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A Comparison of Chromogenic Substrates for Horseradish Peroxidase as a Label in Steroid Enzyme Immunoassay[1)]

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Six chromogenic substrates for horseradish peroxidase (HRP) as an enzyme label were compared with regard to the sensitivity obtainable with a testosterone enzyme immunoassay system. The chromogens tested were 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), *o*-phenylenediamine (OPD), 5-aminosalicylic acid (5-AS), 3-amino-9-ethylcarbazole (AEC), 3,3′,5,5′-tetramethylbenzidine (TMB) and 3-methyl-2-benzothiazolinone hydrazone (MBTH); for comparison, a fluorimetric assay using 3-(*p*-hydroxyphenyl)propionic acid was also carried out. An HRP-labeled antigen was prepared by the *N*-succinimidyl ester method. The bound and free enzyme-labeled antigens were separated by a double antibody method. A dose–response curve with a satisfactory sensitivity was obtained in each system by the use of a minimum amount of the HRP label at an appropriate dilution of anti-testosterone antiserum ($K_a = 2 \times 10^{10}\,M^{-1}$). The amounts of testosterone needed to displace 50% of the bound label ranged from 9 to 150 pg. The sensitivity decreases in the order: TMB > OPD > ABTS > 5-AS > MBTH > AEC. The assay using the non-mutagenic substrate, TMB, gave a high sensitivity, comparable to that of the fluorimetric method.

Keywords——enzyme immunoassay; testosterone; horseradish peroxidase; 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); *o*-phenylenediamine; 5-aminosalicylic acid; 3-amino-9-ethylcarbazole; 3,3′,5,5′-tetramethylbenzidine; 3-methyl-2-benzothiazolinone hydrazone; 3-(*p*-hydroxyphenyl)propionic acid

In recent years, numerous enzyme immunoassays have been developed using various enzymes as labels. Enzymes currently used in the heterogeneous enzyme immunoassay of steroid hormones are horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucose oxidase, glucose-6-phosphate dehydrogenase, glucose dehydrogenase, glucoamylase, penicillinase, and urease. One important factor which determines the sensitivity of an enzyme immunoassay is the sensitivity of the final enzyme assay stage. Previously, in order to obtain a practical basis for selecting the enzyme, we have compared the immunological properties of the labeled antigens prepared with the former three enzymes in fluorimetric enzyme immunoassay systems.[2)]

HRP catalyzes the oxidation of a hydrogen-donating substrate by peroxide. This enzyme is frequently used as an enzyme label and its activity can be measured by using various chromogenic substrates. Some of these chromogens have been shown to be carcinogenic or mutagenic in test systems[3)]; 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and *o*-phenylenediamine (OPD), which are mutagenic, are the most commonly used chromogens. It is desirable to employ a safe substrate, if a satisfactory assay sensitivity can be obtained. The use of 5-aminosalicylic acid (5-AS),[4)] 3-amino-9-ethylcarbazole (AEC),[4b)] 3,3′,5,5′-tetramethylbenzidine (TMB),[5)] or 3-methyl-2-benzothiazolinone hydrazone (MBTH)[6)] as safer chromogens has been reported. However, little information is available concerning the relation between chromogen structure and sensitivity in heterogeneous enzyme immunoassay for steroid hormones or other haptenic compounds. This paper deals with the sensitivities

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obtainable with testosterone enzyme immunoassay systems using ABTS, OPD and the four safer substrates.

Materials and Methods

Materials——HRP (EC 1.11.1.7) (grade I-C, Reinheits-Zahl 3.1, 263 units/mg) was obtained from Toyobo Co., Ltd. (Osaka). 5-AS, TMB, MBTH hydrochloride, and 3-dimethylaminobenzoic acid were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo); ABTS diammonium salt, OPD and AEC were from Nakarai Chemicals, Ltd. (Kyoto). 5-AS was recrystallized from water containing sodium bisulfite.[4a] The *N*-succinimidyl ester of 4-hydroxytestosterone 4-hemiglutarate was prepared by the method previously established in these laboratories.[7] Anti-testosterone antiserum used was that elicited in a rabbit by immunization with the conjugate of 4-hydroxytestosterone 4-hemiglutarate with bovine serum albumin.[8] Goat anti-rabbit immunoglobulin G (IgG) antiserum and normal rabbit serum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo). These sera were diluted with 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin and 0.9% NaCl (assay buffer).

Preparation of HRP-Labeled Antigen——This was carried out in the manner described previously.[2b] In short, a solution of the testosterone *N*-succinimidyl ester (773 μg) in dioxane (0.2 ml) was added to a solution of HRP (2 mg) in PB (0.4 ml) at 0 °C, and the mixture was gently stirred at 4 °C for 4 h. After dialysis against cold PB, an aliquot of the conjugate solution was transferred to a test tube; the solution was stored at 4 °C at a concentration of 500 μg/ml, adjusted with assay buffer. For the immunoassay procedure, the solution was diluted with assay buffer containing 0.5% normal rabbit serum.

Enzyme Immunoassay Procedure——This was carried out in duplicate or triplicate in a glass test tube (10 ml) as follows: a solution of testosterone (1—500 pg) in assay buffer (0.1 ml) and HRP-labeled testosterone in assay buffer (0.1 ml) containing normal rabbit serum were added to diluted anti-testosterone antiserum (0.1 ml), and the mixture was incubated at 4 °C for 4 h. Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1 : 30 with assay buffer containing 0.3% ethylenediaminetetraacetic acid was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4 °C for 16 h. After addition of assay buffer (1.5 ml), the resulting solution was centrifuged at 3000 rpm for 10 min, and the supernatant was removed by aspiration. The immune precipitate was washed once with assay buffer and used for measurement of the enzymic activity. At the same time, the procedure without addition of testosterone or the first antibody was carried out to provide B_0 and non-specific binding (background) values.

Measurement of Enzymic Activity——Fluorimetric measurement was carried out in the manner described in the previous paper.[2b] In colorimetry, the immune precipitate was diluted with a chromogen solution (1.8 ml), vortex-mixed, and preincubated at 37 °C for 3 min. Hydrogen peroxide (0.02% for TMB or 0.05% for other chromogens, 0.2 ml) was added to the resulting solution, and the mixture was incubated for 1 h. The reaction was terminated by addition of a stopping reagent (2 ml). The absorbance was then measured at an appropriate wavelength; the background was estimated as a percentage of the optical density for B_0, using distilled water as the zero reference. The chromogen solutions, stopping reagents and wavelengths employed were as follows:

ABTS: 0.91 mM in 0.05 M citric acid–phosphate buffer (pH 4.2); reaction stopped with 0.01% NaN_3; absorbance read at 418 nm.

OPD: 4.6 mM in 0.05 M acetate buffer (pH 6.0); reaction stopped with 0.5 M H_2SO_4; absorbance read at 492 nm.

5-AS: 6.5 mM in 0.05 M acetate buffer (pH 6.0); reaction stopped with 0.5 M NaOH; absorbance read at 500 nm.

AEC: 0.48 mM in 0.05 M acetate buffer (pH 5.0) containing 10% dimethyl sulfoxide; reaction stopped with 0.4 M NaOH–EtOH (1 : 3); absorbance read at 500 nm.

TMB: 0.42 mM in 0.05 M acetate–citric acid buffer (pH 5.5) containing 3% dimethyl sulfoxide; reaction stopped with 0.5 M H_2SO_4; absorbance read at 450 nm.

MBTH: 0.12 mM in 0.05 M citric acid–phosphate buffer (pH 7.0) containing 6 mM 3-dimethylaminobenzoic acid; reaction stopped with 0.5 M H_2SO_4; absorbance read at 595 nm.

Results and Discussion

The purpose of this work was to assess six substrates for HRP in the testosterone enzyme immunoassay system. The chromogens tested here were ABTS, OPD, 5-AS, AEC, TMB and MBTH; selection of the experimental conditions, such as substrate solution and stopping reagent, used in the measurement of enzymic activity, was based on the previous findings.[4-6,9] In the case of MBTH, the oxidative coupling with 3-dimethylaminobenzoic acid was carried out to form an indamine dye.[6] For comparison, the enzymic activity was also measured by a fluorimetric method using 3-(*p*-hydroxyphenyl) propionic acid (HPPA).[2b]

An enzyme-labeled antigen was prepared by the active ester method. The *N*-succinimidyl

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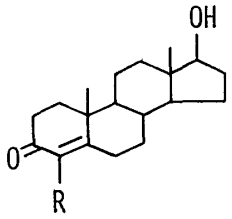

testosterone: R=H
labeled antigen: R=OCO(CH$_2$)$_3$CO–HRP

Chart 1

ester of 4-hydroxytestosterone 4-hemiglutarate was reacted with HRP in phosphate buffer (pH 7.3)–dioxane. The activated ester should react readily with free amino groups of the enzyme. The steroid/HRP molar ratio of 30 was used for the coupling reaction. The conjugate was dialyzed against the buffer to remove the unreacted steroid. No significant loss of enzymic activity was observed under the coupling conditions used. Spectrometric analysis showed that the average number of steroid molecules incorporated per HRP molecule (degree of substitution) was *ca.* 2.[2b]

For the present purpose, anti-testosterone antiserum used must have a high binding affinity responsive to substrate difference, since the sensitivity of heterogeneous enzyme immunoassay is influenced by the affinity constant of the antibody as well as the amount of

TABLE I. Inhibition of Bound Enzymic Activity of the HRP-Labeled Antigen by 50 pg of Testosterone

Substrate	Amount of label (ng)	Antiserum dilution	Inhibition (%)	Absorbance for B_0[a]
ABTS	10	1:20000	69	0.46 (10)
		1:30000	67	0.25 (18)
		1:40000	63	0.20 (23)
	5	1:20000	77	0.39 (12)
		1:30000	75	0.23 (20)
		1:40000	71	0.19 (25)
	2	1:10000	67	0.43 (10)
		1:20000	79	0.23 (19)
		1:40000	87	0.14 (33)
OPD	5	1:40000	74	0.45 (11)
		1:60000	77	0.29 (17)
		1:80000	67	0.20 (26)
	2	1:30000	83	0.29 (16)
		1:40000	81	0.24 (19)
		1:60000	81	0.18 (25)
	1	1:10000	56	0.30 (12)
		1:20000	75	0.25 (14)
		1:30000	78	0.18 (20)
TMB	5	1:40000	88	0.53 (4)
		1:60000	93	0.32 (6)
		1:80000	90	0.16 (12)
	2	1:30000	92	0.32 (5)
		1:40000	96	0.26 (6)
		1:60000	98	0.11 (15)
	1	1:10000	70	0.33 (5)
		1:20000	94	0.22 (7)
		1:30000	99	0.14 (11)
HPPA	1	1:40000	95	— (9)
		1:60000	98	— (18)

a) Figures in parentheses indicate background (%).

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TABLE II. Inhibition of Bound Enzymic Activity of the HRP-Labeled Antigen by 200 pg of Testosterone

Substrate	Amount of label (ng)	Antiserum dilution	Inhibition (%)	Absorbance for B_0[a]
AEC	100	1:1000	9	0.75 (8)
		1:2000	41	0.45 (14)
		1:5000	66	0.09 (70)
	50	1:1000	20	0.66 (9)
		1:2000	64	0.40 (15)
		1:5000	69	0.17 (34)
	20	1:1000	16	0.40 (15)
		1:2000	66	0.22 (27)
MBTH	50	1:2000	52	0.96 (8)
		1:5000	52	0.39 (19)
		1:10000	46	0.22 (33)
	20	1:2000	51	0.62 (13)
		1:5000	67	0.43 (18)
		1:10000	66	0.22 (35)
	10	1:1000	19	0.45 (17)
		1:2000	39	0.38 (20)
		1:5000	67	0.31 (24)
5-AS	20	1:5000	73	0.65 (9)
		1:10000	71	0.32 (19)
		1:15000	66	0.20 (31)
	10	1:5000	75	0.48 (12)
		1:10000	75	0.32 (18)
		1:15000	73	0.20 (28)
	5	1:2000	53	0.41 (15)
		1:5000	74	0.33 (18)
		1:10000	79	0.23 (26)

a) Figures in parentheses indicate background (%).

labeled antigen. The anti-steroid antiserum employed in this work was that elicited in a rabbit by immunization with the conjugate of 4-hydroxytestosterone 4-hemiglutarate with bovine serum albumin, that is, a homologous system.[10] This antiserum showed a sufficient affinity for testosterone ($K_a = 2 \times 10^{10}$ M^{-1}) in the radioimmunoassay procedure.[8] The bound and free enzyme-labeled antigens were separated by a double antibody method, and the enzymic activity of the immune precipitate was determined. The immunoreactivity of the HRP-labeled antigen (1 ng) with the anti-testosterone antiserum diluted 1:500 was *ca.* 70%, as determined by the fluorimetric assay.[2b]

Sensitivities obtainable with the assay systems were tested by examining the inhibition of enzymic activity caused by the addition of 50 or 200 pg of testosterone per tube, in which various amounts of the HRP label and dilutions of the anti-steroid antiserum were used. The assays were assessed in terms of the absorbance for B_0 and non-specific binding (background). The criteria that the optical density obtained upon 1 h incubation and the background should be at least 0.2 and less than 20%, respectively, were employed in this work.

The results obtained with the assays using ABTS, OPD and TMB, together with those with the fluorogenic substrate HPPA, are listed in Table I. One nanogram of the label corresponds to *ca.* 7 pg of testosterone, since no significant effect of the steroid/HRP molar ratio, namely degree of substitution, on the sensitivity of the assay has been observed.[2b] It is clear that the assay systems with these three chromogens are all highly sensitive. The sensitivity obtained with TMB was comparable to that with fluorogenic HPPA. The assay

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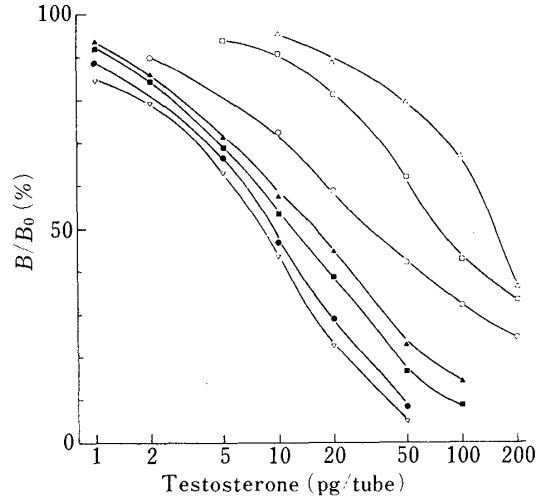

Fig. 1. Dose–Response Curves for Testosterone Enzyme Immunoassays Using TMB (●), OPD (■), ABTS (▲), 5-AS (○), MBTH (□), AEC (△) and HPPA (▽)

TABLE III. Sensitivity of Testosterone Enzyme Immunoassay Using Various Substrates for HRP

Substrate	Sensitivity[a] (pg)	Amount of label (ng)	Antiserum dilution
TMB	9	2	1:30000
OPD	12	2	1:30000
ABTS	16	5	1:20000
5-AS	30	10	1:10000
MBTH	75	20	1:5000
AEC	150	50	1:2000
HPPA	8	1	1:40000

a) The amount of testosterone needed to displace 50% of the bound label in the dose-response curve.

using OPD was somewhat more sensitive than that with ABTS. The results with the less sensitive substrates, 5-AS, AEC and MBTH, are listed in Table II. These assays needed larger amounts of the labeled antigen, resulting in a decrease in the sensitivity. Nevertheless, satisfactory sensitivities were obtained with these chromogens.

Dose–response curves for testosterone enzyme immunoassays under the optimal conditions for each system are shown in Fig. 1, and the related data are listed in Table III. The sensitivity was expressed as the amount of testosterone needed to displace 50% of the bound label in each dose–response curve. In these assays, the absorbance values for B_0 obtained upon 1 h enzymic reaction ranged from 0.3 to 0.4. It can be seen that the assay using TMB is highly sensitive, showing the sensitivity value of 9 pg. The decreasing order of sensitivity is as follows: TMB > OPD > ABTS > 5-AS > MBTH > AEC. This result was predicted by inspection of the minimal detectable amounts of native HRP with the substrates. In fact, a higher absorbance for B_0 was obtained with a more sensitive substrate in most cases, when the amount of the labeled antigen and the dilution of the antiserum were fixed. It should be noted that the assay using TMB was more sensitive than that with OPD, in spite of the use of the same immunoreaction conditions, *i.e.* 2 ng of the label at 1:30000 dilution in Table III. The sensitivity of the assay using 5-AS or MBTH seems to be comparable to that of the radioimmunoassay.[8] Thus, the present work offered a practical basis for selecting the substrate for HRP in the assay system commonly used for measurement of steroid hormones.

In general, the choice of chromogen is based on various criteria, which have been summarized by Ngo and Lenhoff.[6a] The chromogens tested in the present work have already

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been studied with regard to the sensitivity of enzyme immunoassay systems as well as mutagenicity; the safer substrates, TMB, 5-AS, MBTH and AEC, have been examined in comparison with mutagenic OPD or ABTS.[4-6] However, little work has been done on direct comparison of these substrates, including fluorogenic HPPA. To our knowledge, only one report has dealt with a hapten enzyme immunoassay system, in which TMB and OPD were compared using an anti-hapten antiserum coated on microtitration plate.[5]

The information obtained here should be helpful in the further development of enzyme immunoassay for steroid hormones and other haptenic compounds. It may be possible to improve the sensitivity of an enzyme immunoassay, if more suitable conditions or methods for HRP detection with the chromogen are available. A comparative study of the sensitivity of enzyme immunoassays using colorimetric substrates for alkaline phosphatase, β-galactosidase and HRP will be reported elsewhere.

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