QUANTITATIVE CHEMILUMINESCENT DETECTION OF PLANT PEROXIDASES USING A COMMERCIAL KIT ORIGINALLY DESIGNED FOR BLOTTING ASSAYS

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Introduction

Peroxidases are widely spread enzymes in plants, microbes and animal tissues. The broad substrate specifity, multifunctional properties and availability of peroxidases from different sources allows us to apply these enzymes in various biological and biochemical processes. From this point of view horseradish peroxidase (HRP, EC 1.11.1.7)) is the most studied and used enzyme. Colorimetric[1], electrochemical[2] and chemiluminescent methods[3,4] are used for the detection of peroxidase activity. Classical colorimetric methods are generally based on the monitoring of the formation of a coloured product from a colourless oxygen acceptor[5]. These compounds include benzidine, *o*-tolidine, *o*-toluidine, pyrogallol, *o*-dianisidine, *o*-phenylendiamine, guaiacol, 4-chloro-1-naphthol and many others. The chemiluminescent method is based on the oxidation of cyclic diacylhydrazides accompanied by the emission of light[6]. One of the most clearly understood systems is the HRP/hydrogen peroxide-catalysed oxidation of luminol in alkaline conditions. Disadvantages of the normal chemiluminescent method are overcome by the enhanced chemiluminescent method (ECL). ECL is achieved by performing the oxidation of luminol with HRP in the presence of chemical enhancers (e.g. certain phenols, naphthols, etc.)[7]. The main advantages of ECL are that the light emission is intense, easily measured and the peroxidase activity can be assayed in seconds. Also in this field the most studied peroxidase is HRP. ECL has provided the basis for a convenient and sensitive assay for peroxidase and peroxidase conjugates as a qualitative endpoint reaction in blotting assays, DNA – probe assays and immunoassays.

In the present study we describe the use of the ECL method for the estimation of peroxidase activity using the commercial ECL Amersham kit originally designed for qualitative Western blotting detections. This kit was used for the analysis of HRP and peroxidases isolated from plant cells of different plant species cultivated *in vitro*. Chemiluminescent detection was compared with photometric detection of peroxidase activity using two chromogenic substrates, guaiacol and pyrogallol.

Experimental

Materials and Methods

Plant cells: Strains of *in vitro* cultures of various species – *Solanum aviculare* KK1N, *Solanum nigrum* SNC9O, *Atropa belladonna* R1BC and *Armoracia rusticana* K62K were obtained from the Collection of Plant Tissue Cultures of the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the ČR. The plant cells were cultivated on the medium of Linsmeier and Skoog [8] at 26 °C in the dark[9].

Peroxidase extraction: Soluble peroxidase activities were determined in crude extracts obtained by the procedure already described[10].

Photometric procedures: Guaiacol and pyrogallol (both Sigma) were used for the detection of activity of commercial HRP (Sigma) and the estimation of the reproducibility of photometric method in comparison with ECL analysis. The reaction with both chromogenic substrates was measured at 25 °C. Reaction mixtures prepared with guaiacol contained 2.6 ml of 13 mM guaiacol in 0.1 M phosphate buffer pH 6.5, 0.1 ml of 5 mM-H_2O_2 and 0.1 ml of the enzyme[11]. The pyrogallol mixture was prepared according to the Sigma Manual and it contained 0.32 ml of 5 % pyrogallol, 0.16 ml of 147 mM-H_2O_2, 0.32 ml of phosphate buffer pH 6.5, 2.1 ml H_2O and 0.1 ml of the enzyme. In both cases reactions were initiated by the addition of the HRP sample and the change in absorbance was continuously monitored at 470 nm with guaiacol, and at 590 nm with pyrogallol.

ECL procedure: Reagents A and B of the Amersham kit designed for Western blotting were mixed in the ratio 1:1 and 20 µl of this mixture was added to 200 µl of glycine – NaOH buffer pH 10. The chemiluminescent reaction was started by the addition of 20 µl of the peroxidase sample of appropriate dilution. Luminescence expressed in RLU (relative luminescent units) of the reaction mixture was measured immediately after addition of the sample of peroxidase during several seconds with a luminometer Biocounter 1500 (Celsis LUMAC, The Netherlands).

Results and Discussion

The reproducibility of three different methods for detection of peroxidase activity was compared. Peroxidase activity was estimated using guaiacol or pyrogallol as substrates for peroxidase or by the chemiluminescent method (see Table I). As a standard sample of peroxidase pure HRP purchased from Sigma Company in lyophilised form was used. It was dissolved in 0.1 mol.l^{-1} phosphate buffer pH 6.4 at an initial concentration of 1 mg protein/ml. This solution was further diluted according to the requirements of the chosen methods.

The data in Table I show that the lowest standard deviation of the measurement of peroxidase activity was obtained by the chemiluminescent method. This method has other advantages, it is very sensitive[3,4,12] and, compared to the spectrophotometric method, coloured samples do not interfere and the whole procedure is much faster. Using the reagents of the kit (A and B) purchased from Amersham the error of the method which normally needs fresh and individual mixing of the reagents before each procedure (luminol with the enhancer solutions

Table I
Comparison of the reproducibility of spectrophotometric and chemiluminescent methods for the determination of horseradish peroxidase (HRP) activity. (Original concentration of the commercial HRP was 1 mg.ml^{-1} diluted in phosphate buffer pH 6.4, activities were estimated as described in Materials and Methods.)

Spectrophotometric		Chemiluminescent
guaiacol (nkat/mg protein)	pyrogallol (pgl.U[a]/mg protein)	luminol (RLU[b]/mg protein)
1147	18.8	4.6×10^{10}
1086	18.4	4.5×10^{10}
1142	23.1	4.4×10^{10}
964	28.2	4.0×10^{10}
1297	24.1	4.2×10^{10}
1178	23.1	4.4×10^{10}
$\phi^c = 1159\pm84.5$	$\phi^c = 22.6\pm2.7$	$\phi^c = 4.3\times10^{10}\pm1.6\times10^{9}$
$\Delta = 7$ %	$\Delta = 12$ %	$\Delta = 4$ %

[a] Purpurogallin units, [b] relative luminescent units, [c] the average value of measured activities, Δ standard deviation

and hydrogen peroxide in required concentrations in proper buffer) is also lower. According to the producer's manual the shelf life of the ECL kit is one year.

In further experiments other parameters of the chemiluminescent method with the Amersham ECL kit were followed. The dependence of the enzyme activity on the concentration of lyophilised HRP is shown in Figure 1. Activity measurement was linear from 500 to 4×10^8 RLU/ml of the enzyme activity, i.e. values corresponding to concentrations 0.2–15 μg protein/ml. These activities would correspond to 0.26–6.2 U/mg estimated by guaiacol, the values which would not be straightway measurable with chromogens. Consequently the chemiluminescent assay is applicable for a wider peroxidase concentration range than methods which use the colour change of some chromogenic substrates. It is clear that spectrophotometric methods are not so sensitive as chemiluminiscent ones; on the other hand, the enzyme amount suitable for the reaction with chromogens to allow first-order reaction has to be much lower than 15 μg protein/ml.

As an example the method was used for the estimation of peroxidase activities isolated from fresh tissues of different plant species. Peroxidase activities were determined using the same procedure as was used for measurement of HRP activity. In natural samples the enzyme activity can be affected by certain effects. One is the possible interference of heme-containing enzymes, other proteins etc. The possible interference of catalase present in natural samples has been discussed[4]. The authors determined simultaneously peroxidase and catalase activities based on the additions of peroxidase standards with 25 natural samples isolated from various fruits and vegetables. It was shown that peroxidase activities measured by the chemiluminescent method are in good agreement with those obtained using the guaiacol method and maximal intensity of chemiluminescence was influenced only negligibly. Similar results were obtained in our experiments (data not shown).

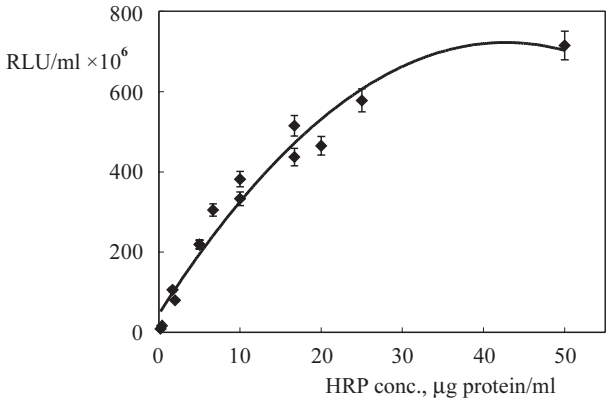

Fig. 1. **Dependence of the activity of horseradish peroxidase (Sigma)**, measured by the chemiluminescent method, on its concentration

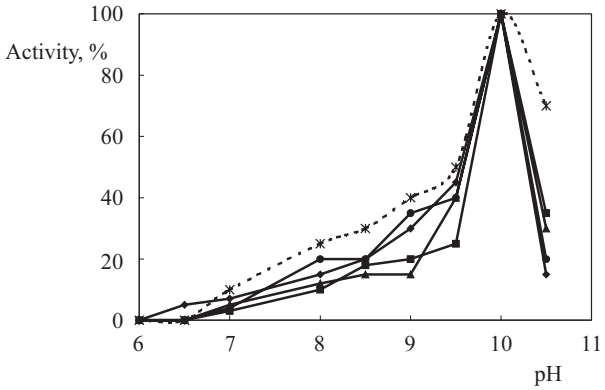

Fig. 2. **pH optimum of the chemiluminescent reaction catalysed by peroxidases isolated from different plant species**, HRP × commercial (Sigma) horseradish (*Armoracia rusticana*) peroxidase preparation, K62K ◆ strain of *A. rusticana* callus, R1BC ■ strain *Atropa belladonna* callus, SNC9O ● hairy root clone of S*olanum nigrum*, KK1N ▲ strain of *S. aviculare* callus

An important parameter of the chemiluminescent reaction is pH (Ref.[12]). Light emission is highly dependent on the pH of the reaction mixture but peroxidases usually have much lower optimum pH than is the optimum for the light emission. Figure 2 shows that the chemiluminescent reaction catalysed by peroxidases from four different plant tissues exhibited very high and the same pH optimum (pH 10) as the reaction catalysed by pure HRP. Many authors measuring peroxidase activities by chemiluminescent methods used reaction pH 7.0–8.0, which was definitely less advantageous. According to our findings the use of lower reaction pH did not give accurate results. The use of the high pH can also negatively influence the interference of other enzymes which could affect reaction conditions of the chemiluminiscent procedure.

The chemiluminescent method which uses the reagents from the Amersham ECL kit offers a good alternative to the well known colorimetric methods still widely exploited today and it significantly improves the chemiluminescent procedure which normally uses separate mixing of all components of the reaction mixture. From the results presented above it can be seen that the use of the described method increases the reproducibility of the assay of peroxidase activity compared to

colorimetric methods, and its use also accelerates the whole procedure. The wide range of measurable peroxidase concentrations reduces the number of sample dilutions required, thus decreasing the experimental error and leading to time saving. Large series of samples can be analysed rapidly. Also the sensitivity of the chemiluminescent method and its reasonable price must be taken into account.

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M. Macková[a], E. N. Ferri[b], K. Demnerová[a], and T. Macek[c] *([a]Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, [b]Institute of Chemical Science, University of Bologna, Italy, [c]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague):* **Quantitative Chemiluminiscent Detection of Plant Peroxidases Using a Commercial Kit Originally Designed for Blotting Assays**

The paper describes an improvement of chemiluminescent analysis of plant peroxidases by the use of a commercial kit originally designed for qualitative Western blotting assays. The chemiluminescent method using the reagents of the Amersham ECL kit offers a good alternative to the well known and widely exploited colorimetric or chemiluminescent methods using luminol. The proposed procedure significantly improves the chemiluminescent enzyme assay with labour-consuming separate mixing of all components by application of standard and ready-to-use solutions.