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Substrate Specificity of African Oil Palm Tree Peroxidase

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Abstract—The optimal conditions for catalysis by the peroxidase isolated from leaves of African oil palm tree (AOPTP) have been determined. The pH optimum for oxidation of the majority of substrates studied in the presence of AOPTP is in the interval of 4.5-5.5. A feature of AOPTP is low pH value (3.0) at which the peroxidase shows its maximal activity toward 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS). Increasing the buffer concentration changes the AOPTP activity, the degree of the effect depending upon the chemical structure of the substrate. Under optimal conditions of AOPTP catalysis, the values of second order rate constant characterizing efficiency of enzymatic oxidation of substrates have been calculated. It was shown that among 12 peroxidase substrates studied, ABTS and ferulic acid are the best substrates for AOPTP. The results show that substrate specificities of AOPTP and royal palm tree peroxidase are similar, but different from substrate specificity of other plant peroxidases.

Key words: peroxidase, palm tree, substrate specificity

Peroxidase (EC 1.1.11.7) is one of the most widely distributed heme-containing enzymes of plants. Peroxidase is used in practical applications. This enzyme is employed most widely in enzyme-linked immune assay using the enzyme as a label of antibodies and antigens, and electrochemical biosensors [1, 2]. Peroxidases can be also used for removal from industrial waste water of aromatic amines and phenols including chloro-substituted phenols, for decolorizing industrial dyes, in organic synthesis for production of low and high molecular weight compounds, etc. [3-6].

The most studied and, hence, used peroxidase is one isolated from horseradish roots (*Armoracia rusticana*) [7]. However, a need for peroxidases with different substrate specificity and higher thermal and pH stability stimulated the search and study of novel plant and fungal peroxidases. Therefore, investigations on purification and properties of peroxidases from soybeans, tobacco, peanut, alfalfa, *Arthromyces ramosus*, etc. have been carried out [8-11].

Recently we reported the purification of a novel peroxidase isolated from African oil palm tree leaves (AOPTP) [12]. This species of palm tree is cultivated widely in tropical countries for palm oil production. The peroxidase exhibits unusually high stability under different denaturing conditions [13], this being very attractive for its application. The study of luminol oxidation by hydrogen peroxide in the presence of AOPTP showed that in the course of the reaction the enzyme produced a long-term chemiluminescent signal. The results indicate that this biocatalyst is not inactivated by radical products [14]. In the present work the study of catalytic properties of the peroxidase isolated from palm tree *Elaeis guineensis* has been continued and its substrate specificity has been determined.

MATERIALS AND METHODS

Peroxidase (RZ = 3.0) was purified from leaves of African oil palm tree *Elaeis guineensis* as described previously [12] with subsequent chromatography on a Superose 12 column (Pharmacia, Sweden) using 5 mM Tris buffer, pH 8.0, as the eluent.

2,2′-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), *o*-dianisidine, *o*-phenylenediamine, ferrocyanide, catechin, guaiacol, KI, citric acid, and NaH_2PO_4 were from Sigma (USA), catechol, phenol, and H_2O_2 (30%) from Merck (Germany), pyrogallol from Aldrich (USA), ferulic acid, veratryl alcohol, and vanillin from Fluka (Switzerland).

AOPTP substrate specificity was studied using colorimetric detection of products formed in the course of

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Table 1. Experimental conditions for determination of k_{app} for the substrate oxidation by hydrogen peroxide in the presence of African oil palm tree peroxidase

Substrate (AH_2)	λ, nm	ε, $M^{-1} \cdot cm^{-1}$	pH	[Citrate-phosphate buffer], M	[H_2O_2], mM	[AH_2], mM
ABTS	414	31100	3.0	0.05	0.8	0.016
Ferulic acid	318	6000	5.0	0.04	1.5	0.06
o-Dianisidine	420	30000	5.5	0.10	3.0	0.1
o-Phenylenediamine	445	11100	5.5	0.02	2.0	0.75
Guaiacol	470	5200	5.5	0.07	3.5	9.0
Pyrogallol	420	2640	6.0	0.05	4.0	5.5
Catechol	295	1700	4.5	0.04	5.0	75.0

oxidation of substrates by H_2O_2. Guaiacol, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt, catechol, pyrogallol, *o*-dianisidine, *o*-phenylenediamine, veratryl alcohol, vanillin, KI, (+)catechin, ferulic acid, ferrocyanide, and phenol were used as substrates. All assays were carried out in 10-100 mM phosphate-citrate buffer, pH 2.8-7.0. When optimizing the AOPTP catalysis conditions, the concentration of H_2O_2 and substrates in reaction medium were varied in the interval of 0.09-11 and 5-150 mM, respectively. The concentration of hydrogen peroxide was measured spectrophotometrically (ε_{240} = 43.5 $M^{-1} \cdot cm^{-1}$ [15]). Second order rate constant (k_{app}) values of enzymatic oxidation of the substrates by hydrogen peroxide were determined under optimal conditions measured in separate experiments (Table 1).

RESULTS AND DISCUSSION

We recently used leaves of the African oil palm tree *Elaeis guineensis* as a source for purification and partially characterization of anionic peroxidase [12]. The study of enzymatic oxidation of luminol in the presence of AOPTP showed that this reaction proceeds with the generation of long-term chemiluminescent signal. Moreover, in contrast to horseradish peroxidase (HRP), the magnitude of the AOPTP produced signal did not depend upon a presence of "enhancers" in the reaction medium [14]. These data stimulated the study of substrate specificity of the palm peroxidase and its comparison with that of peroxidases isolated from other sources.

The substrate specificity of AOPTP has been examined using some well-known peroxidase substrates (hydrogen donors). It should be noted that some of them, namely, veratryl alcohol, $K_4Fe(CN)_6$, phenol, catechin, vanillin, and KI were not oxidized by AOPTP under any reaction conditions and, therefore, the main part of this work was carried out using the following substrates: ferulic acid, *o*-dianisidine, *o*-phenylenediamine, ABTS, guaiacol, catechol, and pyrogallol.

It is well known that the optimal conditions for catalysis by different peroxidases are not identical [16, 17]. Therefore, the effect of pH on the enzymatic oxidation of substrates studied was first examined. As seen in Table 2, for many of the substrates the maximal activity of palm peroxidase occurred in the pH range of 4.5-5.5. Only for ABTS and pyrogallol the optimal pH values were outside this range.

In the case of pyrogallol, the AOPTP activity increased with decreasing acidity of the reaction medium. At the same time, we detected a sharp increase of reaction rate of nonenzymatic (spontaneous) oxidation of pyrogallol. Thus, further work with this substrate was carried out at pH 6.0, as recommended by Sigma in the technical protocol for determination of peroxidase activity toward pyrogallol.

The maximal AOPTP activity measured with ABTS is observed at pH 3.0 (Table 2), whereas for horseradish peroxidase the pH optimum is 2 units higher [17]. The low value of the pH optimum characteristic for AOPTP suggests that in contrast to HRP, which is inactivated rapidly already at pH 4.0 [18], the palm peroxidase is stable in acidic media.

At the study of luminol oxidation by hydrogen peroxide in the presence of AOPTP it was observed that AOPTP activity depends upon buffer concentration [14]. The data obtained here showed that in the case of other substrates the AOPTP activity varies also on changing the concentration of citrate-phosphate buffer. Moreover, the extent of the effect depends on the chemical nature of the studied substrate (Table 3). So, in the case of ferulic acid and *o*-phenylenediamine the AOPTP activity varies slightly in the buffer concentration range from 10 to 100 mM. The influence of the buffer concentration was more striking for *o*-dianisidine, guaiacol, pyrogallol, and catechol. However, the largest effect of the concentration of buffer

Table 2. pH-Dependence of African oil palm tree peroxidase activity measured with different substrates

pH	Activity, %					
	ABTS	ferulic acid	*o*-dianisidine	*o*-phenylenediamine	guaiacol	catechol
2.8	57	—	—	—	—	—
3.0	100	41	—	—	—	50
3.5	67	—	—	2	—	—
4.0	46	79	20	8	—	72
4.5	9	—	65	40	64	100
5.0	11	100	72	96	95	87
5.5	—	—	100	100	100	34
6.0	—	62	80	94	90	31
6.5	—	—	34	—	72	—
7.0	—	35	30	—	47	19

Note: 10 mM phosphate-citrate buffer was used.

Table 3. Effect of buffer concentration on enzymatic activity of African oil palm tree peroxidase measured with different substrates

Buffer concentration, mM	Activity, %						
	ABTS	ferulic acid	*o*-dianisidine	*o*-phenylenediamine	guaiacol	pyrogallol	catechol
10	62	92	75	95	63	80	86
20	80	94	78	100	88	70	90
30	80	100	86	95	80	80	93
40	76	100	91	82	84	70	100
50	100	98	97	95	83	100	93
70	92	97	97	89	100	70	86
100	18	92	100	92	74	90	80

Note: On varying concentration of phosphate-citrate buffer pH values optimal for each substrate were used.

on the AOPTP activity was obtained for ABTS (Table 3). These results suggest that the studied substrates having different chemical structures react with different regions of the active site of the palm peroxidase.

Under optimal conditions (Table 1) the efficiency of catalytic oxidation of the studied substrates by hydrogen peroxide in the presence of AOPTP was evaluated. It is well known that peroxidase catalysis proceeds by a "ping-pong" mechanism (Eqs. (1)-(3)) with formation of two intermediate compounds, Cpd I and Cpd II:

$$E + H_2O_2 \xrightarrow{k_1} \text{Cpd I} + H_2O, \quad (1)$$

$$\text{Cpd I} + AH_2 \xrightarrow{k_2} \text{Cpd II} + AH^{\cdot}, \quad (2)$$

$$\text{Cpd II} + AH_2 \xrightarrow{k_3} E + AH^{\cdot} + H_2O. \quad (3)$$

The reaction with the lowest rate which limits the overall catalytic process is one of Cpd II with substrate (AH_2) (Eq. (3)). Therefore, to evaluate efficiency of catalysis of peroxidase the value of the second order rate constant (k_3) should be calculated:

Table 4. Comparative substrate specificity of plant peroxidases

Substrate	k_{app}, $\mu M^{-1} \cdot sec^{-1}$						
	African oil palm tree	royal palm tree [16]	soya*	horseradish*	tobacco*	peanut*	lucern*
ABTS	17	52	0.36	4.0	1.1	0.37	1.0
Ferulic acid	18	63	—	—	—	—	—
o-Dianisidine	3.9	0.97	0.39	4.3	2.0	2.0	2.4
o-Phenylenediamine	0.85	2.9	0.04	0.49	0.032	0.22	0.042
Guaiacol	0.21	1.2	0.64	1.6	0.51	2.4	15
Pyrogallol	0.049	0.17	—	—	—	—	—
Catechol	0.049	0.23	—	—	—	—	—

* k_{app} values are calculated from the literature data [8].

$$k_3 = \frac{v}{[\text{Cpd II}]\,[AH_2]}, \quad (4)$$

where v is measuring the rate of oxidation of substrate. Because at optimal concentration of hydrogen peroxide the concentration of compound II is approximately equal to initial concentration of peroxidase [19], the k_3 value will be equal to the k_{app} value:

$$k_3 \approx k_{app} = \frac{v}{[E]_0\,[AH_2]}. \quad (5)$$

A similar approach was previously used successfully in [16, 19].

To minimize the difference between values of Cpd II concentration and initial concentration of peroxidase, the optimal concentration of H_2O_2 should be determined. At H_2O_2 concentration lower than optimal the Cpd II concentration in the reaction medium is decreased and, hence, the k_{app} value is lower. At higher concentrations of H_2O_2 some side reactions produce Cpd III and compound P670 [20] and, hence, due to inactivation of peroxidase a decrease in k_{app} values is observed. In Table 1 optimal concentrations of hydrogen peroxide are presented. These concentrations have been used further for determination of k_{app} values for studying substrates.

Although k_{app} value should not depend on concentration of substrate, in practice this rule is fulfilled only in a substrate concentration range lower than a defined concentration. Use of higher concentrations results in a decrease in k_{app} values. This is observed due to additional side reactions resulting in formation of enzyme–substrate complexes whose composition is $E(AH_2)_n$ and, hence, an inhibition of AOPTP. Thus, for correct determination of k_{app} the optimal concentration of substrate at which inhibition does not occur should be determined for each substrate (Table 1).

The k_{app} values characteristic of the efficiency of the enzyme oxidation of the studied substrates in the presence of AOPTP are presented in Table 4. The poorest substrates are catechol and pyrogallol, phenolic compounds containing two and three hydroxy groups in their chemical structures, respectively. Such compounds as guaiacol (monophenol), *o*-dianisidine, and *o*-phenylenediamine (aromatic amines) are oxidized by AOPTP at a higher rate. However, the best substrates for AOPTP are ABTS and ferulic acid. If the high k_{app} value measured with ABTS (synthetic substrate) has only a theoretical importance, the high value determined with ferulic acid suggests that *in vivo* the anionic AOPTP participates effectively in lignification.

Comparison of k_{app} values determined for peroxidases isolated from leaves of African oil and royal palm trees showed that although AOPTP towards many substrates is a more active and effective catalyst than the latter peroxidase, substrate specificity of these enzymes is similar. Furthermore, comparison of substrate specificity of AOPTP and peroxidases from other plants (Table 4) demonstrate catalytic features of AOPTP that in combination with its high stability mentioned previously create good perspectives for its practical application.

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REFERENCES

1. Tijssen, P. (1985) *Practice and Theory of Enzyme Immunoassay*, Elsevier, Amsterdam.
2. Lindgren, A., Ruzgas, T., Gorton, L., Csöregi, E., Bautista Ardila, G., Sakharov, I. Yu., and Gazaryan, I. G. (2000) *Biosensors Bioelectronics*, **15**, 491-497.

3. Lui, W., Choilli, A. L., Nagarajan, R., Kumar, J., Tripathy, S., Bruno, F. F., and Samuelson, L. (1999) *J. Am. Chem. Soc.*, **121**, 11345-11355.
4. Ferrari, R. P., Laurenti, E., and Trotta, F. (1999) *J. Biol. Inorg. Chem.*, **4**, 232-237.
5. Adam, W., Lazarus, M., Saha-Moller, C. R., Weichold, O., Hoch, U., Haring, D., and Schreier, P. (1999) in *Advances of Biochemical Engineering and Biotechnology* (Scheper, Th., ed.) Vol. 63, Springer-Verlag, Berlin-Heidelberg, pp. 74-108.
6. Egorov, A. M., Gavrilova, E. M., and Sakharov, I. Yu. (2000) in *Integrated Plant Systems* (Greppin, H., et al., eds.) University of Geneva, pp. 369-385.
7. Dunford, H. B. (1999) *Heme Peroxidases*, Willey-VCH, New York.
8. Gazaryan, I. G., Reshetnikova, M. A., Doseeva, V. V., and Bekker, E. G. (1995) *Biochemistry (Moscow)*, **60**, 767-772.
9. Barber, K. R., Rodriguez Maranon, M. J., Shaw, G. S., and van Huystee, R. B. (1995) *Eur. J. Biochem.*, **232**, 825-833.
10. McEldoon, J. P., and Dordick, J. S. (1996) *Biotechnol. Progr.*, **12**, 555-558.
11. Farhangrazi, Z. S., Copeland, B. R., Nakayama, T., Amachi, T., Yamazaki, I., and Powers, L. S. (1994) *Biochemistry*, **33**, 5647-5652.
12. Sakharov, I. Yu., Castillo L. J., Areza, J. C., and Galaev, I. Yu. (2000) *Bioseparation*, **9**, 125-132.
13. Sakharov, I. Yu. (2001) *J. Inorg. Chem.*, **86**, 415.
14. Sakharov, I. Yu. (2001) *Biochemistry (Moscow)*, **66**, 515-519.
15. Rasmussen, Ch. B., Dunford, H. B., and Welinder, K. G. (1997) *Biochemistry*, **36**, 9453-9463.
16. Sakharov, I. Yu., Vesga, B. M. K., Galaev, I. Yu., and Pletyushkina, O. Yu. (2001) *Plant Sci.*, **161**, 853-860.
17. Childs, R. E., and Bardsley, W. G. (1975) *Biochem. J.*, **145**, 93-103.
18. Lui, W., Kumar, J., Tripathy, S., Senecal, K. J., and Samuelson, L. (1999) *J. Am. Chem. Soc.*, **121**, 71-78.
19. Ryabov, A. D., and Goral, V. N. (1997) *J. Biol. Inorg. Chem.*, **2**, 182-190.
20. Nicell, J. A., and Wright, H. (1997) *Enzyme Microb. Technol.*, **21**, 302-310.