New fluorimetric assay of horseradish peroxidase using sesameol as substrate and its application to EIA

Hidetoshi Arakawa*, Shigeo Nakabayashi, Ken-ichi Ohno, Masako Maeda

School of Pharmacy, Showa University, Shinagawa-ku, Tokyo 142-8555, Japan

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Abstract Horseradish peroxidase (HRP) is generally used as a label enzyme in enzyme immunoassay (EIA). The procedure used for HRP detection in EIA is critical for sensitivity and precision. This paper describes a novel fluorimetric assay for horseradish peroxidase (HRP) using sesameol as substrate. The principle of the assay is as follows: sesameol (3,4-methylenedioxy phenol) is reacted enzymatically in the presence of hydrogen peroxide to produce dimeric sesameol. The dimer is fluorescent and can be detected sensitively at ex. 347 nm, em. 427 nm.

The measurable range of HRP was 1.0 \times 10^{-18} to 1.0 \times 10^{-15} mol/assay, with a detection limit of 1.0 \times 10^{-19} mol/assay. The coefficient of variation (CV, \(n=8\)) was examined at each point on the standard curve, with a mean CV percentage of 3.8%. This assay system was applied to thyroid stimulating hormone (TSH) EIA using HRP as the label enzyme.

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1. Introduction

Imunoassays using antigen–antibody reactions can selectively isolate and specifically analyze target components in a biological sample, despite being a complex matrix. In 1958, a radioimmunoassay (RIA) for the measurement of blood insulin was developed by Berson and Yallow [1]; this technique was subsequently used for high sensitivity assays of various substances. However, RIA is suboptimal due to biohazard and the need for special facilities including waste treatment. These difficulties encouraged the rapid development of non-radioactive immunoassays. In a marked improvement, EIA was developed by Engvall using enzymes as the labeling substance [2]. Various EIAs have since been developed using fluorescent material as the substrate and are currently used for diagnosis in numerous diseases. The sensitivity of the immunoassay depends on the detection method used for the labeling enzyme. Thus, both the enzyme used and its substrate are important for diagnostic accuracy.

At present, various enzymes such as horseradish peroxidase (HRP), alkaline phosphatase (ALP), and β-D-galactosidase are used as label. Among these enzymes, HRP is most frequently used as label in EIA and DNA-probe assays. The procedure used for detection of HRP is critical due to...
the sensitivity and the precision of these assays. Currently, highly sensitive fluorescent methods using 3-(4-hydroxyphenyl) propionic acid (HPPA) [3,4] as well as chemiluminescent methods using luminol [5] have been employed for detecting HRP labels in EIA. This paper describes a novel fluorimetric assay of HRP using sesamol as substrate. The principle of the assay is as follow: sesamol (3,4-methylenedioxy phenol) is reacted enzymatically in the presence of hydrogen peroxide to produce dimeric sesamol. The sesamol dimer is fluorescent and is detected sensitively. The detection limit of HRP was 1.0 × 10^{-18} mol/assay. This assay system was applied to TSH EIA using HRP as the label enzyme. Serum TSH levels could be assayed sensitively and precisely by the proposed method.

2. Materials and methods

2.1. Materials

The fluorescence intensity was measured with an FP-6500 Spectrofluorometer (JASCO Inc., Tokyo, Japan). HRP (RZ; 3.15) was purchased from Toyobo Co. (Tokyo, Japan). Sesamol, hydrogen peroxide, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and diethylenetriamine-N,N,N',N''-pentaaacetic acid (DETPAC) were purchased from Wako (Tokyo, Japan). TSH, anti-TSH antibody, and anti-TSH–HRP conjugate were donated by Toso Co. (Tokyo, Japan). Other chemicals were of analytical reagent grade.

2.2. Methods

2.2.1. Radical scavenging assay for sesamol

1 mM FeSO₄ 50 μL, 1 mM DETAPAC 50 μL, 0.1 M phosphate buffer (pH 7.4)(PB) 50 μL, 1% DMPO 20 μL, 10 mM sesamol 50 μL (or water as blank), and 0.003% H₂O₂ 30 μL were mixed in a glass tube, and the reaction mixture was transferred to an ESR cuvette and measured by electron spin resonance (ESR) (a JEOL JES REIX spectrometer equipped with 100 kHz field modulation) after 1 min of mixing.

2.2.2. Fluorimetric assay of HRP

20 μL HRP sample was added to a reaction well containing 100 μL of 0.1 M citrate buffer (pH 7.5) and mixed with 10 μL of 10 mM sesamol solution and 10 μL of 0.05% H₂O₂. The reaction was incubated for 60 min at 25 °C, and then 100 μL of dimethyl sulfoxide (DMSO) was added as enhancer of fluorescence intensity before the measurement of fluorescence at an excitation wavelength of 347 nm and an emission wavelength of 427 nm.

2.2.3. Fluorimetric EIA of TSH

TSH standard (20 μL) or serum sample was added to a microtiter plate coated with anti-TSH antibody. Next, 100 μL of HRP-labeled anti-TSH antibody solution was added and the mixture was incubated overnight at 25 °C. After washing three times the plate with saline, 100 μL of 0.1 M citrate buffer (pH 7.5), 10 μL of 10 mM sesamol, and 10 μL of 0.05% H₂O₂ were added. After incubation of 60 min at 25 °C, 100 μL of DMSO was added and the fluorescence intensity was measured with a Wallac multilabel counter 1420.

3. Results and discussion

HRP has been widely used as the label enzyme in EIA and DNA probe assays. A sensitive assay of HRP is required for developing better performing diagnostic tests. At present, colorimetric assay using 3,3',5,5'-tetramethylbenzidine [6] and chemiluminescent HRP assays using luminol with p-iodophenol [7] as enhancer are most commonly used. Other high-sensitivity fluorimetric assays have also been reported. In this paper, we report a new fluorimetric assay for HRP using sesamol. Sesamol is a general reagent which can be extracted from sesame or chemically synthesized. Previously, we developed a high-sensitivity NO assay using sesamol [8,9]. In our NO research, we found that sesamol also reacts with an OH radical to produce strong fluorescence. The reactivity of sesamol with the OH radical was evaluated by radical scavenging assay, with the OH radical being generated from hydrogen peroxide via Fenton reaction. The resultant OH radical was measured by ESR after reaction with sesamol. DMPO was used as spin-trapping reagent.

Fig. 1 shows the ESR spectrum of Fenton’s reaction with and without sesamol. ESR spectrum obtained without sesamol showed peak-height and -shape (1:2:2:1) of typical OH-DMPO adduct. Moreover, these peaks were greatly reduced by the addition of sesamol. This results shows that sesamol reacts not only with NO but also with the OH radical. The principle of this assay is shown in Fig. 2.

In this paper, we demostrate a high-sensitivity fluorimetric HRP assay using sesamol as substrate in the presence of hydrogen peroxide. In preliminary tests, we used phosphate buffer (PB) for HRP assay. However, HRP assay using PB did not show a linear standard curve. Therefore, citrate buffer was used. The reason for this remains unclear.

![Figure 1](image1.png)

Figure 1 OH radical scavenging activity of sesamol. (A) ESR spectrum of Fenton’s reaction without sesamol. (B) ESR spectrum of Fenton’s reaction with sesamol.

![Figure 2](image2.png)

Figure 2 Principle of the fluorometric assay for HRP using sesamol.
3.1. Determination of fluorimetric assay condition

The effects of various factors ((1)–(3)) such as concentrations of sesamol, buffer pH, reaction temperature, and time on fluorimetric HRP assay were examined as follows in order to establish optimal assay conditions.

(1) Based on the method of HRP assay described in the methods section, sesamol concentration was determined in the range of 0.1–100 mM. The fluorescence intensity increased with increasing sesamol concentration. However, blank values obtained when water was used in place of HRP also increased with the concentration. Therefore, S/N ratio decreased as a result. The maximum value of S/N ratio was obtained at 10 mM.

(2) The optimal concentration of hydrogen peroxide was studied similarly in the range of 0.005–0.5% H$_2$O$_2$. The optimal concentration of H$_2$O$_2$ was 0.05% as a result.

(3) The reaction temperature, reaction time, buffer pH, and buffer concentration were studied by the method described above, and determined as described in the method conditions.

3.2. A standard curve for HRP

A standard curve for HRP was obtained according to the procedure outlined. As shown in Fig. 3, a standard curve was obtained in the range of 2.0 $\times$ 10$^{-19}$ to 1.0 $\times$ 10$^{-15}$ mol/assay. The coefficient variation (CV) percentages ($n=8$) were 1.8 to 4.8%, with a mean of 3.5%. The detection limit (blank+2SD) was 2.0 $\times$ 10$^{-18}$ mol/assay. The sensitivity obtained by the proposed method corresponded to that obtained by fluorimetric assay using HPPA as substrate.

3.3. Application of fluorimetric HRP assay in EIA

The new fluorimetric assay for HRP was applied to detect the HRP conjugate in EIA. Immunoassay was carried out according to the procedure described in the method. The measurable range for TSH was 0.005 to 1.28 µIU/mL. The detection limit was 0.008 µIU/mL (blank+2SD). Variations of this EIA were determined in a precision, a dilution curve, and a correlation experiment using serum sample. The precision of EIA was evaluated by CV% ($n=8$) of each concentration of TSH on standard curve. The CVs were 1.8–2.5%, with a mean of 1.9%. Dilution curve of serum was studied using three different serum samples. Serum sample was diluted by PBS containing 0.01% BSA and measured by EIA. As shown in Fig. 4, linearity through the origin was obtained for three samples. In addition, correlation coefficient versus chemiluminescent immunoassay (CLIA) was high ($r=0.972$, $n=116$) as shown in Fig. 5. From the above results, this EIA is considered to be applicable to clinical practice.

![Figure 3](image-url) Standard curve of HRP. HRP (2 $\times$ 10$^{-19}$ to 1 $\times$ 10$^{-15}$ mol/assay) is measured by fluorimetric assay using sesamol as substrate.

![Figure 4](image-url) Dilution curves of TSH in serum samples. Serum sample was diluted with saline. 1, 0.5, 0.25, 0.125, 0.0625 are correspond to 1-fold, 2-fold, 4-fold, 8-fold, 16-fold respectively.

![Figure 5](image-url) Correlation analysis with sesamol-fluorimetric EIA (proposed method) and chemiluminescent immunoassay (CLIA). Serum samples ($n=116$) were measured by sesamol-fluorimetric EIA and chemiluminescent immunoassay. The obtained values were compared.
4. Conclusion

We developed a fluorimetric assay of HRP using sesamol as substrate. This assay system of HRP is highly sensitive. Sesamol is extracted from sesame oil and can be purchased as an inexpensive general reagent. Though the sensitivity was the same as that of HPPA fluorimetric assay for HRP, furthermore, the sensitivity of the new assay can be further improved by purification of substrate and use of a improved enhancement method. Further studies on both enhancement of sensitivity and the applications of this system are currently in progress in our laboratory.

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