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Sensitive Fluorimetric Assay for Human Thyroid Peroxidase Using *p*-Cresol as a Substrate

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A sensitive fluorimetric method using *p*-cresol as a hydrogen-donating substrate is described for th assay of thyroid peroxidase in a crude preparation of human thyroid tissue. The fluorescence of the product formed enzymatically from *p*-cresol is measured at 410 nm with excitation at 325 nm in an alkaline solution (pH 12.3). The specific activities of thyroid peroxidase are 2 to 5 times higher in tissues from patients with Graves' disease than in normal tissues from patients with adenoma of the thyroid. This method is simple and sensitive enough to allow the enzyme assay to be carried out with as little as 10 mg of thyroid tissue.

Keywords——fluorimetric assay; thyroid peroxidase; *p*-cresol; fluorogenic substrate; human thyroid; Graves' disease

Thyroid peroxidase (TPO) (donor: hydrogen peroxideoxidoreductase, EC 1.11.1.7.) is a hemoprotein enzyme bound to particulate membranes of thyroid tissue, and catalyzes the oxidation of some organic compounds or iodide in the presence of hydrogen peroxide.[1-5] It has been shown that this enzyme plays an essential role in the biosynthesis of thyroid hormone, *i.e.*, the iodination of tyrosyl residues of thyroglobulin and the coupling of the diiodotyrosine.[6,7] Therefore, the measurement of TPO activity in human thyroid is necessary for the study of thyroid disorders.[8-10] Several methods have been reported for the assay of TPO activity based on spectrophotometric determination of enzymatic oxidation of guaiacol or iodide[11-13] and radiochemical assay of iodination of tyrosyl residues in bovine serum albumin or thyroglobulin.[14] Although conventional spectrophotometric methods using guaiacol as the substrate for the assay of TPO activity are simple and widely used, the sensitivity of the methods is not sufficient to measure the TPO activity in minute tissue samples obtained by needle biopsy or in cultured thyroid cells.

We have developed a sensitive fluorimetric method for the micro-assay of TPO activity in a crude preparation of human thyroid tissue. This method is based on the enzymatic formation of a fluorescent product from *p*-cresol in the presence of hydrogen peroxide under the optimum reaction conditions for the enzyme. Several phenolic compounds other than *p*-cresol, such as homovanillic acid, *p*-hydroxyphenylacetic acid, 3-(*p*-hydroxyphenyl)propionic acid, tyrosine and tyramine, were also examined for possible use as fluorogenic substrates in the assay of TPO of human thyroid tissue, since these compounds have been reported to provide highly fluorescent 2,2′-dihydroxydiphenyl derivatives in a catalytic reaction of horseradish peroxidase with hydrogen peroxide.[15-19] Of the compounds tested, *p*-cresol was the most useful fluorogenic substrate for the assay of TPO in preparation of human thyroid tissue.

Experimental

Chemicals and Apparatus——Deionized and distilled water was used. *p*-Cresol (Tokyo Chemicals, Japan) was purified by distillation. A hydrogen peroxide solution (Wako, Japan) was standardized by acid permanganate titration. Horseradish peroxidase (258 purpurogallin units/mg, RZ; 3.1) was obtained from Toyobo Co. Japan. Other chemicals were of reagent grade.

The fluorescence spectra and intensities were measured with a Shimadzu RF-503 spectrofluorimeter using quartz cells of 1.0 × 1.0-cm optical path-length. The slit widths were set at 5.0 nm in both the exciter and analyzer. The fluorescence excitation and emission maxima are uncorrected.

Sample Preparation——Thyroid tissues were obtained from individuals with Graves' disease and adenoma of the thyroid during operation, and stored at −70 °C until use. The tissue (about 100 mg) was cut into small pieces with scissors, washed with saline and homogenized in 5.0 ml of 50 mM sodium phosphate buffer (pH 7.5) with an Omega electric homogenizer at 1200 rpm for 3 min. After centrifugation at 800 *g* for 10 min, 2.0 ml of the supernatant was centrifuged at 105000 *g* for 30 min. The resulting precipitate was suspended in 2.0 ml of the phosphate buffer. The above procedure was carried out at 0—4 °C. The subcellular particles present in the suspension was then solubilized according to Nakashima and Taurog[13] with slight modifications. Briefly, 0.1 ml of 72 mM sodium deoxycholate and 0.05 ml of 1.4 mg/ml crystalline trypsin (ICN, 3000 NF units/mg) were added to 2.0 ml of the suspension, and left for 15 min at room temperature, then 0.05 ml of 0.28 mg/ml soybean trypsin inhibitor was added. A portion (0.1 ml) of the resulting solution was used as a sample for assay in the incubation mixture described below. The protein concentration was about 30 μg per 0.1 ml of the sample solution, as measured by the method of Lowry *et al.*[20] with bovine serum albumin as the standard protein.

Assay Procedure for TPO——The incubation mixture consisted of 0.1 ml of the sample solution, 0.5 ml of 18.5 mM *p*-cresol in 50 mM sodium phosphate buffer (pH 7.5) and 0.1 ml of 0.53 mM hydrogen peroxide. The mixture was incubated at 37 °C for 30 min, and 0.25 ml of 64 mM β-mercaptoethanol was added to stop the reaction. The pH of the mixture was adjusted to 12.3 by adding 3.0 ml of 0.2 M trisodium phosphate. For the blank, the same procedure was carried out except that the order of addition of hydrogen peroxide and β-mercaptoethanol was reversed and incubation was omitted. Horseradish peroxidase (0.2 mU in 0.1 ml of 50 mM sodium phosphate buffer, pH 7.5) was used in place of the TPO sample in the incubation mixture as the standard enzyme in order to define the unit of TPO activity in the sample. The fluorescence intensities of the sample (ΔF_{test}), blank (ΔF_{blank}), the horseradish peroxidase (ΔF_{HRP}) and its blank ($\Delta F_{blank\text{-}HRP}$) were measured at 410 nm with excitation at 325 nm. The activity was calculated as follows.

$$\Delta F_{test} - \Delta F_{blank} = \Delta F_{sample}$$

$$\Delta F_{HRP} - \Delta F_{blank\text{-}HRP} = \Delta F_{standard}$$

$$\text{Activity unit (mU)} = \Delta F_{sample} / \Delta F_{standard} \times 0.2$$

Specific activity was defined as the activity per mg protein in the incubation mixture.

Results and Discussion

Selection of *p*-Cresol as a Fluorogenic Substrate

Horseradish peroxidase has *in vitro* properties similar to those of TPO in the catalytic reactions for the formation of thyroid hormone from 3,5-diiodotyrosine and the oxidation of iodide in the presence of hydrogen peroxide.[21,22] Thus, the phenolic compounds tested (*p*-cresol, homovanillic acid, *p*-hydroxyphenylacetic acid, 3-(*p*-hydroxyphenyl)propionic acid, tyrosine and tyramine), which are substrates of horseradish peroxidase, might be useful as fluorogenic substrates of TPO of human thyroid.

However, the compounds tested, other than *p*-cresol, were not suitable for the assay of TPO in crude preparations of human thyroid tissue because the compounds were non-enzymatically oxidized in the presence of thyroid tissue and hydrogen peroxide. For this experiment, two preparations of human thyroid tissue from an individual with Graves' disease, obtained by the procedure described in Experimental, were employed as crude TPO samples. One is the homogenate after removal of cell debris and nuclei, and the other is the solubilized particulate fraction obtained from the homogenate by ultracentrifugation. The inactivation of TPO in these preparations was carried out by heating at 98 °C for 20 min. Under these conditions, horseradish peroxidase was completely inactivated. As shown in Fig. 1, 3-(*p*-

hydroxyphenyl)propionic acid, *p*-hydroxyphenylacetic acid, homovanillic acid, tyrosine and tyramine were unexpectedly oxidized by the inactivated preparation of the homogenate in the presence of hydrogen peroxide and gave strong fluorescences depending on the incubation time. The same results were observed with the inactivated preparation of the particulate fraction, but the oxidation rates for the substrates tested (excluding *p*-cresol) were approximately 1/10 or less of those in the inactivated preparation of the homogenate. In addition, the deproteinized preparation of the homogenate, which was obtained by neutralization after the preparation had been treated with perchloric acid, also showed fluorescence development from substrates such as 3-(*p*-hydroxyphenyl)propionic acid in the presence of hydrogen peroxide. In this case, the rate of the catalytic reaction was approximately half of that in the heated homogenate. For these non-enzymatic oxidations, both the inactivated preparation and hydrogen peroxide were required. The excitation and emission spectra of the fluorescence produced from these compounds by the above non-enzymatic oxidation were identical to those in the catalytic reaction of horseradish peroxidase. In view of these observations, some heat-stable substance (other than TPO) which oxidizes the phenolic compounds tested, except for *p*-cresol, may be present in the preparations of human thyroid, though the mechanism of the non-enzymatic oxidation of these compounds remains unknown.

On the other hand, *p*-cresol was not oxidized non-enzymatically in the presence of these preparations with hydrogen peroxide (Fig. 1). A similar result was obtained with guaiacol, used in the conventional spectrophotometric method, and also Hosoya *et al.*[11] showed that TPO purified partially from pig thyroid tissues was completely inactivated by heating. Thus, *p*-cresol was employed as a favorable fluorogenic substrate for the assay of TPO activity in crude preparations of human thyroid.

Assay of TPO Activity in Human Thyroid Tissue

The particulate fraction rather than the homogenate is preferred as an enzyme sample for the assay of the endogenous TPO activity in thyroid tissue from patients with hyperthyroidism. Recently, inhibitory effects of several antithyroid drugs have been recognized on TPO activity.[23,24] Drugs used in clinical therapy would be present in the homogenate. The TPO activity was recovered in the particulate fraction obtained by the procedure described in Experimental. The specific activity of TPO in the solubilized particulate fraction was approximately ten times higher than that in the supernatant and three times higher than that before solubilization. Moreover, the enzyme activity in the sample did not change for at least 3.0 h when stored at 0—4 °C.

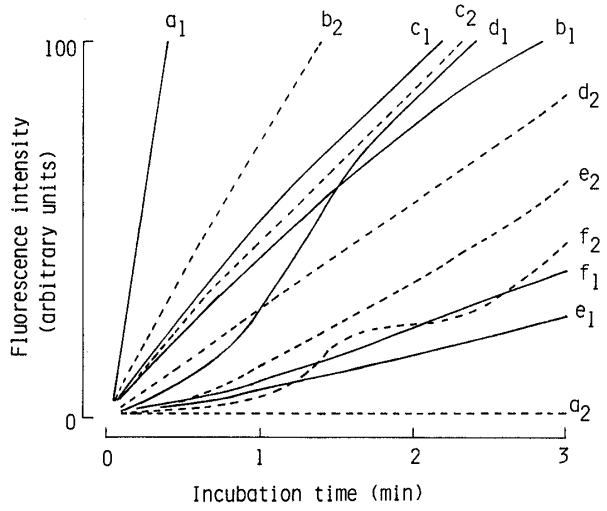

Fig. 1. Fluorescence Development from Various Substrates in the Presence of Thyroid Homogenate and Heated Homogenate

Portions (0.1 ml) of the preparations from human thyroid tissue were added to reaction mixtures (3 ml each) consisting of 50 mM Tris-HCl buffer (pH 8.5) containing the substrate (3—8 mM) and hydrogen peroxide (0.01—0.6 mM). The fluorescence intensity from each substrate was measured at various incubation times at 25 °C. Initial background fluorescence was substracted. The concentrations (mM) of the substrate and hydrogen peroxide in each final reaction mixture, and the excitation and emission wavelengths (nm) in the fluorescence spectra are noted in parenthesis. a_1—f_1, thyroid homogenate; a_2—f_2, heated thyroid homogenate. a, *p*-cresol (7.4, 0.01; 325/410); b, 3-(*p*-hydroxyphenyl)propionic acid (8.0, 0.5; 317/405); c, *p*-hydroxyacetic acid (5.5, 0.5; 315/415); d, homovanillic acid (4.5, 0.5; 315/425); e, tyrosine (3.0, 0.6; 325/410); f, thyramine (4.0, 0.6; 325/410).

The excitation and emission maxima of the fluorescence produced enzymatically from *p*-cresol by TPO in the sample are at 325 and 410 nm, respectively. These maximum wavelengths did not shift over the pH range of 6.0 to 13.0 in the final incubation mixture. The fluorescence intensity of the fluorophore was the strongest between pH 12.0 and 12.5 (Fig. 2); the pH of the final incubation mixture was adjusted to 12.3 by the addition of 0.2 M trisodium phosphate after the enzyme reaction.

The enzyme reaction was not completely stopped by the change of pH to 12.3 but was completely stopped by heating at 98 °C for 10 min or by the addition of β-mercaptoethanol at a concentration above 32 mM. Therefore 64 mM β-mercaptoethanol was used to stop the reaction. In the assay procedure, the enzyme reaction mixture for the blank was not incubated because the incubation had no effect on the blank value after the addition of β-mercaptoethanol.

The enzyme was most active in the pH range from 7.0 to 8.5 in both 50 mM sodium phosphate buffer and 50 mM Tris-HCl buffer (Fig. 3); the phosphate buffer of pH 7.5 was used for the standard procedure. The Michaelis constants (K_m) were 3.5 mM for *p*-cresol and 0.045 mM for hydrogen peroxide (Fig. 4). Maximum and constant enzyme activity was obtained in the presence of 12—20 mM *p*-cresol and 0.07—0.08 mM hydrogen peroxide; 13.2 mM *p*-cresol and 0.076 mM hydrogen peroxide were used in the enzyme reaction.

The TPO activity was proportional to the amount of the sample protein in the incubation mixture up to at least 100 μg. In addition, low enzyme activity in a sample containing as little as 1.0 μg of the protein could be assayed because the fluorescence of the final incubation mixture gave an intensity of twice the blank level. In contrast, the spectrophotometric method[12] using guaiacol requires at least 100 μg of protein in the sample for the reliable assay of the TPO activity.

The precision of the method was examined by performing twenty separate analyses on a sample solution. The coefficient of variation was 2.0% for the TPO activity of 27.3 mU/mg protein.

The unit of TPO activity was expressed in terms of that of horseradish peroxidase since this should facilitate comparisons of substrate specificity.

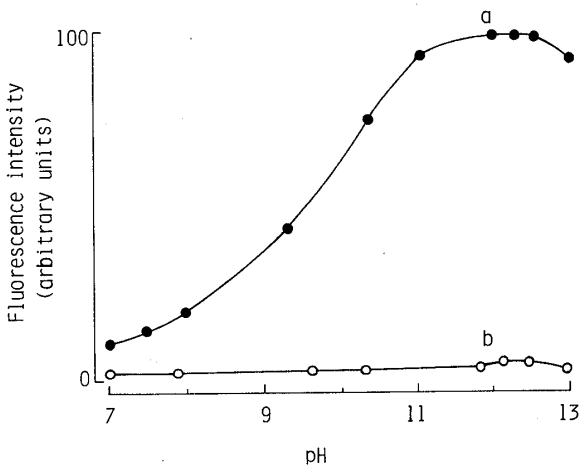

Fig. 2. Effect of pH in the Final Incubation Mixture on the Fluorescence Developed

a, portions (0.1 ml) of a sample from a patient with Graves' disease were treated according to the standard procedure, but the final incubation mixtures were adjusted to various pHs with 0.2 M sodium dihydrogen phosphate or 0.2 M sodium hydroxide; b, blank corresponding to a.

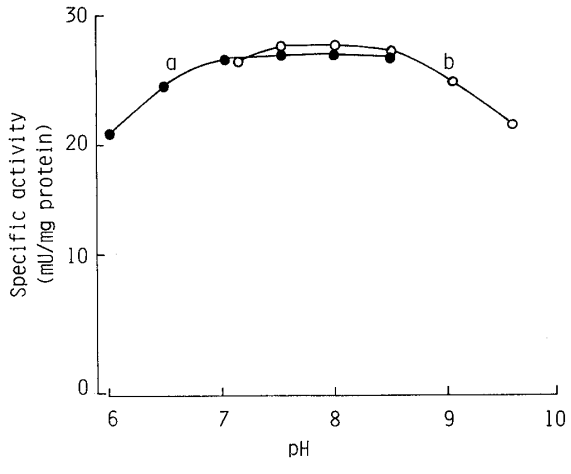

Fig. 3. Effect of pH of the Incubation Mixture on TPO Activity

a, 50 mM sodium phosphate buffer (pH 6.0—8.5); b, 50 mM Tris-HCl buffer (pH 7.2—9.6).

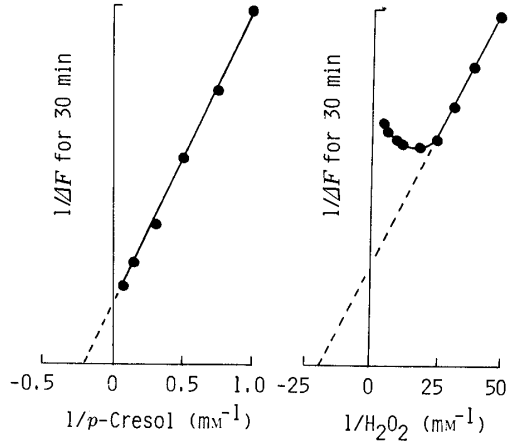

Fig. 4. Lineweaver–Burk Plots of the Dependence of TPO Activity on *p*-Cresol (Left) and Hydrogen Peroxide (Right)

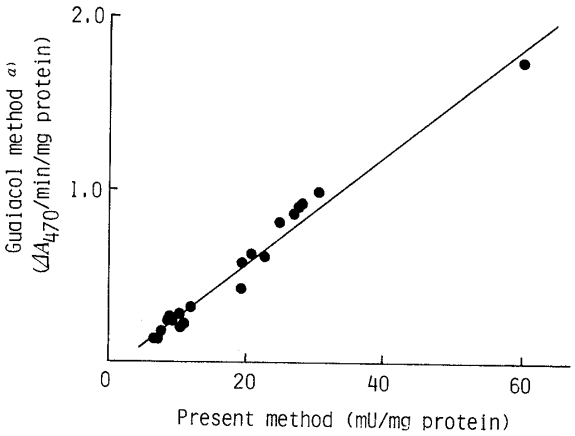

Fig. 5. Correlation between TPO Activities in Human Thyroid Tissue Obtained by the Present Method and the Guaiacol Method

a) The unit is the absorbance increase (ΔA) at 470 nm in 1.0 min per mg protein.

TABLE I. TPO Activities in Thyroid Tissues of Patients with Graves' Disease and in Normal Thyroid Tissues of Patients with Thyroid Adenoma

Graves' disease			Normal[a]		
Subject	(sex[b])	Specific activity (mU/mg protein)	Subject	(sex[b])	Specific activity (mU/mg protein)
H. F.	(F)	25.1	M. M.	(F)	12.1
U. Y.	(F)	26.9	Y. M.	(F)	10.7
H. H.	(F)	62.7	Y. S.	(F)	9.5
A. K.	(F)	27.6	S. K.	(F)	10.4
S. I.	(F)	30.1	S. K.	(F)	7.2
K. Y.	(M)	19.5	S. T.	(M)	11.1
S. H.	(F)	22.6	T. K.	(F)	6.6
Y. I.	(F)	20.7	S. F.	(F)	7.6
T. N.	(M)	27.3	M. H.	(F)	8.9
M. T.	(M)	19.3	S. I.	(F)	8.2
A. F.	(F)	39.3	Y. Y.	(F)	12.0
H. K.	(F)	25.3	H. H.	(F)	6.0
N. A.	(M)	39.6	Y. K.	(F)	6.9
S. E.	(F)	34.9	T. T.	(F)	7.5
M. Y.	(F)	37.0	T. I.	(M)	10.7
T. O.	(F)	20.7	T. K.	(F)	8.7
Y. O.	(F)	37.8	Y. F.	(F)	10.7
F. M.	(F)	33.2	M. K.	(F)	9.7
Mean ± S.D.		30.5 ± 10.6	Mean ± S.D.		9.1 ± 1.9

a) Obtained from histologically normal portions of tissues from patients with thyroid adenoma.
b) F, female; M, male.

The specific activities of TPO in thyroid tissues from patients with Graves' disease ($n=18$) and those in the normal portions of thyroid tissues obtained from patients with thyroid adenoma ($n=18$) were measured (Table I). The enzyme activity of human thyroid with hyperthyroidism was elevated several-fold as compared with the normal activity. The results

are in agreement with the published data[9] obtained by the guaiacol and iodide oxidation methods. Thus, a high level of TPO activity in thyroid tissue of patients with hyperthyroidism may be associated with the increased biosynthesis of thyroid hormone in the tissue.[8-10]

The enzyme activity obtained by the present fluorimetric method was also compared with that obtained by the spectrophotometric method[11] using guaiacol. A good correlation was found, as shown in Fig. 5. The correlation coefficient (r) is 0.982 ($n=20$) and the regression equation for the present method (X) against the guaiacol method (Y) is $Y=0.31\ X-0.41$.

The present study is the first application of the fluorimetric method using *p*-cresol to the assay of TPO activity in human thyroid tissue. The method is simple and about 100 times more sensitive than the conventional spectrophotometric methods using guaiacol as the substrate; the sensitivity is sufficient to permit the assay of TPO activity in very small samples, such as those obtained in needle biopsy, and in cultured thyroid cells.

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