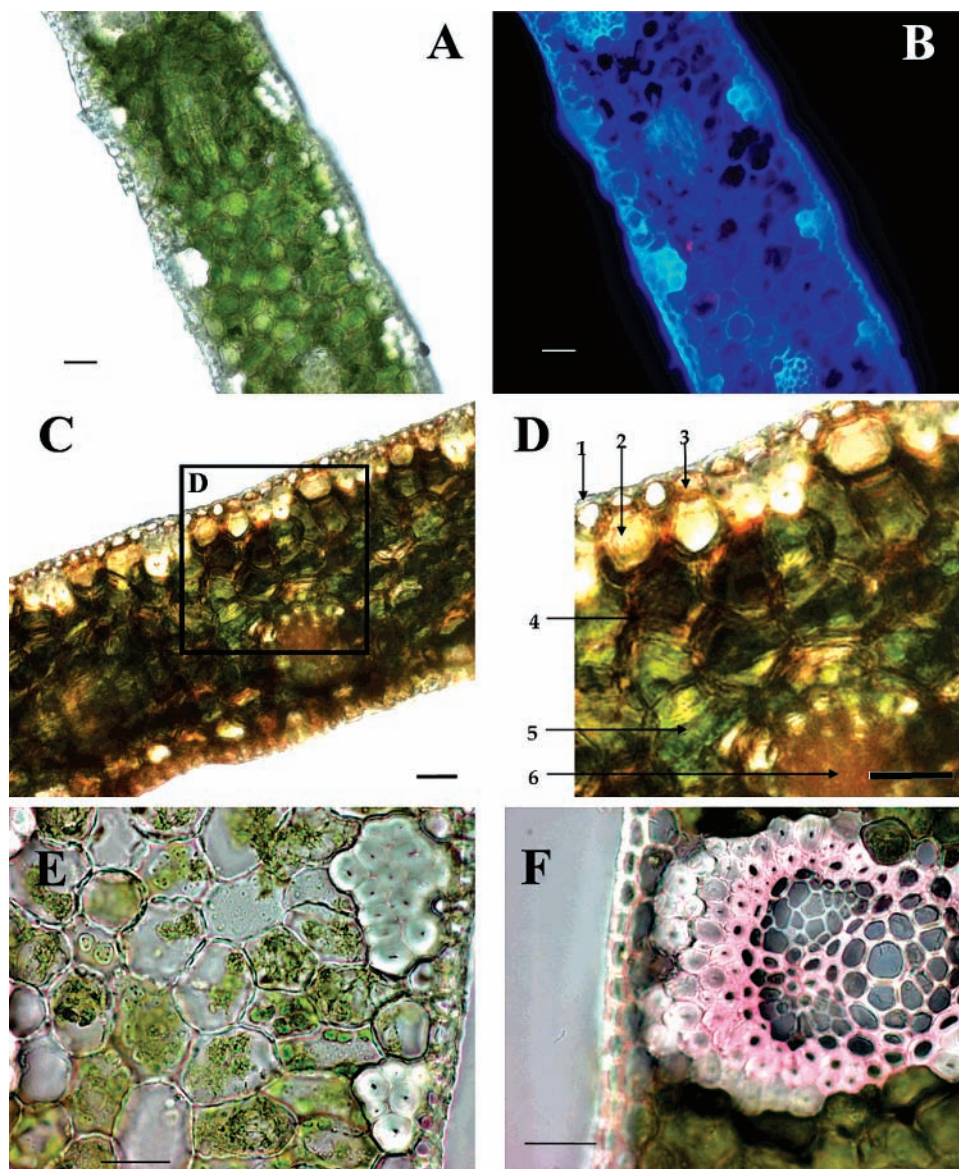
Figure 1. Histochemical staining of windmill palm leaf sections to monitor the localization of peroxidase activity, phenolics, and lignin: (**A**) control section of the palm leaf treated with the substrate solution without H_2O_2; (**B**) autofluorescence of phenols (blue staining) in cuticula, epidermis, hypoderma, conducting bundles, and cell walls; (**C**) sections stained with H_2O_2/DAB reagent; (**D**) same as (**C**) but in higher magnification [peroxidase activity (brown staining) was localized in epidermis, hypoderma, transmitting veins, and cell walls]; (**E**, **F**) localization of lignin (pink staining) in the cells of epidermis, hypoderma, conducting bundles, and cell walls. Bar = 20 μm. 1, cuticula; 2, epidermis; 3, hypoderma; 4, cell wall; 5, mesophyll cells; 6, conducting bundles.

chlorophyll-containing mesophyll cells had no peroxidase at all. In the control experiments no brown color was observed (**Figure 1A**). Note that in the hypodermal and epidermal cells, the peroxidase activity is localized both inside the cells and on the cell walls. The similar localization of peroxidase activity was observed in the royal palm leaf (data not shown). Previously, using an immunohistochemical method, the peroxidase was detected in areas adjacent to the epidermis in the oil palm leaf (*14*). Therefore, the palm trees cultivated in different climate areas have the same localization of peroxidase activity.

In parallel, the distribution of some phenolics was investigated in the windmill palm leaf. The light blue (positive) staining was observed in cuticle, epidermis, hypoderma, conducting bundles, and cell walls (**Figure 1B**). Thus, the peroxidase activity and phenols have similar localization, except cuticula exhibiting no peroxidase activity. Like peroxidase, lignin (pink staining) was localized in the cells of epidermis, hypoderma, bundles, and cell walls (**Figure 1E,F**).

Table 2. Purification of Windmill Palm Tree Peroxidase

procedure	protein, mg	specific activity,[a] units/mg	total activity,[a] units	purifi- cation	yield, %
PEG/ammonium sulfate	43200	128	5.5×10^6	1.0	100
phenyl-Sepharose	5040	842	4.3×10^6	6.6	77
Sephacryl S200	248	4094	1.0×10^6	31.9	20
DEAE-Toyopearl	132	6230	8×10^5	48.6	15

[a] The enzyme activity was measured toward guaiacol.

Purification. The peroxidase was isolated from windmill palm tree leaves and purified to homogeneity. The results are summarized in **Table 2**. WPTP with a specific activity of 6230 units/mg of protein, measured toward guaiacol, was obtained in 15% overall yield.

The purified WPTP migrated in SDS electrophoresis as a single band with a molecular mass of 50 kDa. This was similar
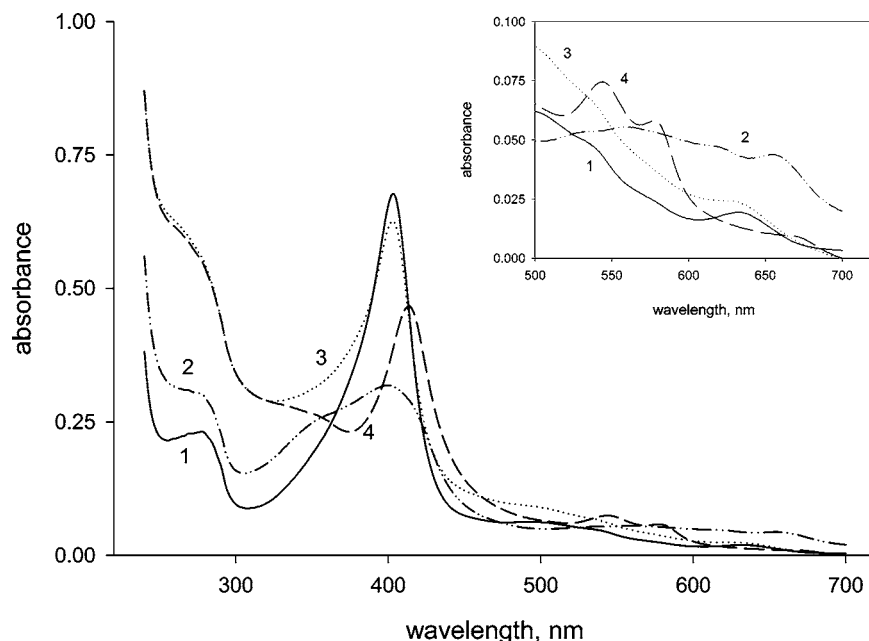
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Figure 2. Spectra of WPTP and its derivatives: (1) resting form; (2) compound **I** obtained after addition of a 1.3-fold excess of hydrogen peroxide to WPTP; (3) compound **I** after its incubation for 16 h; (4) compound **III** obtained after addition of a 500-fold excess of H_2O_2 to WPTP.
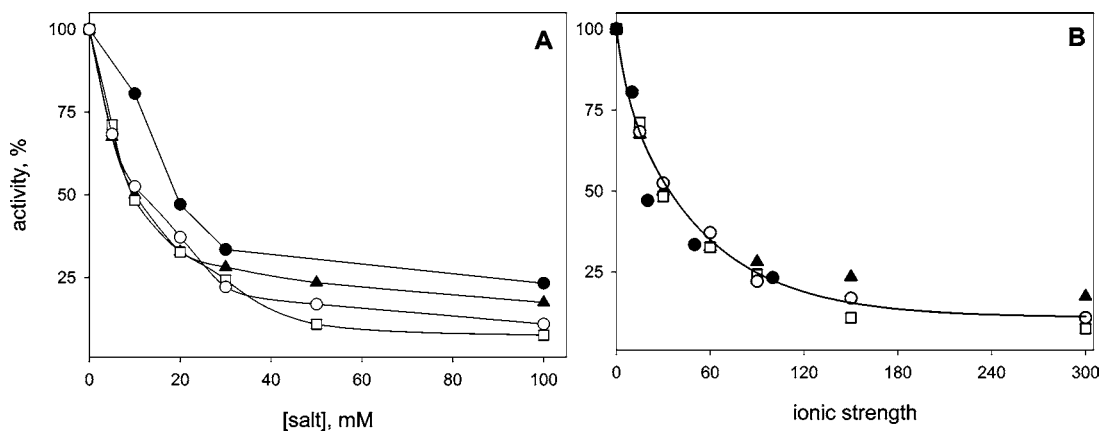

Figure 3. Effect of concentration (**A**) and ionic strength (**B**) of some salts on WPTP activity measured toward ABTS: (●) NaCl; (▲) Na_2SO_4; (○) $CaCl_2$; (□) $MgCl_2$.

to that for royal palm peroxidase (RPTP) and was lower than that determined for African oil palm tree peroxidase (AOPTP) (57 kDa) (*12−15*). Isoelectrofocusing confirmed the high purity of the obtained protein (data not shown). WPTP is an anionic enzyme with a p*I* value of 3.5.

The spectrum of WPTP (resting form) with a Soret band at 403 nm (**Figure 2**, curve 1) was typical of plant peroxidases (*26*). It also had a CT band at 632 nm. These bands in combination with the Q-band at 493 nm suggested the presence of a high-spin heme. The RZ value (A_{403}/A_{280}) for purified WRPP was 3.0. This value did not change after the enzyme storage at −18 °C for at least 6 months.

Calcium Content. Many plant peroxidases have been reported to contain in their structure two cations of calcium (*27*). The Ca^{2+}-binding sites are known as proximal and distal according to their location relative to the porphyrin plane. The Ca^{2+} cations are important for both the activity and thermal stability of the enzyme (*28, 29*). The Ca^{2+} content in purified WPTP was measured by atomic absorption spectroscopy. Contrary to other secretory peroxidases, WPTP had 6.5 calcium cations/molecule. Besides WPTP, only anionic peanut peroxidase was reported to have an elevated content of calcium (5 Ca^{2+}/molecule) (*25*).

Reactions with Hydrogen Peroxide. The addition of a 1.3-fold excess of hydrogen peroxide to WPTP at pH 5.0 initiated the transformation of the resting form of the enzyme to compound **I** (**Figure 2**, curve 2). The obtained spectrum of compound **I** had a characteristic shoulder at 350 nm and bands at 555 and 655 nm. Note that compound **II** was not detected in this reaction.

Like compound **I** of anionic tobacco peroxidase (*7*), compound **I** of WPTP was stable and its spectrum remained unchanged for 30 min. However, the compound **I** spectrum slowly recovered to that of the WPTP resting form, and the complete recovery of the Soret band was observed only after 16 h. Note that we did not observe the complete recovery of the resting form spectrum, because UV absorbance of compound **I** was notably higher than that of the WPTP resting form (**Figure 2**, curve 3). The precise reason for this effect is not clear and is currently under investigation.

Upon addition of a 500-fold excess of H_2O_2, the characteristic spectrum of compound **III** was detected (**Figure 2**, curve 4). As in the case of compound **I**, the UV absorbance of compound **III** was higher than that of the WPTP resting form.

N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence of WPTP was determined (**Table 3**). Comparison

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Table 3. N-Terminal Amino Acid Sequences[a] of Some Plant Peroxidases

plant peroxidase	enumeration of amino acids																				homology, %
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
WPTP	D	L	Q	I	G	F	Y	X	Q	S	X	P	S	A	E	S	L	V	X	Q	100
HRP-C	Q	L	S	S	N	F	Y	A	T	K	C	P	N	A	L	S	T	I	K	S	30
ToP	Q	L	S	A	T	F	Y	D	T	T	C	P	N	V	T	S	I	V	R	G	30
SbP	Q	L	D	P	S	F	Y	R	D	T	C	P	R	V	H	S	I	V	R	E	30
GDwP	Q	L	R	V	G	F	Y	S	K	S	C	P	H	A	E	S	I	I	T	E	45
RP	Q	L	S	A	T	F	Y	D	T	S	C	P	N	A	L	S	T	I	K	S	35
SPP-*a2*	D	E	A	C	V	F	S	A	V	K	E	V	V	V	A	A	I	N	A	R	10

[a] Where X is the unidentified amino acid residue.

Table 4. Substrate Specificity of Windmill Palm Tree Peroxidase

substrate (AH$_2$)	λ, nm	ϵ, M^{-1} cm^{-1}	[H$_2$O$_2$], mM	[AH$_2$], mM	pH	[buffer[a]], M	k_{app}, M^{-1} s^{-1}
ABTS	414	31100	3.0	0.005	3.0	0.01	6.0×10^7
o-dianisidine	420	30000	12.5	0.2	5.2	0.09	5.0×10^6
ferulic acid	318	6000	1.0	0.02	5.0	0.02	3.0×10^6
guaiacol	470	5200	5.6	0.4	5.5	0.05	1.8×10^6
o-phenylene-diamine	445	11100	1.0	0.6	5.0	0.10	5.5×10^4

[a] Citrate–phosphate buffer was used.

of the N-terminal 20-member peptide fragments of different plant peroxidases showed that highest homology between WPTP and peroxidases isolated from monocotyledonous plants, such as great duckweed (*30*) and rice (*31*), was observed. Contrary to other plant peroxidases containing pyroglutamate at the N terminus, in this position WPTP as well as isozyme *a2* of sweet potato peroxidase (SPP) (*32*) had an Asp residue (**Table 3**).

Substrate Specificity. Substrate specificity of WPTP was examined using some well-known peroxidase substrates (**Table 4**). The catalytic efficiency of WPTP was evaluated in terms of the "ping-pong" mechanism characteristic of plant peroxidases (*33*).

$$E + H_2O_2 \rightleftharpoons \text{compound } \mathbf{I} + H_2O \qquad (1)$$

$$\text{compound } \mathbf{I} + AH_2 \rightleftharpoons \text{compound } \mathbf{II} + AH^{\bullet} \qquad (2)$$

$$\text{compound } \mathbf{II} + AH_2 \rightleftharpoons E + AH^{\bullet} + H_2O \qquad (3)$$
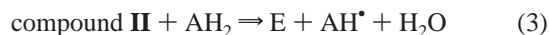
E is the ferric enzyme (resting form), compounds **I** and **II** are the oxidized intermediates of peroxidase, and AH$_2$ and AH$^{\bullet}$ are the electron donor substrate and the radical product of its one-electron oxidation, respectively.

As mentioned above, the reduction of compound **I** (eq 2) is the rate-limiting step in WPTP catalysis and, hence, we used the values of the second-order rate constant (k_{app}) for the reaction between compound **I** and hydrogen donor substrates (AH$_2$) to evaluate WPTP catalytic efficiency. The constant was calculated from eq 4.

$$\text{rate} = k_{app}[\text{compound } \mathbf{I}][AH_2] \qquad (4)$$

Because reaction 2 is rate-limiting, we used in calculations the initial concentration of peroxidase instead of compound **I** concentration. The use of k_{app} is more informative compared to the term "specific activity", which depends on AH$_2$ concentration.

Analysis of the substrate specificity of novel peroxidase was carried out under conditions favorable for other palm peroxidases (*20*). Comparison of the k_{app} values revealed that the best

WPTP substrate was ABTS (**Table 4**). The k_{app} values for other peroxidase substrates, such as *o*-dianisidine, guaiacol, and ferulic acid, were 10–30-fold lower than that for ABTS. Among the studied substrates, *o*-phenylenediamine was the worst one.

Analysis of the obtained results showed that the efficiency of WPTP catalysis toward ABTS, *o*-dianisidine, and guaiacol was similar to that characteristic of other palm peroxidases. However, WPTP catalyzed the oxidation of ferulic acid and *o*-phenylenediamine with notably lower k_{app} values than AOPTP and RPTP did.

It is also known that anionic peroxidases, including AOPTP and RPTP, efficiently catalyze the oxidation of luminol in the absence of enhancers (*34–37*). In contrast, WPTP was a poor biocatalyst in this reaction (data not shown). Thus, WPTP substrate specificity is distinct from those of other palm peroxidases.

Effect of Salts. Earlier, it was reported that palm peroxidase activity depended on the concentration of the citrate–phosphate buffer used (*13, 20*). Here we studied the effect of some salts on WPTP activity and showed that the increasing concentrations of NaCl, Na$_2$SO$_4$, CaCl$_2$, and MgCl$_2$ in the reaction mixture resulted in the increase of WPTP inhibition. Furthermore, the effect depended upon the chemical structure of the used salt (**Figure 3A**). However, when the results were plotted in the coordinates "activity" versus "ionic strength", a single curve was obtained for all salts (**Figure 3B**). This indicated that the inhibiting effect was not specific and depended only on ionic strength.

Stability. The unique feature of palm peroxidases is their extremely high stability (*16–18*). Like other palm peroxidases, WPTP also showed a high thermal stability. The enzyme retained its catalytic activity after 1 h of incubation at temperatures up to 80 °C at pH 7.5 (data not shown). Under acidic conditions WPTP had a lower stability. The kinetic study of WPTP thermal inactivation in the temperature range of 61–67 °C and at pH 3.0 showed that the denaturation reactions obeyed first-order kinetics (**Figure 4**). Thermal inactivation energy was calculated using the values of WPTP inactivation constants (k_{inac}). The obtained value of 76 kcal/mol was lower than that estimated for AOPTP (104 kcal/mol) (*16*), but much higher as compared to that reported for HRP under the same conditions (38 kcal/mol) (*38*).

We also studied guanidine-induced inactivation of WPTP. **Figure 5** shows that the peroxidase activity measured toward *o*-dianisidine did not alter up to 4 M guanidine and that only at higher concentrations was the enzyme inactivated. Under the same conditions the HRP-C stability was much lower (*16*).

Unexpectedly, we found that WPTP stability in the presence of guanidine depended on the chemical nature of used substrates. Therefore, when the peroxidase activity was measured toward ABTS, WPTP stability was lower than that with the use of
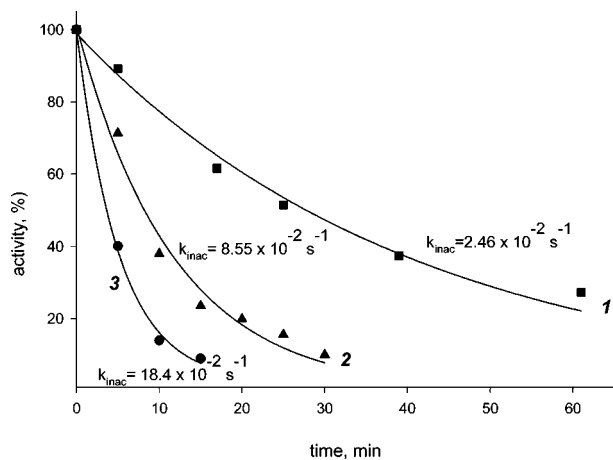
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Figure 4. Kinetics of thermal inactivation of WPTP at (1) 61 °C, (2) 64 °C, and (3) 67 °C. Experimental conditions: $[E] = 6.8 \times 10^{-8}$ M; 10 mM citrate–phosphate buffer (pH 3.0). Activity was measured using ABTS as a substrate.
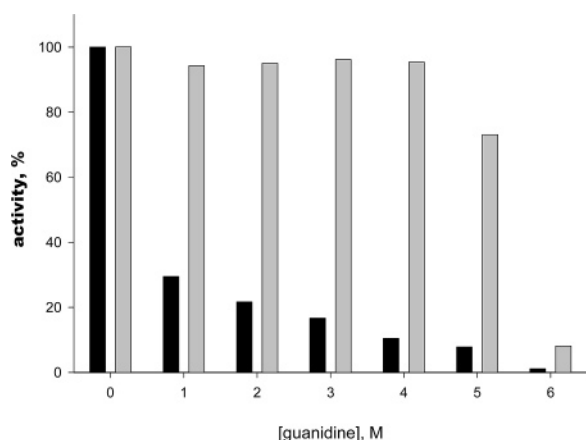

Figure 5. Effect of guanidine concentration on the activity of WPTP. Experimental conditions: the enzyme ($[E] = 6.8 \times 10^{-8}$ M) was incubated in 10 mM Tris-HCl buffer (pH 7.0) containing guanidine at different concentrations for 20 h. The residual activity was measured using *o*-dianisidine (gray bars) and ABTS (black bars) as substrate.

o-dianisidine (**Figure 5**). In the case of ABTS even in 1 M guanidine we observed 70% inactivation of WPTP.

Although the effect of the chemical nature of the tested substrates on the guanidine-induced WPTP inactivation is not clear and should be studied in detail, we suppose that this can be related to the difference in interaction of *o*-dianisidine and ABTS with WPTP. Unfortunately, for *o*-dianisidine and ABTS there is no information about their interaction with peroxidase. Presently, there are only X-ray data for crystal complexes of HRP-C with ferulic and benzhydroxamic acids. The results showed that binding sites of HRP-C for these substrates were not identical (*27*). Therefore, the observed difference in WPTP stability with respect to guanidine treatment may be related to the different stabilities of WPTP fragments responsible for the binding of *o*-dianisidine and ABTS.

ABBREVIATIONS USED

WPTP, windmill palm tree peroxidase; HRP-C, isozyme *c* of horseradish peroxidase; RPTP, royal palm tree peroxidase; AOPTP, African oil palm tree peroxidase; SbP, soybean peroxidase; SPP, sweet potato peroxidase; ToP, tobacco peroxidase; GDwP, great duckweed peroxidase; RP, rice peroxi-

dase; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); DAB, 3,3′-diaminebenzidine.

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