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Soybean Peroxidase-Catalyzed Oxidation of Luminol by Hydrogen Peroxide

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Anionic soybean peroxidase *Glycine max* (SbP) is shown to efficiently catalyze luminol oxidation by hydrogen peroxide. Contrary to horseradish peroxidase, the presence of *p*-iodophenol in the reaction medium affects slightly the efficiency of SbP catalysis. A maximal intensity of chemiluminescence, produced through this enzymatic reaction, was detected at pH 8.4−8.6. Contrary to anionic palm tree peroxidase, in the presence of SbP, chemiluminescence intensity increases with the reaction buffer concentration. The detection limit of SbP in the reaction of luminol oxidation is 0.3×10^{-12} M. Therefore, high sensitivity in combination with the long-term chemiluminescent signal is indicative of good prospects for application of this enzyme in enzyme immunoassay with chemiluminescent detection.

KEYWORDS: Peroxidase; soy; chemiluminescence; luminol; hydrogen peroxide; enhancement

INTRODUCTION

Peroxidase (EC 1.1.11.7) is one of the most widely distributed plant enzymes. In vivo this enzyme is involved in various physiological processes, such as the decarboxylation of indolyl-3-acetic acid, cell wall formation, lignification, and suberization (*1*−*3*). Peroxidases have been isolated from various plant sources (*4*−*8*), but only the cationic isoenzyme *c* of the peroxidase purified from horseradish roots (HRP-C) has been studied in detail.

Presently, HRP-C is widely used in practice. This enzyme is applied for the removal of aromatic amines and phenols from industrial waters, in organic synthesis, for bleaching of industrial dyestuffs, etc. (*9*−*12*). However, the widest application of the peroxidase is in an enzyme-linked immunosorbent assay (ELISA) as enzyme for antibody or antigen labeling (*13*). In ELISA, the peroxidase activity is commonly measured using the reactions of substrate oxidation by hydrogen peroxide with colorimetric detection of the obtained products. As substrates, tetramethylbenzidine and *o*-phenylenediamine are usually used. However, after discovery of the "enhanced chemiluminescence reaction" (ECR), based on HRP-C-catalyzed oxidation of luminol in the presence H_2O_2 and enhancers (*14*), various immunoenzymatic kits with chemiluminescence detection of peroxidase activity have been developed. A wide use of ECR is due to its higher sensitivity compared to immunological methods based on colorimetric detection. It should be noted that in the absence of enhancers, HRP-C produces a low chemiluminescent signal. The mode of action of enhancers, among which *p*-iodophenol is the most popular, was reviewed in detail previously (*15*, *16*).

In a kinetic study of enzymatic oxidation of luminol by hydrogen peroxide the detected intensity of chemiluminescence is unstable. At the first step, for a short time, the intensity increases, reaching its maximum, and then quenches quickly. Later it was shown (*16*) that chemiluminescence quenching is a result of chemical inactivation of HRP-C by radical products forming through the reaction. The change in the intensity causes the overestimation of analytes using ECR.

On the other hand, recently (*17*) we showed that the luminol oxidation by hydrogen peroxide could be efficiently catalyzed by anionic peroxidase isolated from leaves of African oil palm tree (AOPTP) in the absence of any enhancer. Furthermore, the feature of this peroxidase is the production of a long-term signal of chemiluminescence through luminol oxidation. Therefore, this fact permits us to look forward to the construction of new highly sensitive immunoenzymatic kits without using enhancers and stabilizing additives. This raises the question of whether only AOPTP can oxidize luminol in the absence of enhancers producing the long-term chemiluminescence signal or whether other anionic peroxidases can catalyze luminol oxidation in the same manner. To resolve this, the present paper describes the reaction of oxidation of luminol by hydrogen peroxide catalyzed by commercially available anionic peroxidase isolated from shells of soybeans (*Glycine max*).

MATERIALS AND METHODS

Peroxidases of soybeans (RZ 1.5) and horseradish roots (RZ 3.0) were purchased from Enzymol International (Columbus, OH) and Biozyme (Blaenavon, Wales) and used without further purification.

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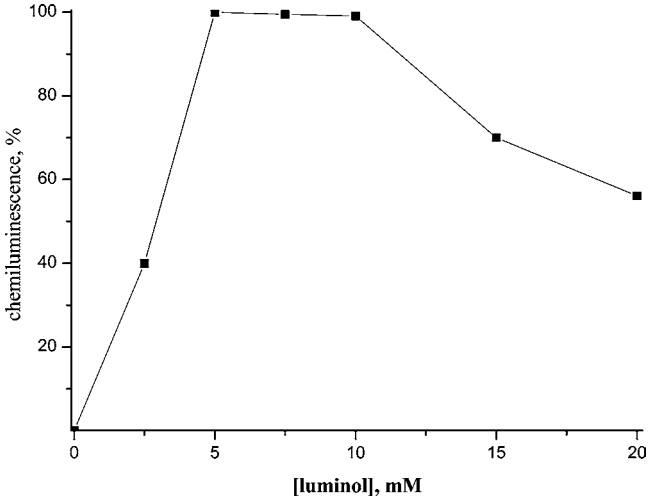

Figure 1. Effect of luminol concentration in reaction medium on chemiluminescence intensity produced through soybean peroxidase-catalyzed oxidation of luminol by hydrogen peroxide. Conditions: [peroxidase] = 2.5×10^{-10} M; [H_2O_2] = 8 mM; 100 mM Tris buffer (pH 8.4); chemiluminescence intensity was recorded 60 min after the start of the reaction.

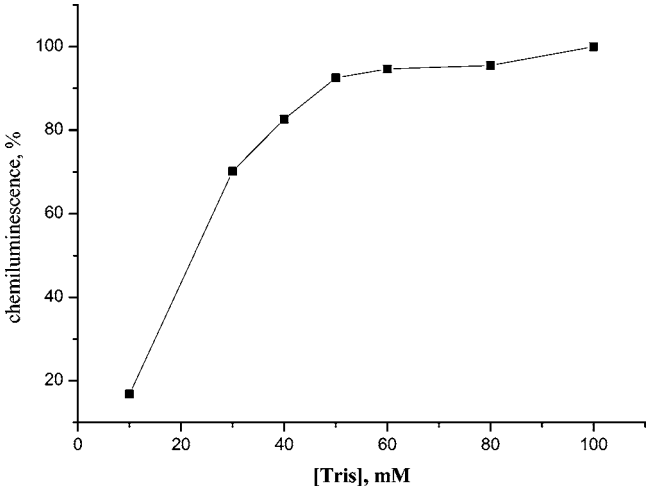

Figure 2. Effect of Tris buffer concentration on chemiluminescence intensity produced through soybean peroxidase-catalyzed oxidation of luminol by hydrogen peroxide. Conditions: [peroxidase] = 2.5×10^{-10} M; [luminol] = 10 mM; [H_2O_2] = 8 mM; Tris buffer (pH 8.4); chemiluminescence intensity was recorded 80 min after the start of the reaction.

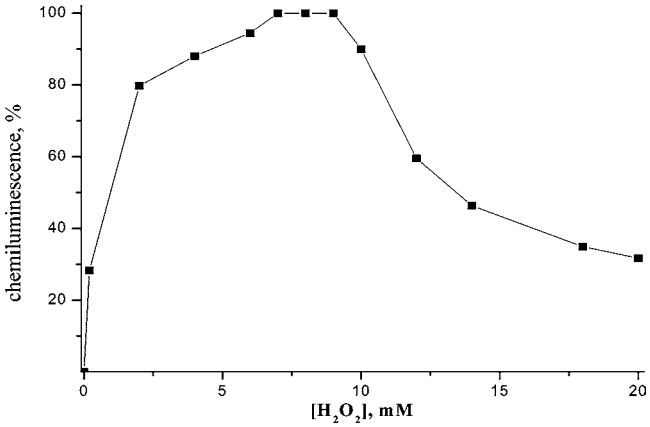

Figure 3. Effect of H_2O_2 concentration in reaction medium on chemiluminescence intensity produced through soybean peroxidase-catalyzed oxidation of luminol by hydrogen peroxide. Conditions: [peroxidase] = 2.5×10^{-10} M; [luminol] = 10 mM; 100 mM Tris buffer (pH 8.4); chemiluminescence intensity was recorded 60 min after the start of the reaction.

Luminol−HCl, *p*-iodophenol, and Tris were obtained from Sigma (St. Louis, MO), and H_2O_2 (30%) was from Merck (Darmstadt, Germany).

Catalytic luminol oxidation was assayed as follows: 250 μL of 10−100 mM Tris (pH 7.6−9.0) containing 0.1−20 mM hydrogen peroxide and 2.5−20 mM luminol were added into wells of transparent polystyrene strips or 96-well polystyrene microtiter plates (MaxiSorp plates, Nunc, Roskilde, Denmark) for enzyme immunoassay, and then the reaction was initiated by adding peroxidase solution. Chemiluminescence kinetics was measured at room temperature using a LumiScan luminometer (Immunotech, Russia).

RESULTS AND DISCUSSION

Anionic soybean peroxidase was purified first by Gillikin and Graham in 1991 (*18*). This enzyme is a glycoprotein with a molecular mass of 37000 Da and belongs to a family of secretory plant peroxidases (*19*). Like other secretory peroxidases, it catalyzes oxidation of its substrates according to the "ping-pong" mechanism:

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

$$EI + A \rightarrow EII + A^{\bullet}$$

$$EII + A \rightarrow E + A^{\bullet} + H_2O$$

where E, EI, and EII indicate peroxidase and peroxidase intermediate compounds and A and $A^{\bullet}$ 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*).

For the determination of favorable conditions for SbP-catalyzed oxidation of luminol by hydrogen peroxide, it was shown that a maximal value of chemiluminescence was detected when the reaction proceeded at pH 8.4−8.6. This value is equal to those for the same reaction catalyzed by AOPTP and HRP-C (*17*, *21*). However, not all plant peroxidases show the same pH optimum. Therefore, for tobacco peroxidase, the value of the pH optimum is higher (pH 9.3) (*22*).

For the optimization of luminol concentration, it was found that contrary to AOPTP, for which the chemiluminescent signal increased with luminol concentration in reaction medium reaching saturation, this reaction had another character for SbP (**Figure 1**). Therefore, in the concentration interval up to 5 mM luminol, the chemiluminescence intensity increases with the substrate concentration in the feed. At further increase of the substrate concentration to 12 mM, the variation of the luminol concentration does not affect the chemiluminescence. When luminol solutions with concentrations >12 mM are used, the intensity gradually decreases. On the basis of the obtained results, luminol was used at the concentration of 10 mM in the following work.

Earlier in the study of the same reaction catalyzed by palm peroxidase, the effect of buffer concentration on chemiluminescence intensity was reported (*17*). There the intensity increased with decreasing buffer concentration and reached its maximum in 10−20 mM Tris solutions. Studying SbP, we found a reverse dependence, where the light intensity increased with the buffer concentration, reaching its maximum in 60−100 mM Tris buffer (**Figure 2**). The reasons for such inversion are not

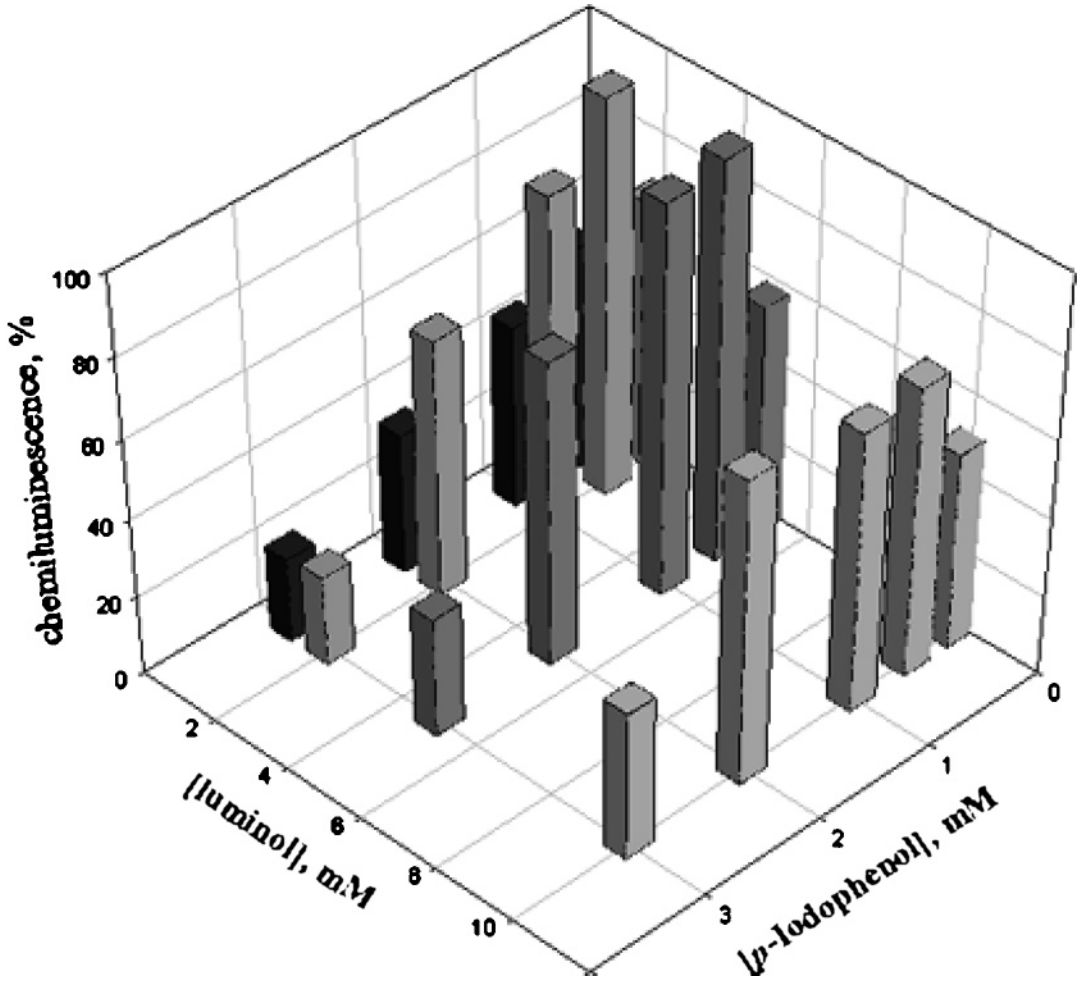

Figure 4. Effect of luminol and enhancer (*p*-iodophenol) concentrations in reaction medium on chemiluminescence intensity produced through soybean peroxidase-catalyzed oxidation of luminol by hydrogen peroxide. Conditions: [peroxidase] = 1.7×10^{-10} M; [H_2O_2] = 8 mM; 100 mM Tris buffer (pH 8.4); chemiluminescence intensity was recorded 15 min after the start of the reaction.

yet clear, but the obtained results allow the reaction of SbP-catalyzed oxidation of luminol in concentrated buffers to be carried out effectively, which is an advantage of this enzyme for its application in ELISA.

In the presence of SbP, the variation of hydrogen peroxide concentration affects chemiluminescence intensity. The maximum chemiluminescent signal was shown to be measured at a H_2O_2 concentration of ~8 mM (**Figure 3**). Further increase in the concentration of this substrate in the reaction medium attenuates the light intensity. It should be emphasized that the use of SbP as a catalyst of luminol oxidation allows using peroxide solutions at higher concentrations than for other peroxidases (*17*, *21*, *22*).

As mentioned above, HRP-C can catalyze the luminol oxidation only in the presence of enhancers. These compounds induced up to a 1000-fold increase in light intensity over the unenhanced reactions (*14*). Contrary to HRP-C, SbP is only slightly sensitive to enhancers. At varying *p*-iodophenol concentrations from 0.1 to 3.0 mM in the reaction medium, the chemiluminescent signal slightly changed (**Figure 4**). The maximum enhancement effect, observed in the presence of 0.5−1.0 mM *p*-iodophenol, was 3.7 times (**Figure 5**, curves a and c). The ability to efficiently catalyze the luminol oxidation appears to be a feature of all anionic peroxidases (*14*, *17*, *22*).

The kinetic curves of luminol oxidation by hydrogen peroxide, catalyzed by SbP and HRP-C, are presented in **Figure 5**. The catalysis by the peroxidases, measured under favorable conditions for each enzyme, was carried out in the presence and absence of *p*-iodophenol as enhancer. As reported in the literature, in the absence of the enhancer, HRP-C does not produce chemiluminescence (**Figure 5**, curve a). For ECR in the presence of HRP-C, the intensity reaches its maximum for a short time and then begins to quickly drop (**Figure 5**, curve b). In the presence of SbP the kinetic curves for change in chemiluminescence intensity show an alternative character (**Figure 5**, curves c and d). As can be seen, both in the presence and absence of the enhancer, the chemiluminescent signal, obtained in the SbP-catalyzed reaction, changed little with time. Furthermore, although in the presence of *p*-iodophenol the maximum value of the chemiluminescent signal was slightly higher, in absence of the enhancer it was more stable. Because in enzyme immunoassay the precision of analysis depends on the accuracy of the measurements, we believe that the use of SbP-catalyzed reaction, carried out in the enhancer absence, is more preferable than that with the enhancer.

The mean standard curve of SbP using chemiluminescent detection under the favorable conditions is present in **Figure 6**. The relative standard deviation (RSD) and recovery values at SbP concentrations of 10, 40, and 100 pM were 5 and 101.0 ± 5.0%, 8.8 and 108.0 ± 9.5%, and 9.1 and 106.1 ± 9.6%, respectively. The best fitting of the experimental data was achieved with the linear function $y = 440x - 3240$ ($r^2 = 0.993$, $n = 6$). The detection limit for SbP was defined as the enzyme concentration required for chemiluminescence twice

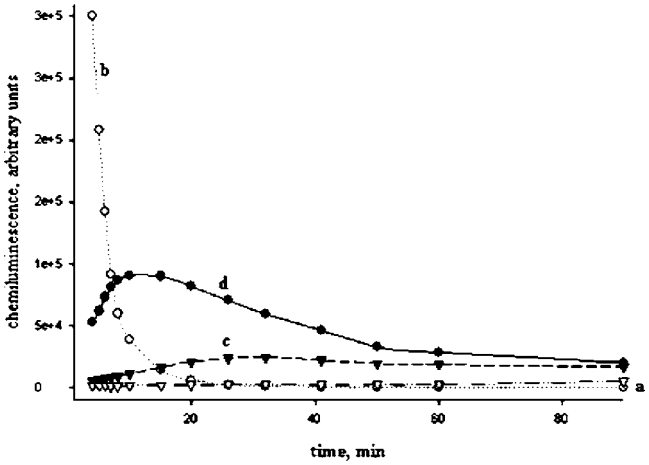

Figure 5. Kinetic curves of chemiluminescence intensity through luminol oxidation by hydrogen peroxide in the presence of horseradish and soybean peroxidases. Conditions: *(in the presence of enhancer)* (curve b) [HRP-C] = 1.8×10^{-10} M, [H_2O_2] = 1.5 mM, [luminol] = 2 mM, 50 mM Tris buffer (pH 8.4), [*p*-iodophenol] = 1 mM; (curve d) [SbP] = 1.7×10^{-10} M, [H_2O_2] = 8 mM, [luminol] = 4 mM, 100 mM Tris buffer (pH 8.4), [*p*-iodophenol] = 1 mM; *(in the absence of enhancer)* (curve a) [HRP-C] = 1.8×10^{-10} M, [H_2O_2] = 1.5 mM, [luminol] = 2 mM, 50 mM Tris buffer (pH 8.4); (curve c) [SbP] = 1.7×10^{-10} M, [H_2O_2] = 8 mM, [luminol] = 4 mM, 100 mM Tris buffer (pH 8.4).

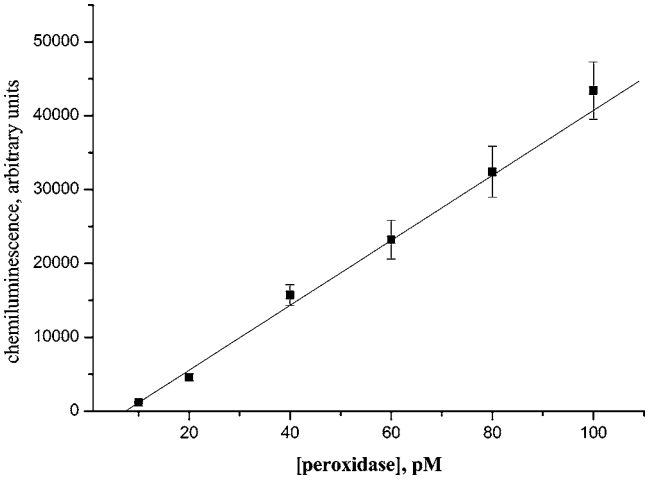

Figure 6. Calibration curve for determination of soybean peroxidase using the reaction of luminol oxidation. Conditions: [H_2O_2] = 8 mM; [luminol] = 10 mM; 100 mM Tris buffer (pH 8.4); chemiluminescence intensity was recorded 60 min after the start of the reaction.

that of the chemiluminescence of the same solution but without the enzyme. The detection limit value for SbP (0.3 pM) is less than those reported previously for HRP-C (1 pM for ECR) and AOPTP (2 pM in enhancer absence) and is near that reported for tobacco peroxidase (0.1 pM) (*22*). It should be also noted that the dependence of SbP concentration versus the light intensity is linear in a sufficiently wide interval of the enzyme concentration. Therefore, the results obtained allow us to look forward to successful application of SbP in the enzyme immunoassay with chemiluminescent detection.

Thus, the present work demonstrated that SbP was able to catalyze luminol oxidation. This reaction can be carried out with similar efficiencies both in the presence and in the absence of *p*-iodophenol. Furthermore, the chemiluminescent signal, formed through the enzymatic reaction, did not change with reaction time. Comparison of the obtained results with those reported previously for other anionic peroxidases showed that, although catalyses of luminol oxidation by the two anionic peroxidases were similar, each peroxidase had some advantageous features.

ABBREVIATIONS USED

SbP, soybean peroxidase; HRP-C, isozyme *c* of horseradish peroxidase; AOPTP, African oil palm tree peroxidase; ELISA, enzyme-linked immunosorbent assay; ECR, enhanced chemiluminescence reaction.

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