Chem. Pharm. Bull.
33(12)5393—5398(1985)

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

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(Received May 22, 1985)

Enzyme labeling of a steroid with alkaline phosphatase by the *N*-succinimidyl ester method was investigated. The activated ester of 4-hydroxytestosterone 4-hemiglutarate was treated with alkaline phosphatase to give a labeled antigen. Various molar ratios of steroid to enzyme, ranging from 5 to 200, were employed. The loss of enzymic activity was less than 20% under the coupling conditions used. Satisfactory immunoreactivities with an anti-testosterone antiserum in the enzyme immunoassay procedure were obtained with the labeled antigens prepared at molar ratios higher than 20. The effect of steroid/enzyme molar ratio in the labeling on the sensitivity of the testosterone assay was then examined. It was found that the sensitivity of the assay is significantly influenced by the molar ratio, and a higher ratio results in a decrease in assay sensitivity. A dose-response curve with a reasonable sensitivity could be obtained by the use of the labeled antigen prepared at a molar ratio of 30. The active ester method proved to be useful for the preparation of alkaline phosphatase-labeled antigens as well as for β-galactosidase and horseradish peroxidase labelings, because of its simplicity and excellent reproducibility.

Keywords——enzyme immunoassay; steroid enzyme labeling; *N*-succinimidyl ester method; testosterone; alkaline phosphatase; anti-testosterone antiserum; immunoreactivity; sensitivity

Enzyme-labeled antigens for use in the heterogeneous 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 enzyme labeling. Enzymes currently used as labels are β-galactosidase, horseradish peroxidase, alkaline phosphatase (AP), glucose oxidase, glucoamylase, glucose-6-phosphate dehydrogenase, penicillinase and urease. The choice of enzyme is based on various criteria such as purity and stability.[2] In the preparation of an enzyme-labeled antigen, the coupling method employed seems rather important, since this influences the sensitivity and reproducibility of enzyme immunoassays. In order to obtain a practical basis for selecting the enzyme, it is necessary to perform comparative studies using the same coupling method, haptenic steroid derivative, and anti-steroid antiserum. There is an excellent review dealing with enzyme labeling of antibodies with the former four enzymes.[3] We have previously shown that the *N*-succinimidyl ester method offers satisfactory reproducibility, using β-galactosidase and horseradish peroxidase as labels.[4] Further, the two enzymes have been compared with regard to the effects of steroid/enzyme molar ratio in the labeling on the immunoreactivity of the labeled antigen with an anti-steroid antiserum and on the assay sensitivity.[4b]

AP is frequently used as an enzyme label, 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 AP-testosterone conjugates by the active ester method, and with the immunological properties of the antigens in an enzyme immunoassay system.

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Materials and Methods

Materials——AP (EC 3.1.3.1) from calf intestine (enzyme label for enzyme immunoassay, 2500 units/mg, Lot No. 1144524) was obtained as a solution (1 mg/0.1 ml) from Boehringer–Mannheim Yamanouchi Co. (Tokyo). The *N*-succinimidyl ester of 4-hydroxytestosterone 4-hemiglutarate was prepared by the method previously established in these laboratories.[5] Anti-testosterone antiserum used was that reported in the previous paper.[6] Goat anti-rabbit IgG antiserum and normal rabbit serum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo).

Buffer Solution——A 0.05 M phosphate buffer (pH 7.3) (PB) was used in the enzyme labeling. In the enzyme immunoassay procedure, three solutions containing 0.1% gelatin, 0.9% NaCl and 0.1% NaN_3 were used; these were PB (buffer A), a 0.05 M borax buffer (pH 7.3) (buffer B) and a 0.05 M carbonate buffer (pH 10) (buffer C).

Preparation of Testosterone–AP Conjugates——Dioxane solutions (0.1 ml) containing calculated amounts of the testosterone *N*-succinimidyl ester corresponding to steroid/AP molar ratios of 5, 10, 15, 20, 30, 50, 100 and 200 (M_r of AP, 116500)[7] were each added to a solution of AP (100 μg) in PB (0.2 ml) at 0 °C, and the mixture was gently stirred at 4 °C for 4 h. After addition of PB (0.5 ml), the resulting solution was dialyzed against cold PB (1 l × 4) for 2 d. A 0.7 ml aliquot of the dialyzed conjugate solution was transferred to a test tube; the solutions were stored at 4 °C at a concentration of 100 μg/ml, adjusted with buffer A containing additional gelatin (0.5%).

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 180 was added before dialysis to another steroid–enzyme conjugate solution prepared at a molar ratio of 20. The recovery of enzymic activity in the coupling procedure was also tested using the native enzyme in PB as a control. The 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 buffer A or buffer B each containing 0.5% normal rabbit serum.

Determination of the Number of Testosterone Molecules Incorporated per AP Molecule——Spectrometric analysis was carried out with the steroid–enzyme conjugate prepared at a molar ratio of 30 by use of 500 μg of AP. The absorbances at 280 and 253 nm of the conjugate were compared with those of AP and 4-hydroxytestosterone 4-hemiglutarate as controls in PB. The constants used were as follows: M_r of AP, 116500; ε value for the steroid, 13000 (253 nm).

Immunoreactivity——The enzyme immunoassay procedure was carried out in duplicate or triplicate in a glass test tube (10 ml) as follows: AP-labeled testosterone (5 ng) in buffer B (0.1 ml) containing normal rabbit serum and buffer A (0.1 ml) were added to anti-testosterone antiserum (0.1 ml) diluted 1 : 500 with buffer A, and the mixture was incubated at 4 °C for 4 h. Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1 : 30 with buffer A 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 buffer C (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 buffer C (1.5 ml) 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 (1 ng) was also carried out to obtain 100% enzymic activity.

Antibody Dilution Curve——The enzyme immunoassay procedure was carried out in the manner described above, except for the use of buffer A instead of buffer B as a solvent for the AP-labeled antigen, and of various dilutions of the anti-testosterone antiserum.

Inhibition of the Binding of Enzyme-Labeled Antigen to Antibody by Addition of Testosterone——A solution of testosterone (50 or 500 pg) in buffer A (0.1 ml) and enzyme-labeled testosterone (5 ng) in buffer A (0.1 ml) containing normal rabbit serum 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——The immune precipitate was diluted with a 0.05 M carbonate buffer (pH 10) (2 ml) containing 4-methylumbelliferyl phosphate (0.005%) and $MgCl_2$ (0.01%), vortex-mixed, and incubated at 37 °C for 30 min. The reaction was terminated by addition of a 0.5 M phosphate buffer (pH 10.4) (2 ml). The fluorescence intensity was then measured at 450 nm with excitation at 360 nm. The background was estimated as a percentage of the intensity for B_0; the zero reference used consisted 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 in buffer B, and the reaction was terminated after a 10-min incubation. The fluorescence intensity obtained with the latter was corrected for both the incubation time and the amount of the label, and this was defined as 100% enzymic activity.

Results and Discussion

The purpose of this work was to examine the immunoreactivity of the testosterone–AP

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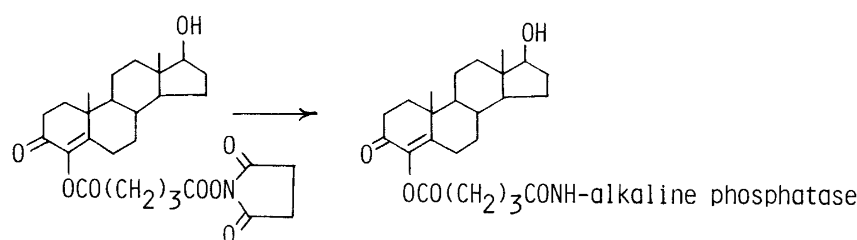

Fig. 1. Preparation of Enzyme-Labeled Antigen

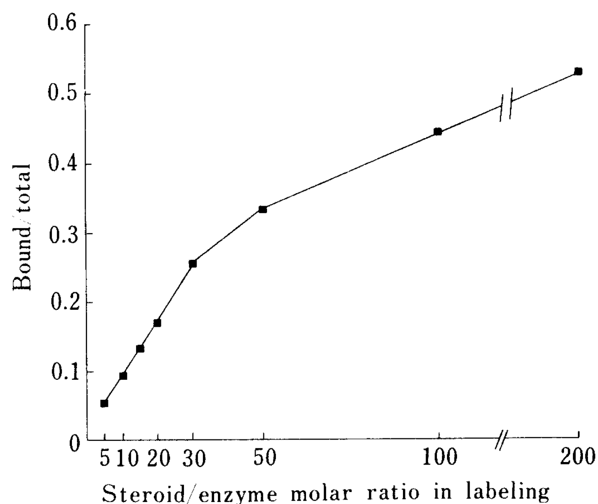

Fig. 2. Immunoreactivities of AP-Labeled Antigens with the Anti-testosterone Antiserum

conjugate prepared by the *N*-succinimidyl ester method with the anti-testosterone antiserum in the enzyme immunoassay procedure, and the sensitivity of the testosterone assay using the labeled antigen. 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 AP to give enzyme-labeled antigens (Fig. 1). The enzyme labeling was carried out by mixing the activated ester with the enzyme in phosphate buffer (pH 7.3)–dioxane. The activated ester should react readily with free amino groups of the enzyme. Various molar ratios of the steroid to enzyme, ranging from 5 to 200, were used. The reaction mixtures were dialyzed against the buffer to remove the unreacted steroid. 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 AP molecule (degree of substitution) was *ca.* 1 for the labeled antigen prepared at a molar ratio of 30.

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.[8] The bound and free enzyme-labeled antigens were separated by a double antibody method. The enzymic activity of immune precipitate was determined by a fluorophotometric method using 4-methylumbelliferyl phosphate as a substrate.

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 are shown in Fig. 2. The binding ability increased with increasing molar ratio; satisfactory immunoreactivities were obtained with the labeled antigens prepared at molar ratios higher than 20. It should be noted that the unreacted steroid in the enzyme labeling was effectively removed by the dialysis: this conclusion was based on the fact that the immunoreactivities of the labeled antigens prepared at a molar ratio of 20 with and without

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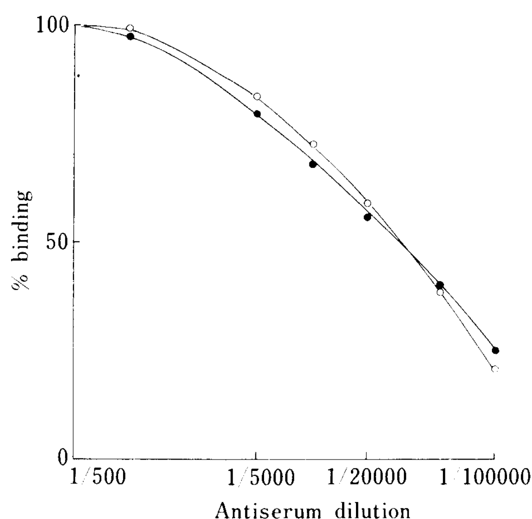

Fig. 3. Antibody Dilution Curves with the AP-Labeled Antigens Prepared at Molar Ratios of 20 (●) and 50 (○)

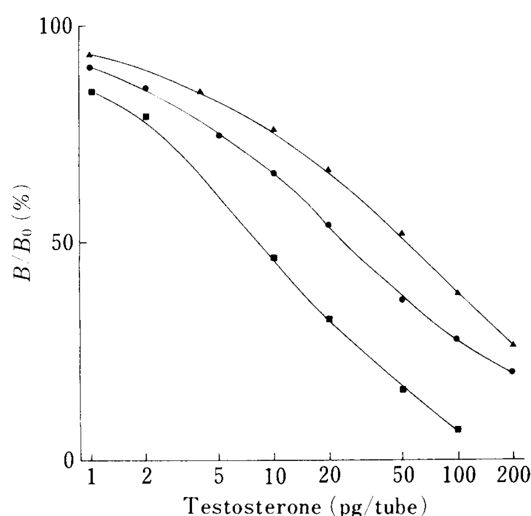

Fig. 4. Dose-Response Curves for Testosterone Enzyme Immunoassays Using AP (●)-, Horseradish Peroxidase (■)- and β-Galactosidase (▲)-Labeled Antigens

The initial dilution of anti-testosterone antiserum used was 1 : 40000.

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 on the sensitivity of the testosterone 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 results obtained with the AP-labeled antigens prepared at molar ratios of 20 and 50 are 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 : 30000 may be suitable for obtaining dose-response curves. In practice, however, various dilutions (20—80% bindings) were employed in the comparative study of sensitivity. Similar antibody dilution curves were obtained in the cases of other labeled antigens. Sensitivities obtainable with the assay systems were tested by examining the inhibition of enzymic activity caused by the addition of 50 pg or 500 pg of testosterone per tube, *i.e.* the extent of inhibition at the corresponding points of steroid amount in the dose-response curve, as shown in Fig. 4. 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. With all the assay systems, good parallelism of inhibition was obtained between the experiments using 50 and 500 pg of testosterone. The results obtained in the former are listed in Table I. The data showed that the sensitivity was significantly influenced by the molar ratio and a higher ratio resulted in a decrease in assay sensitivity. The use of a smaller amount of the enzyme label prepared at molar ratios of 100 and 200 was also examined, but was not very effective in terms of sensitivity.

A typical dose-response curve for the testosterone enzyme immunoassay using the AP-labeled antigen prepared at a molar ratio of 30, together with those reported for the assays using β-galactosidase and horseradish peroxidase as labels, where the same anti-steroid antiserum was used,[4b)] is shown in Fig. 4. These were the "best" standard curves obtained with

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

Molar ratio	Antiserum dilution	Inhibition (%)	NSB[a] (%)
5	1:5000	26	16
	1:10000	65	25
10	1:10000	59	15
	1:20000	71	16
15	1:10000	57	9
	1:20000	67	12
	1:40000	80	15
20	1:10000	58	9
	1:20000	67	10
	1:40000	72	15
30	1:10000	55	6
	1:20000	65	7
	1:40000	68	10
50	1:10000	43	6
	1:20000	50	7
	1:60000	56	10
100	1:20000	43	4
	1:40000	45	6
	1:60000	52	7
200	1:20000	33	4
	1:40000	35	6
	1:60000	41	7

a) Non-specific binding (background).

each assay system. In the case of the AP label, the amount of testosterone needed to displace 50% of the bound label was 25 pg. With the label prepared at a molar ratio of 200, it was estimated to be 120 pg (data not shown). It can be seen that the sensitivity of the present assay is higher than that of the assay using β-galactosidase, but is less than that obtained with the horseradish peroxidase label. The minimal detectable amount of testosterone, that is, twice the standard deviation of the zero determination (B_0, $n=10$), was 2 pg. Based on a balance between sensitivity and precision, we recommend steroid/AP molar ratios ranging from 20 to 40. In general, however, it is desirable to estimate the degree of substitution, 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 AP-labeled antigen as well as for β-galactosidase and horseradish peroxidase labels. Enzyme labeling of steroids or other haptens with AP has been carried out by the mixed anhydride[9] and carbodiimide[10] methods, which are not always satisfactory with respect to reproducibility. With the carbodiimide method, a significant loss of enzymic activity has been reported.[10a] The immunoreactivity of the AP-labeled antigen prepared by the present method, in which a high enzymic activity was recovered, was found to increase reasonably with increasing steroid/enzyme molar ratio. The effect of the molar ratio in the labeling on the assay sensitivity was not unexpected. It seems likely that a 1 : 1 steroid–enzyme conjugate is suitable for obtaining a high sensitivity, since the immunoreaction between this antigen and the antibody molecule is efficiently responsive to a minimum amount of the antigen to be measured; a higher degree of substitution results in a decrease in assay sensitivity. This may be the case in the present assay, because of the availability of 42 lysine

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residues[7] for labeling. A more marked effect and no significant effect have previously been observed with β-galactosidase and horseradish peroxidase, respectively.[4b] These results are reasonably related to the numbers of lysine residues in the enzyme molecules; the values have been reported to be 115 for β-galactosidase[11] and 5—6 for horseradish peroxidase.[12]

Thus, the sensitivities of the testosterone immunoassays using β-galactosidase, horseradish peroxidase[4b] and AP as labels, in spite of the use of homologous systems, were found to be comparable to or higher than that of the radioimmunoassay.[6] This indicates that the anti-steroid antiserum used here has weak, if any, binding affinity for the bridge between the enzyme and steroid in the labeled antigen. In general, however, it is often necessary to design an enzyme immunoassay system using different haptenic derivatives, *i.e.* a heterologous system,[8] since an anti-steroid antiserum contains various antibodies showing different specificities and at least a portion of the antibody population has an affinity for not only the steroid molecule but also the bridge portion. Hence, a homologous assay system does not provide satisfactory assay sensitivity. It is of interest to know whether the bridging phenomenon is influenced by the enzyme used. This is under investigation.

The findings obtained here should be useful in the development of hapten enzyme immunoassays. A comparative study of the sensitivity obtainable with enzyme immunoassay systems using colorimetric substrates for β-galactosidase, horseradish peroxidase and AP will be reported elsewhere. Application of the active ester method to other enzymes which are currently employed in enzyme immunoassays, and studies on bridging phenomena in various assay sytems are in progress in these laboratories.

Acknowledgement This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, which is gratefully acknowledged.

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