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Long-Term Chemiluminescent Signal Is Produced in the Course of Luminol Peroxidation Catalyzed by Peroxidase Isolated from Leaves of African Oil Palm Tree

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Abstract—Optimal conditions were found for the oxidation of luminol by hydrogen peroxide in the presence of peroxidase isolated from leaves of the African oil palm tree *Elaeis guineensis* (AOPTP). The pH range for maximal chemiluminescence intensity (8.3-8.6) is similar for AOPTP, horseradish, and *Arthromyces ramosus* peroxidases and slightly different from that for tobacco peroxidase (9.3). Increasing the buffer concentration decreases the chemiluminescence intensity. As in the case of other anionic peroxidases, the catalytic efficiency of AOPTP does not depend on the presence of enhancers (4-iodophenol and 4-hydroxycinnamic acid) in the reaction medium. The detectable limit of AOPTP assayed by luminol peroxidation is $2 \cdot 10^{-12}$ M. The long-term chemiluminescence signal produced during AOPTP-dependent luminol peroxidation is a characteristic feature of the African oil palm enzyme. This feature in combination with its very high stability suggests that AOPTP will be a promising tool in analytical practice.

Key words: peroxidase, oil palm tree, chemiluminescence, luminol

Peroxidase (EC 1.1.11.7) is one of most widely distributed plant enzymes. *In vivo* it is involved into various physiological processes such as decarboxylation of indolyl-3-acetic acid, cell wall formation, lignification, suberization, etc. [1-4]. Commercially available horseradish peroxidase is widely employed for various practical purposes. It is used for removal from industrial waters of aromatic amines and phenols (including chlorinated phenols), in organic synthesis, and for bleaching of industrial dyestuffs [5-8]. Horseradish peroxidase is widely used in enzyme-linked immunoassay for antibody or antigen labeling [9]. It is also used for detection of nucleic acids [10]. Advantages of the use of peroxidase include its high activity and stability. This is very important both for analyses and for long term storage of peroxidase conjugates.

Oxidation of substrates by hydrogen peroxide with colorimetric detection of the oxidation products is usually employed for the determination of peroxidase activity. Guaiacol, ammonium 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonate), *o*-dianisidine, and pyrogallol are usually used as the substrates [11-14]. However, after the discovery of enhanced chemiluminescence which is observed during luminol oxidation by horseradish peroxidase in the presence of H_2O_2 and enhancers [15], new kits for enzyme-linked immunoassay with chemiluminescent detection of peroxidase activity were developed. This method is sensitive, which explains its wide use. Subsequently, it was shown that anionic peroxidases from tobacco leaves [16] and *Arthromyces ramosus* [17, 18] also effectively catalyze luminol oxidation, and the measured chemiluminescence was almost enhancer-independent. In the present study we have investigated luminol peroxidation catalyzed by a new anionic peroxidase isolated from leaves of the African oil palm tree *Elaeis guineensis*.

MATERIALS AND METHODS

Peroxidase was isolated from leaves of the African oil palm tree *Elaeis guineensis* as described in [19]. The specific activity of the peroxidase with guaiacol as substrate was 4300 U per mg, and the RZ value was 3.0. Luminol-HCl, 4-iodophenol, 4-hydroxycinnamic acid, and Tris were purchased from Sigma (USA). Boric acid and H_2O_2 (30%) were produced by Merck (Germany).

Catalytic luminol oxidation was assayed as follows. Components of the reaction mixture (250 μl of 10-100 mM Tris or borate buffer containing 0.1-8.0 mM hydrogen peroxide and 2-14 mM luminol) were added to opaque wells of teflon plates (Dynatech, USA) and the reaction was initiated by adding peroxidase solution. The kinetics of chemiluminescence was measured at room temperature using a luminometer (Amersham, USA). The effect of enhancers on the chemiluminescence of the catalytic

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luminol oxidation was studied using 0.5 mM 4-iodophenol and 4-hydroxycinnamic acid.

RESULTS AND DISCUSSION

Peroxidases catalyze the oxidation of luminol by hydrogen peroxide. The overall process can be illustrated by the following scheme:

$$E + H_2O_2 \rightarrow EI + H_2O ,$$

$$EI + L \rightarrow EII + L^{\cdot} ,$$

$$EII + L \rightarrow E + L^{\cdot} + H_2O ,$$

where E, EI, and EII are the ferric peroxidase and intermediate compounds of peroxidase, and L and $L^{\cdot}$ are luminol and its radical product of one electron oxidation, respectively. The radical product of luminol oxidation is then converted into 3-aminophthalate, and this process results in light emission [20].

This reaction has been studied for various peroxidases. Horseradish peroxidase is a poor catalyst of this reaction [21], and increase of its efficacy requires the presence of so-called "enhancers". For example, addition of some phenols (4-iodophenol, 4-hydroxycinnamic acid, etc.) in the reaction medium is accompanied by a sharp (~1,000-fold and even more) increase in chemiluminescence intensity [15, 21]. This phenomenon, known as the "enhanced chemiluminescence" reaction is employed in many diagnostic kits.

Later chemiluminescent luminol oxidation was studied using anionic peroxidases isolated from tobacco leaves and *Arthromyces ramosus*. In contrast to the basic horseradish peroxidase (p*I* 8.9), anionic peroxidases catalyze luminol oxidation more effectively and they are almost insensitive to enhancers [16-18, 22].

We recently isolated and partially characterized a new anionic peroxidase from oil palm leaves [19]. Since the optimal catalytic conditions for various peroxidases are different, it was important to optimize the conditions for luminol oxidation catalyzed by AOPTP. The maximal chemiluminescence measured in Tris-buffer was registered at pH 8.3-8.6 (Fig. 1). The same pH optimum was found for this reaction catalyzed by *Arthromyces ramosus* peroxidase and also for the reaction of enhanced chemiluminescence catalyzed by horseradish peroxidase [18]. This value differed slightly from the pH optimum for tobacco peroxidase (9.3) [16]. Substitution of Tris for borate did not influence the pH optimum or chemiluminescent intensity of the palm tree peroxidase (data not shown).

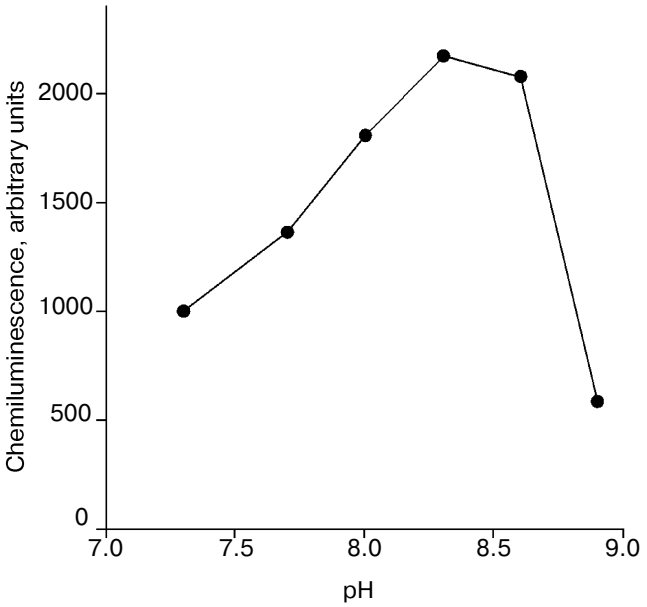

Fig. 1. The pH dependence for chemiluminescence intensity during luminol oxidation by hydrogen peroxide catalyzed by oil palm tree peroxidase. The reaction medium contained 20 mM Tris buffer, 20 pM peroxidase, 9 mM luminol, and 4 mM H_2O_2. Chemiluminescence intensity was registered 42 min after the start of the reaction.

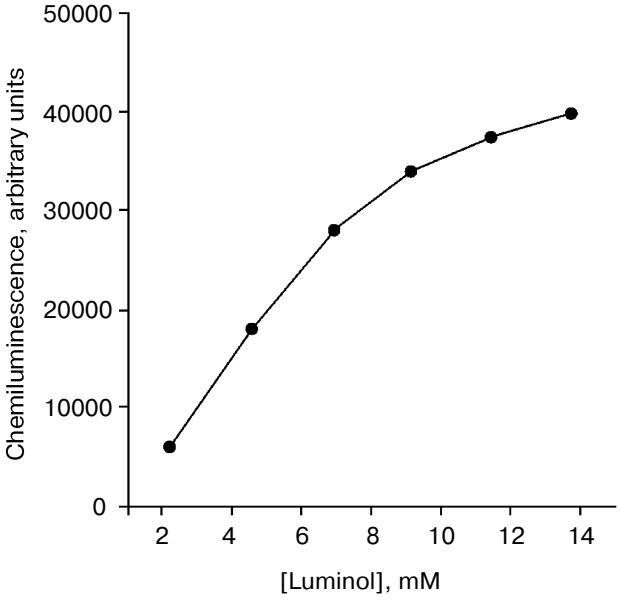

Fig. 2. Dependence of chemiluminescence intensity during luminol oxidation by hydrogen peroxide catalyzed by palm tree peroxidase on luminol concentration. The reaction medium contained 100 mM Tris-buffer, pH 8.3, 180 pM peroxidase, and 4 mM H_2O_2. Chemiluminescent intensity was registered 42 min after the start of the reaction.

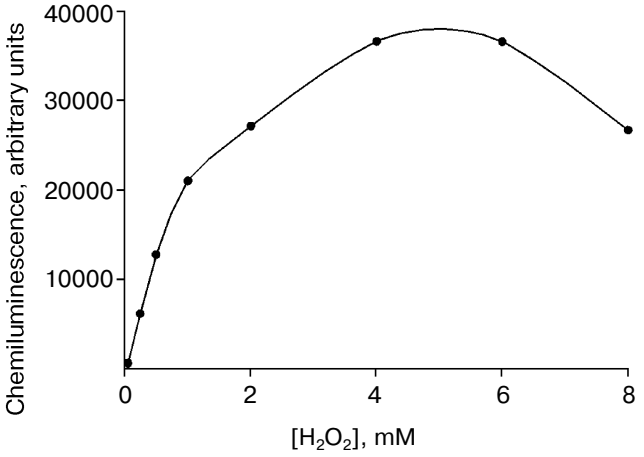

Fig. 3. Dependence of chemiluminescence intensity during luminol oxidation by hydrogen peroxide catalyzed by palm tree peroxidase on hydrogen peroxide concentration. The reaction medium contained 100 mM Tris-buffer, pH 8.3, 180 pM peroxidase, and 9 mM luminol. Chemiluminescence intensity was registered 42 min after the start of the reaction.

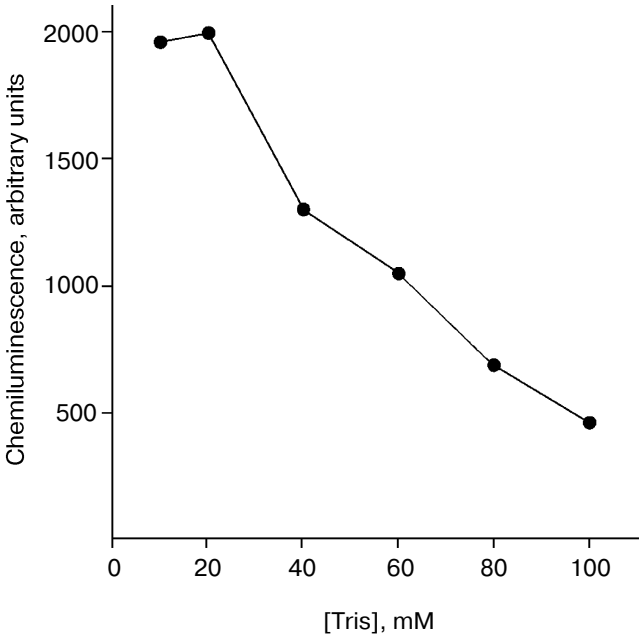

Fig. 4. Dependence of chemiluminescence intensity during luminol oxidation by hydrogen peroxide catalyzed by palm tree peroxidase on concentration of Tris-buffer, pH 8.3. The reaction medium contained 30 pM peroxidase, 9 mM luminol, and 4 mM H_2O_2. Chemiluminescent intensity was registered 20 min after the start of the reaction.

Increasing the luminol concentration in the reaction mixture increased the chemiluminescent signal (Fig. 2). Within the range of luminol concentrations 2-14 mM, the intensity curve tended to saturation. However, due to the limited solubility of luminol, the luminol concentration could not be increased further. In subsequent experiments 9-14 mM luminol concentrations were used.

The dependence of chemuliminescence on hydrogen peroxide concentration was characterized by a maximum at 4-6 mM H_2O_2 (Fig. 3). Further increase in the hydrogen peroxide concentration reduced the chemiluminescence intensity. This is consistent with the well-known fact that hydrogen peroxide is a suicide substrate for peroxidases [23, 24].

The optimal substrate concentrations for the palm tree peroxidase are slightly higher than corresponding values for maximal catalytic efficiency of tobacco peroxidase (2.5 mM luminol, 2 mM H_2O_2 [16]) and *Arthromyces ramosus* peroxidase (1-2 mM luminol, 0.75 mM H_2O_2 [18]).

Buffer concentration was crucial for the manifestation of chemiluminescence intensity during luminol oxidation catalyzed by the palm tree peroxidase (Fig. 4). The intensity of chemiluminescence increased with decreasing buffer concentration, and 10-20 mM Tris-buffer was optimal for maximal chemiluminescence. A similar effect was found with ammonium 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonate) as the substrate for colorimetric detection of the palm tree peroxidase activity (Sakharov, unpublished data). This phenomenon has not been reported for other peroxidases.

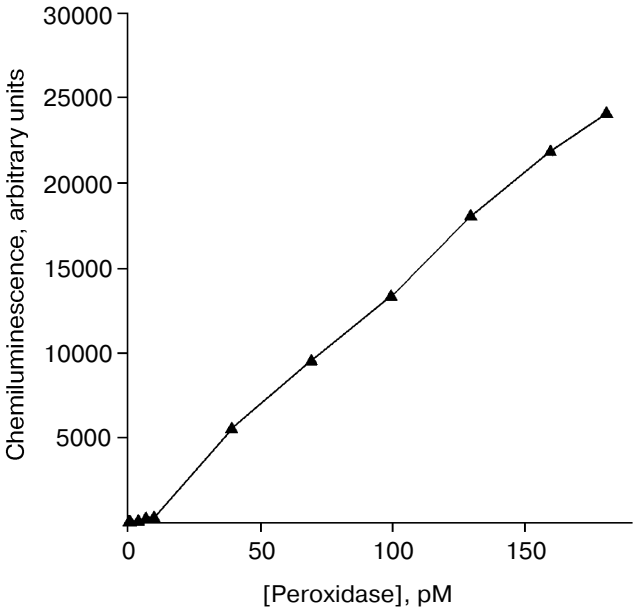

Fig. 5. Sensitivity of the reaction of luminol oxidation by hydrogen peroxide to palm tree peroxidase concentration. The reaction medium contained 100 mM Tris-buffer, pH 8.3, 9 mM luminol, and 4 mM H_2O_2. Chemiluminescence intensity was registered 120 min after the start of the reaction.

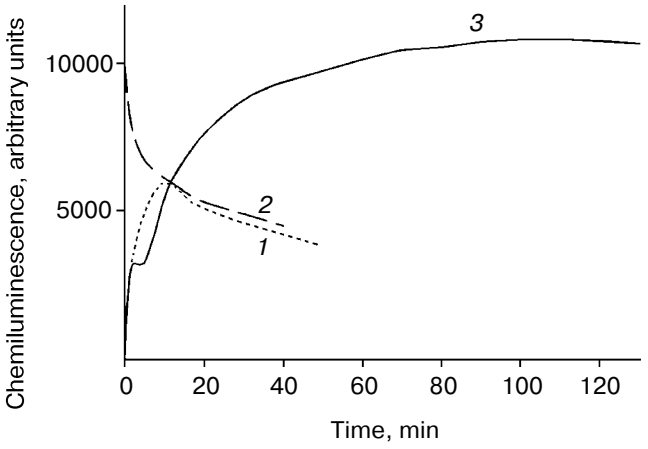

Fig. 6. Typical kinetic curves of chemiluminescence intensity during luminol oxidation by hydrogen peroxide catalyzed by horseradish (*1*), tobacco (*2*), and palm tree (*3*) peroxidases. Data for the horseradish and tobacco enzymes were taken from [16].

Anionic peroxidases are characterized by low sensitivity to enhancers, which can only slightly increase (by 1.5-2-fold) the chemiluminescence [16-18]. The palm tree peroxidase is also characterized by a lack of significant effect of enhancers. For example, in the presence of 0.5 mM 4-iodophenol, the maximal activating effect was 40%, and 4-hydroxycinnamic acid even slightly inhibited (by 10%) the chemiluminescence intensity. Thus, the palm tree anionic peroxidase (p*I* 3.5), like other anionic peroxidases, does not catalyze the reaction of enhanced chemiluminescence.

Optimization of the conditions for enzymatic luminol oxidation allowed the determination of the lower detection limit for the palm tree peroxidase. The latter corresponds to the enzyme concentration required for chemiluminescence twice that of the chemiluminescence of the same solution but without the peroxidase. Peroxidase concentration varied in the range 0.5-180 pM (Fig. 5). The detectable limit of peroxidase was 2 pM. This value slightly exceeds the corresponding parameter determined earlier for horseradish and tobacco peroxidase (1 and 0.1 pM, respectively) [16]. Nevertheless, the sensitivity of this reaction is quite reasonable for use of the palm tree peroxidase in enzyme-linked immunoassay. The linear dependence between concentration of the palm tree enzyme and chemiluminescence intensity over a wide concentration range (10-180 pM) is also an important feature for the practical use of the enzyme.

Figure 6 shows typical kinetic curves of luminol oxidation by hydrogen peroxide in the presence of three different peroxidases. They are characterized by different behavior. For example, in the case of enhanced chemiluminescence in the presence of horseradish peroxidase, the chemiluminescence intensity increases, reaches a maximum, and then decreases (curve *1*, Fig. 6). The decrease in chemiluminescence intensity can be attributed to inactivation of horseradish peroxidase by products of oxidation of enhancers and luminol [25]. In the case of tobacco peroxidase, the initial increase in chemiluminescence intensity is faster (curve *2*, Fig. 6) [16].

In the case of the palm tree peroxidase as the catalyst of luminol oxidation, the intensity of chemiluminescence increased slower, but after reaching a maximum it remained unchanged over a long time interval (curve *3*, Fig. 6). This is an obvious advantage of the palm tree peroxidase over the other peroxidases studied. Taking into consideration its very high stability, the palm tree enzyme is a promising tool for analytical use.

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REFERENCES

1. Lamport, D. T. A (1986) in *Molecular and Physiological Aspects of Plant Peroxidases* (Greppin, H., Penel, C., and Gaspar, T., eds.) University of Geneva, pp. 199-208.
2. Fry, S. C. (1986) *Annu. Rev. Plant Physiol.*, **37**, 165-186.
3. Moerschbacher, B. M. (1992) in *Plant Peroxidases 1980-1990. Topics and Detailed Literature on Molecular, Biochemical, and Physiological Aspects* (Penel, C., Gaspar, T., and Greppin, H., eds.) University of Geneva, pp. 91-99.
4. Gazaryan, I. G. (1992) in *Advances in Science and Technology. Biotechnology Series* [in Russian], Vol. 36, VINITI, Moscow, pp. 4-27.
5. Kauffmann, C., Petersen, B. R., and Bjerrum, M. J. (1999) *J. Biotechnol.*, **73**, 71-74.
6. Ferrari, R. P., Laurenti, E., and Trotta, F. (1999) *J. Biol. Inorg. Chem.*, **4**, 232-237.
7. Adam, W., Lazarus, M., Saha-Moller, C. R., Weichold, O., Hoch, U., Haring, D., and Schreier, P. (1999) in *Adv. Biochem. Engineering Biotechnology* (Scheper, Th., ed.) Vol. 63, Springer-Verlag, Berlin-Heidelberg, pp. 74-108.
8. Kim, S. J., and Shoda, M. (1999) *Appl. Environ. Microbiol.*, **65**, 1029-1035.
9. Tijssen, P. (1985) *Practice and Theory of Enzyme Immunoassay*, Elsevier, Amsterdam.
10. Reddy, L. V., DeSilva, R., Handley, R. S., Schaap, A. P., and Akhavan-Tafti, H. (1999) *Biotechniques*, **26**, 710-714.
11. Sakharov, I. Yu., and Bautista, A. G. (1999) *Food Chemistry*, **65**, 51-54.
12. Abelskov, A. K., Smith, A. T., Rasmussen, Ch. B., Dunford, H. B., and Welinder, K. G. (1997) *Biochemistry*, **36**, 9453-9460.
13. Ugarova, N. N., Rozhkova, G. D., and Berezin, I. V. (1979) *Biochim. Biophys. Acta*, **570**, 31-36.
14. Kwak, S.-S., Kim, S.-K., Lee, M.-S., Jung, K.-H., Park, I.-H., and Liu, J.-R. (1995) *Phytochemistry*, **39**, 981-984.

15. Thorpe, G. H. G., and Kricka, L. J. (1986) *Meth. Enzymol.*, **133** (Pt. B), 331-353.
16. Gazaryan, I. G., Rubtsova, M. Yu., Kapeliuch, Yu. L., Rodriguez-Lopez, J. N., Lagrimini, L. M., and Thorneley, R. N. F. (1998) *Photochem. Photobiol.*, **67**, 106-110.
17. Akimoto, K., Shinmen, Y., Sumida, M., Asami, S., Amachi, T., Yoshizumi, H., Saeki, Y., Shimizu, S., and Yamada, H. (1990) *Analyt. Biochem.*, **189**, 182-185.
18. Kim, B. B., Pisarev, V. V., and Egorov, A. M. (1991) *Analyt. Biochem.*, **199**, 1-6.
19. Sakharov, I. Yu., Castillo, L. J., Areza, J. C., and Galaev, I. Yu. (2000) *Bioseparation*, **9**, 125-132.
20. Thorpe, G. H. G., Kricka, L. J., Moseley, S. R., and Whitehead, T. P. (1985) *Clin. Chem.*, **31**, 1335-1341.
21. Whitehead, T. P., Thorpe, G. H. G., Carter, T. J. N., Groucutt, C., and Kricka, L. J. (1983) *Nature*, **305**, 159-160.
22. Nakamura, M., and Nakamura, S. (1998) *Free Radical Biol. Med.*, **24**, 537-544.
23. Arnao, M. B., Acosta, M., del Rio, J. A., and Garcia-Canovas, F. (1990) *Biochim. Biophys. Acta*, **1038**, 85-89.
24. Baynton, K. J., Bewtra, J. K., Biswas, N., and Taylor, K. E. (1994) *Biochim. Biophys. Acta*, **1206**, 272-278.
25. Kapeliuch, Yu. L., Rubtsova, M. Yu., and Egorov, A. M. (1997) *J. Biolum. Chemilum.*, **12**, 299-308.