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Enzyme Labeling of Steroids by the *N*-Succinimidyl Ester Method. Preparation of Horseradish Peroxidase-Labeled Antigen for Use in Enzyme Immunoassay[1)]

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Enzyme labeling of a steroid with horseradish peroxidase by the *N*-succinimidyl ester method has been investigated in comparison with that with β-galactosidase. The reaction of the activated ester of 4-hydroxytestosterone 4-hemiglutarate with horseradish peroxidase provided a labeled antigen showing high immunoreactivity with an anti-testosterone antiserum in the enzyme immunoassay procedure. The effect of steroid/enzyme molar ratio, ranging from 2 to 60, in the labeling on the assay sensitivity was then examined. It was found that, in contrast to the case of β-galactosidase, the sensitivity of the assay using horseradish peroxidase-labeled antigen is not significantly influenced by the molar ratio. Dose–response curves with high sensitivities could be obtained by the use of these labeled antigens at an appropriate dilution of the antiserum. The active ester method proved to be useful for the preparation of enzyme-labeled antigens because of its simplicity and excellent reproducibility.

Keywords——enzyme immunoassay; enzyme labeling of steroid; *N*-succinimidyl ester method; testosterone; horseradish peroxidase; β-galactosidase; anti-testosterone antiserum; immunoreactivity; sensitivity

Enzyme-labeled antigens for use in the enzyme immunoassay of steroid hormones have usually been prepared by condensation of the carboxyl group of a steroid with the amino groups of lysine residues in an enzyme. The mixed anhydride and carbodiimide methods have mainly been used for the enzyme labeling. The sensitivity and reproducibility of enzyme immunoassays are influenced by the coupling method. Previously, using β-galactosidase (β-GAL) as label, we have shown that the *N*-succinimidyl ester method is satisfactory with respect to reproducibility,[2)] and is useful for the investigation of factors influencing the sensitivity of enzyme immunoassay.[3)] The latter is related to the bridging phenomenon; anti-steroid antiserum used in the assay is elicited with an immunogen haptenized *via* a "chemical bridge," and usually contains antibodies showing affinities for the bridge portion of the enzyme-labeled antigen, and hence, the combination between antiserum and labeled antigen is an important factor determining the assay sensitivity.[4)] We have found that the use of enzyme-labeled steroid prepared from a hapten having a bridge shorter than that used for antibody preparation is advantageous for obtaining increased sensitivity.[5)] Whether the bridge length effect is dependent upon the enzyme used is an interesting problem.

Horseradish peroxidase (HRP) is an enzyme frequently used, but little work has been done on labeling of steroids with this enzyme by the *N*-succinimidyl ester method. This paper deals with the preparation of HRP–testosterone conjugates by the active ester method, and with the immunological properties of the antigens in an enzyme immunoassay system, in comparison with those of β-GAL-labeled antigens.

Materials and Methods

Materials——HRP (EC 1.11.1.7) (grade II, Reinheits-Zahl 3.2, 254 units/mg, Lot No. 3329) and β-GAL (EC 3.2.1.23) from *E. coli* (grade VI, 455 units/mg, Lot No. 122F6803) were obtained from Toyobo Co. (Osaka) and Sigma Chemical Co. (St. Louis, MO), respectively. The *N*-succinimidyl ester of 4-hydroxytestosterone 4-hemiglutarate was prepared by the method previously established in these laboratories.[6] Anti-testosterone antiserum used was that reported in the previous paper.[7] Goat anti-rabbit IgG antiserum and normal rabbit serum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo).

Assay Buffer——A 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin and 0.9% NaCl (gel–PBS) was used in the immunoassay using HRP as a label. In the system using β-GAL, gel–PBS containing 0.1% NaN_3 was used.

Preparation of Testosterone–Enzyme Conjugates——HRP Labeling: Dioxane solutions (0.2 ml) containing calculated amounts of the testosterone *N*-succinimidyl ester corresponding to steroid/HRP molar ratios of 2, 5, 10, 30 and 60 (molecular weight of HRP, 40000) were each 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 addition of PB (1.4 ml), the resulting solution was dialyzed against cold PB (3 l) for 3 d. A 1 ml aliquot of the dialyzed conjugate solution was transferred to a test tube; the solutions were stored at 4 °C at a concentration of 500 μg/ml, adjusted with assay buffer. The remaining conjugate solution was used for determination of the number of steroid molecules incorporated per enzyme molecule.

β-GAL Labeling: This was carried out in a manner similar to the HRP labeling described above. The activated ester was reacted with β-GAL (molecular weight, 540000) at molar ratios of 5, 10, 15, 30 and 60 to give β-GAL-labeled antigens.

Simultaneously, in order to test the efficiency of the dialysis, a calculated amount of 4-hydroxytestosterone 4-hemiglutarate corresponding to a steroid/enzyme molar ratio of 50 was added before dialysis to another steroid–enzyme solution prepared at a molar ratio of 10. The recovery of enzymic activity in the coupling procedure was also tested using the native enzymes in PB as controls. The HRP- and β-GAL-labeled antigens were stable for several months as regards enzymic activity and immunoreactivity under these storage conditions. For the immunoassay procedure, the solution was diluted with assay buffer containing 0.5% normal rabbit serum.

Determination of the Number of Testosterone Molecules Incorporated per HRP Molecule——Spectrometric analysis was carried out by comparing the absorbances at 403 nm and 253 nm of the conjugate with those of HRP and 4-hydroxytestosterone 4-hemiglutarate as controls in the same buffer and by using the following constants: molecular weight of HRP, 40000; ε value for the steroid, 13000 (253 nm).

Immunoreactivity and Antibody Dilution Curve——The enzyme immunoassay procedures were carried out in duplicate or triplicate in glass test tubes (10 ml) as follows: HRP- (1 ng) or β-GAL-labeled testosterone (10 ng) in the buffer (0.1 ml) and assay buffer (0.1 ml) were added to anti-testosterone antiserum (0.1 ml) diluted 1 : 500 or more, 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 the first antibody was carried out to provide a blank value. An experiment using only the enzyme-labeled steroid was also carried out to obtain 100% enzymic activity.

Inhibition of the Binding of Enzyme-Labeled Antigen to Antibody by Addition of Testosterone——A solution of testosterone (50 pg) in assay buffer (0.1 ml) and enzyme-labeled testosterone (HRP label, 1 ng; β-GAL label, 10 ng) in the buffer (0.1 ml) were added to diluted antiserum (0.1 ml), and the mixture was incubated at 4 °C for 4 h. Separation of free and bound enzyme-labeled antigens was carried out just as described above. Simultaneously, the procedure without addition of testosterone or the first antibody was carried out to provide B_0 and non-specific binding (background) values, respectively.

Measurement of Enzymic Activity——HRP: The immune precipitate was diluted with 0.5% 3-(*p*-hydroxyphenyl)propionic acid–PB (1 : 5, 1.2 ml), vortex-mixed, and preincubated at 25 °C for 3 min. Hydrogen peroxide (0.01%, 0.2 ml) was added to the resulting solution, and the mixture was incubated for 40 min. The reaction was terminated by addition of 3% NaN_3–0.5 M NaOH (1 : 1, 2 ml). The fluorescence intensity was then measured at 405 nm with excitation at 320 nm; the background was estimated as a percentage of the intensity for B_0, using the NaN_3–NaOH solution diluted 1 : 1.7 with H_2O as the zero reference.

β-GAL: The immune precipitate was diluted with assay buffer (1 ml) containing 0.2% $MgCl_2$ and 0.7% 2-mercaptoethanol, vortex-mixed, and preincubated at 37 °C for 3 min. 4-Methylumbelliferyl β-D-galactopyranoside (0.007%, 1 ml) in assay buffer was added to the resulting solution, and the mixture was incubated for 60 min. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml) and the fluorescence intensity was measured at 450 nm with excitation at 360 nm. The zero reference used consists of these solutions except the substrate solution.

In the case of the recovery test for enzymic activity or the use of only enzyme-labeled testosterone as described above, these procedures were applied to the enzyme solution, and the reaction was terminated after a 20-min

incubation. The fluorescence intensity obtained with the latter was corrected for the incubation time, and this was defined as 100% enzymic activity.

Results and Discussion

The purpose of this work was to examine the immunoreactivity of the testosterone–HRP conjugate prepared by the *N*-succinimidyl ester method with the anti-testosterone antiserum in the enzyme immunoassay procedure, and the sensitivity of the assay using the labeled antigen. For comparison, the enzyme labeling of testosterone with β-GAL was also carried out.[2] The *N*-succinimidyl ester prepared from 4-hydroxytestosterone 4-hemiglutarate by condensation with *N*-hydroxysuccinimide in the presence of a water-soluble carbodiimide was reacted with HRP and β-GAL to give enzyme-labeled antigens (Fig. 1). The enzyme labeling was carried out by mixing the activated ester with the enzymes in phosphate buffer (pH 7.3)–dioxane. The activated ester should react readily with free amino groups of these enzymes. Various molar ratios of the steroid to enzyme, ranging from 2 to 60 in the HRP labeling and from 5 to 60 in the β-GAL labeling, were used. The reaction mixtures were dialyzed against the buffer to remove the unreacted steroid and *N*-hydroxysuccinimide. The loss of enzymic activity was less than 20% under the coupling conditions used. Spectrometric analysis showed that the average number of steroid molecules incorporated per HRP molecule (substitution degree) ranged from 0.6 to 2.3. In the case of β-GAL, where the values were not precisely determined, substitution degrees were at least 3, 4, 9 and 17 for molar ratios of 10, 15, 30 and 60, respectively.

The anti-testosterone antiserum used in the enzyme immunoassay was that elicited in a rabbit by immunization with the conjugate of 4-hydroxytestosterone 4-hemiglutarate with bovine serum albumin, that is, a homologous assay system.[4] The bound and free enzyme-labeled antigens were separated by a double antibody method. The enzymic activity of immune precipitate was determined by fluorophotometric methods using 3-(*p*-

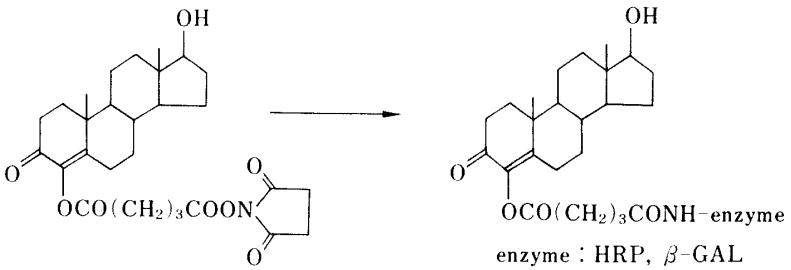

Fig. 1. Preparation of Enzyme-Labeled Antigens

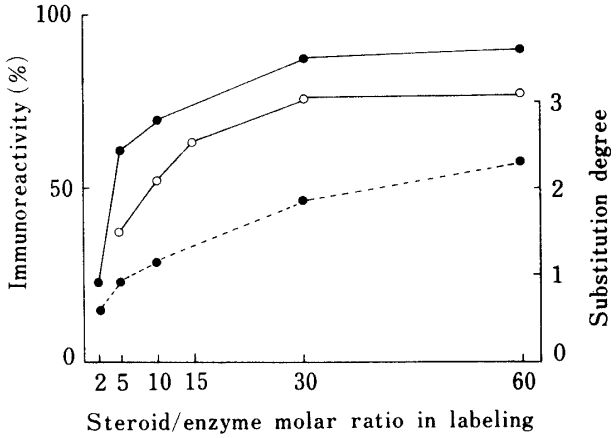

Fig. 2. Immunoreactivities of HRP- (●—●) and β-GAL-Labeled Antigens (○—○) with the Anti-testosterone Antiserum and Substitution Degrees in the HRP-Labeled Antigens (●---●)

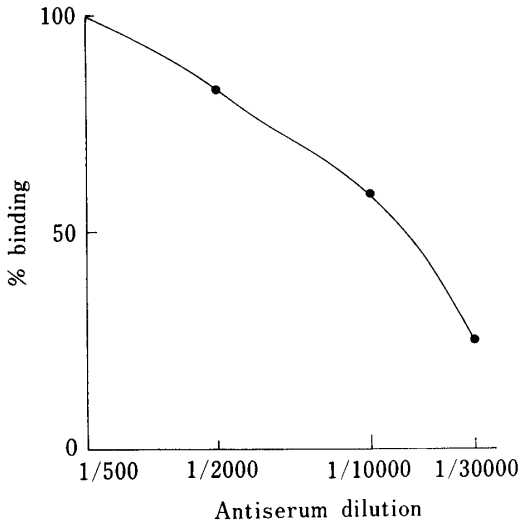

Fig. 3. An Antibody Dilution Curve with the HRP-Labeled Antigen Prepared at a Molar Ratio of 10

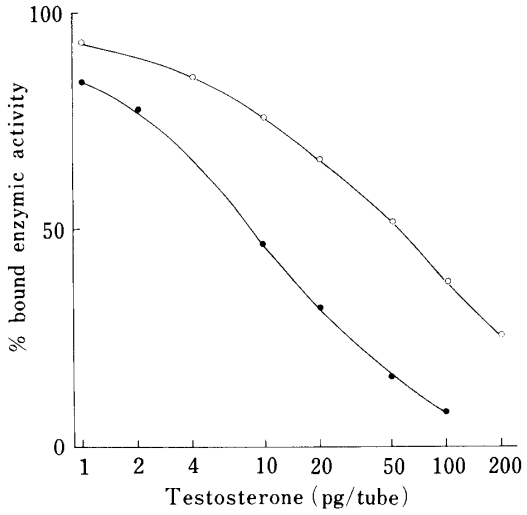

Fig. 4. Dose–Response Curves for Testosterone Enzyme Immunoassays Using HRP- (●) and β-GAL-Labeled Antigens (○) Prepared at a Molar Ratio of 10

Anti-testosterone antiserum diluted 1 : 40000 was used.

hydroxyphenyl)propionic acid for HRP[8] and 4-methylumbelliferyl β-D-galactopyranoside for β-GAL as substrates.

Immunoreactivities of the enzyme-labeled antigens obtained with various steroid/enzyme molar ratios were investigated at 1 : 500 dilution of the anti-testosterone antiserum. The amount of the antigen fixed corresponds to *ca.* 10 pg of testosterone, if the substitution degree is 1. The results, together with the substitution degree in the HRP-labeled antigens, are shown in Fig. 2. The binding ability increased with increasing molar ratio for both enzymes, and reached a plateau at a ratio of 30 in the case of β-GAL; this is in good agreement with the result obtained in the previous study.[2] In the case of HRP, excellent immunoreactivities were observed at molar ratios of 30 and 60. A good correlation between the immunoreactivity and substitution degree was also found. It should be noted that the unreacted steroid in the enzyme labeling was effectively removed by the dialysis; this conclusion was based upon the fact that the immunoreactivities or substitution degrees of the labeled antigens prepared at a molar ratio of 10 with and without addition of 4-hydroxytestosterone 4-hemiglutarate as a tracer were nearly equal, and was further confirmed by comparison of the assay sensitivities obtained with these antigens as described below (data not shown).

The effect of the steroid/enzyme molar ratio, namely substitution degree, on the sensitivity of the assay was then examined. For this purpose, an appropriate dilution of anti-testosterone antiserum for use in enzyme immunoassay was determined by construction of antibody dilution curves. The result obtained with the HRP-labeled antigen prepared at a molar ratio of 10 is shown in Fig. 3. The binding ability was expressed for convenience as a percentage of that obtained with 1 : 500 dilution. The dilution showing 50% binding can be defined as a titer. Therefore, the use of the antiserum diluted approximately 1 : 15000 may be suitable for obtaining a dose–response curve. In practice, however, various dilutions (15—60% bindings) were employed in the comparative study of sensitivity. Similar antibody dilution curves were obtained in the other cases, including β-GAL-labeled antigens. Sensitivities obtainable with the assay systems were tested by examining the inhibition of enzymic activity caused by the addition of 50 pg of testosterone per tube, *i.e.* the extent of inhibition at the point of 50 pg of the steroid in the dose–response curves, as shown in Fig. 4.

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

Molar ratio	Antiserum dilution	Inhibition (%)	NSB[a] (%)
2	1 : 10000	69	7
	1 : 20000	85	10
	1 : 40000	91	18
5	1 : 10000	48	3
	1 : 20000	70	4
	1 : 40000	83	8
10	1 : 10000	75	2
	1 : 20000	75	3
	1 : 40000	86	7
30	1 : 20000	74	3
	1 : 40000	80	6
	1 : 60000	85	8
	1 : 100000	90	19
60	1 : 40000	77	5
	1 : 60000	78	8
	1 : 80000	85	9
	1 : 100000	90	14

a) Non-specific binding (background).

TABLE II. Inhibition of Bound Enzymic Activity of β-GAL-labeled Antigens by 50 pg of Testosterone

Molar ratio	Antiserum dilution	Inhibition (%)	NSB[a] (%)
5	1 : 10000	14	19
	1 : 20000	29	19
	1 : 40000	46	22
10	1 : 10000	27	7
	1 : 20000	38	10
	1 : 40000	40	10
15	1 : 10000	20	5
	1 : 20000	35	10
	1 : 40000	35	10
30	1 : 10000	15	6
	1 : 20000	16	6
	1 : 40000	16	7
60	1 : 10000	5	5
	1 : 20000	6	6
	1 : 40000	7	6

a) Non-specific binding (background).

The assays were assessed in terms of the fluorescence intensity for B_0 and non-specific binding (background), since higher dilution of antiserum tended to cause an increase in the sensitivity, accompanied with an undesired increase in the background. The criterion that the background obtained should be less than 10% was employed in this work. The results obtained with the assays using the HRP- and β-GAL-labeled antigens are listed in Tables I and II, respectively. The data in Table I showed that, according to our criteria, similar sensitivities (83—86% inhibition) were obtained with all the HRP-labeled antigens tested, when an

appropriate dilution of the antiserum was employed. Thus, it is evident that the effect of molar ratio in the HRP labeling on the assay sensitivity is not significant. In contrast, in the case of β-GAL-labeled antigen, the sensitivity was markedly influenced by the molar ratio, in accord with the previous findings.[2,3]

Typical dose–response curves for testosterone enzyme immunoassays using the HRP- and β-GAL-labeled antigens prepared at a molar ratio of 10 are shown in Fig. 4. These were the "best" standard curves obtained with each assay system. It can be seen that the sensitivity of the assay using HRP-labeled antigen is higher than that of the assay system with the β-GAL-labeled antigen. The minimal detectable amounts of testosterone, that is, twice the standard deviation of the zero determination (B_0, $n=10$), were 1 pg and 4 pg in the assays using the HRP and β-GAL labels, respectively. Based on a balance between sensitivity and precision, we recommend steroid/enzyme molar ratios ranging from 10 to 60 in the HRP labeling, and from 10 to 20 in the β-GAL labeling; employment of a molar ratio higher than 60 in the former is not practical. In general, however, it is desirable to estimate the substitution degree, since the labeling rate is influenced by various factors, such as pH, solvent volume, and reactivity of steroid derivatives.

The present work showed that the *N*-succinimidyl ester method is useful in the preparation of HRP-labeled antigen as well as β-GAL label. The difference in the effect of the molar ratio in the enzyme labelings on the assay sensitivity is of interest. It seems likely that a 1 : 1 steroid–enzyme conjugate is suitable for obtaining a high sensitivity, since the immunoreaction between this antigen and antibody molecule is efficiently responsive to a minimum amount of the antigen to be measured; a higher substitution degree results in a decrease in assay sensitivity. This may be the case in the assay using the β-GAL-labeled antigens. With the HRP-labeled antigen, a possible explanation for the lack of effect of the molar ratio on the sensitivity is that a limited number of free amino groups of this enzyme is available. In fact, the number of steroid molecules incorporated per HRP molecule was not very large even when a large excess of the activated ester, for example, a molar ratio of 60, was used, and hence, the range of substitution degree was narrow (Fig. 2). We also speculate that if the steroid molecules in the label having a substitution degree of 2 or more are sterically near each other, they do not bind simultaneously to antibody molecules. Therefore, the effect of the molar ratio may be dependent upon the method of enzyme labeling, the enzyme preparation (lot), and the haptenic steroid derivative used. Enzyme labeling of steroids with HRP has mostly been carried out by the mixed anhydride method, which is not always satisfactory with respect to reproducibility, resulting in inexplainable relationships among the molar ratio, substitution degree and immunoreactivity. There are various reported substitution degrees up to 11,[9] whereas the number of lysine residues in HRP molecule has been reported to be 5—6.[10] To our knowledge, only one report has dealt with the effect of the molar ratio on sensitivity; it was noted that decreasing the substitution degree causes a marked improvement in sensitivity, but the details have not been described.[9a] Thus, we can say at the present time that, under the conditions used here, the sensitivity of the assay using HRP-labeled antigens is not significantly influenced by the molar ratio.

Application of the active ester method to other enzymes which are currently employed in enzyme immunoassays, and studies on the bridging phenomena in assay systems using HRP as a label are in progress in these laboratories.

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