

Enzyme Labeling in Steroid Enzyme Immunoassays. The *p*-Nitrophenyl Ester Method for Alkaline Phosphatase and Glucose Oxidase Labelings

Hiroshi HOSODA,* Katsuyuki FUKUDA, and Junichi GOTO

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan. Received October 29, 1991

The *p*-nitrophenyl ester method was assessed as an enzyme labeling technique. The active ester of a carboxylated testosterone derivative was treated with alkaline phosphatase and glucose oxidase to give labeled antigens, using various molar ratios of steroid to enzyme. Satisfactory immunoreactivities with an anti-testosterone antibody in an enzyme immunoassay system were obtained with the labeled antigens prepared at pH 8.5 by the use of molar ratios higher than 30 and 10, respectively, in the alkaline phosphatase and glucose oxidase labelings.

Keywords enzyme immunoassay; testosterone; steroid enzyme labeling; *p*-nitrophenyl ester method; *N*-succinimidyl ester method; alkaline phosphatase; glucose oxidase

Enzyme-labeled antigens for use in the enzyme immunoassay of steroid hormones have usually been prepared by condensation of the carboxyl groups of a steroid hapten with the amino groups of lysine residues in an enzyme. The sensitivity and reproducibility of enzyme immunoassays are influenced by the coupling method, minimum losses of enzymic activity and immunoreactivity are essential. Thus, the coupling reactions must be efficient under mild conditions. The *N*-succinimidyl (NS) ester method is useful for alkaline phosphatase (AP), β -galactosidase, horseradish peroxidase and glucose oxidase (GOD) labelings, as an alternative to the conventional mixed anhydride and carbodiimide methods.¹⁾ We previously showed that, in steroid enzyme immunoassay systems with β -galactosidase and horseradish peroxidase labels, the activation of the carboxyl group as the *p*-nitrophenyl (*p*-NP) ester²⁾ was the method of choice.³⁾ This paper deals with the *p*-NP ester method in a testosterone assay system using AP and GOD as label enzymes.

Materials and Methods

Materials AP (EC 3.1.3.1) from calf intestine (enzyme label for enzyme immunoassay, 2500 U/mg) and GOD (EC 1.1.3.4) from *Aspergillus niger* (grade I, 289 U/mg) were obtained from Boehringer-Mannheim Yamanoichi Co. (Tokyo); horseradish peroxidase (EC 1.11.1.7) (grade I-C, Reinheits-Zahl 3.15, 250 U/mg) was from Toyobo Co. (Osaka). The NS and *p*-NP esters of 4-(carboxymethylthio)testosterone (T·CMT) were prepared by the methods previously established in these laboratories.³⁾ Anti-testosterone antiserum used was that produced by immunization in the rabbit using the conjugate of 4-hydroxytestosterone 4-hemiglutarate with bovine serum albumin.⁴⁾ Normal rabbit serum and goat anti-rabbit immunoglobulin G antiserum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo). *p*-NP phosphate and 3,3',5,5'-tetramethylbenzidine were obtained from Nacalai Tesque Inc. (Kyoto) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo), respectively.

Buffer Solution 0.05 M Phosphate buffers (PBs) (pH 7.3, 8.0) and a 0.05 M borate buffer (pH 8.5) were used in the enzyme labeling. In the immunoassay, two solutions containing 0.1% gelatin and 0.9% NaCl were used; these were PB, pH 7.3 (buffer A) and a 0.05 M borax buffer, pH 7.3 (buffer B).

Enzyme Labeling of Steroid by the Active Ester Methods AP Labeling: Dioxane solutions (0.1 ml) containing calculated amounts of T·CMT *p*-NP ester corresponding to steroid/AP molar ratios of 10–100 (molecular weight of AP, 116500)⁵⁾ were each added at 0°C to a solution of AP (100 μ g) in borate buffer, pH 8.5, or PBs, pH 7.3 and 8.0 (0.2 ml), and the mixture was gently stirred at 4°C for 4 h. After addition of PB, pH 7.3 (0.5 ml), the resulting solution was dialyzed against the cold PB (2 l) for 2 d. A 0.7 ml aliquot of the dialyzed conjugate solution was transferred to a test tube; the solution was stored at 4°C at a concentration of 100 μ g/ml, adjusted with buffer A containing additional gelatin (0.5%). In a similar manner, the AP labeling by the NS ester method was carried out with

T·CMT NS ester in PBs, pH 7.3 and 8.0.

GOD Labeling: A solution of T·CMT *p*-NP or NS ester (steroid/enzyme molar ratios; 4–100) in dioxane (0.1 ml) was added to a solution of an enzyme (1 mg) (molecular weight of GOD, 160000)⁶⁾ in borate buffer, pH 8.5, or PB, pH 7.3 (0.2 ml) at 0°C, and the mixture was gently stirred at 4°C for 4 h. After addition of PB, pH 7.3 (1.2 ml), the resulting solution was dialyzed against the cold buffer (2 l) for 2 d. A 1 ml aliquot of the dialyzed conjugate solution was transferred to a test tube; the solution was stored at 4°C at a concentration of 500 μ g/ml, adjusted with buffer A. Preliminary experiments for GOD labeling were also carried out at pH 8.0 by the active ester method with a final enzyme concentration of 2.9 μ M, which is equal to that of AP.

Enzyme Immunoassay Procedure This was carried out in triplicate in glass test tubes (10 ml). The standard procedure for immunoreactivity of the AP label is as follows: AP-labeled antigen (10 ng) in buffer B (0.1 ml) containing 0.5% normal rabbit serum was 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 immunoglobulin G 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 B (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 B (1.5 ml), and used for measurement of the enzymic activity (B_0).

In the enzyme immunoassay using GOD as a label, the immune reaction procedure was carried out using buffer A in a manner similar to that described above; the amount of the labeled antigen used was 50 ng. For the bound/free separation, the second antibody in the buffer containing 0.3% ethylenediaminetetraacetic acid was used. In each system, the procedure without addition of the first antibody was carried out to provide non-specific binding values. An experiment using only the enzyme label was also carried out to obtain 100% enzymatic activity (T).

Measurement of Enzyme Activity The enzymatic activity of the immune precipitate or enzyme solution was measured by the methods described earlier.⁷⁾ *p*-NP phosphate was used as a substrate for AP, and the tetramethylbenzidine–horseradish peroxidase system was employed in the detection of GOD activity.

Results and Discussion

The purpose of this work was to assess the *p*-NP ester method as an enzyme labeling technique, in comparison with the NS ester method; optimal coupling reaction conditions were examined for AP and GOD labelings in the testosterone enzyme immunoassay procedure. The carboxylated derivative employed here as a model hapten was T·CMT, since both *p*-NP and NS esters of this compound can be obtained in a pure crystalline form.

The enzyme labeling was carried out by mixing the active ester with the enzyme in buffer–dioxane at 4°C for 4 h (Fig. 1). The active ester should react with free amino groups of these enzymes. Various molar ratios of the steroid to enzyme, ranging from 4 to 100, were used. The reaction

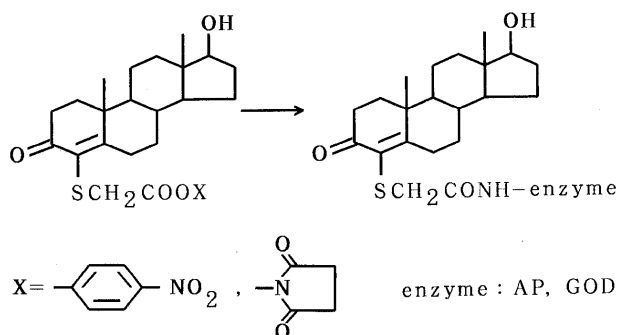


Fig. 1. Enzyme Labeling of T·CMT by the Active Ester Methods

mixtures were dialyzed against PB, pH 7.3, to remove the unreacted steroid and *p*-nitrophenol or *N*-hydroxysuccinimide. The loss of enzymatic activity was less than 20% under the coupling conditions used.

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.⁴⁾ The bound and free enzyme-labeled antigens were separated by a double antibody method. The enzymatic activity of immune precipitate was determined by colorimetric methods.

First, preliminary coupling experiments were carried out at pH 8.0, using an equal molar concentration of AP and GOD. The immunoreactivity of the enzyme-labeled T·CMT showed that high binding abilities were obtained with the labels prepared by the NS ester method. In contrast, the *p*-NP ester method gave much less reactive labels under the conditions tested; although the AP system showed a higher reactivity than the GOD system, more alkaline conditions were needed for the labeling by the *p*-NP ester method in both enzyme systems.

We then explored the practical enzyme labeling conditions in the *p*-NP ester method. The results obtained above, together with the previous findings,³⁾ suggested that a pH to be tested for coupling was 8.5 with reaction time of 4 h. The immunoreactivities obtained with the two enzyme labels are shown in Fig. 2, in which the reactivities of the labels prepared at pH 7.3 by the NS or *p*-NP ester method were also presented. It can be seen that satisfactory immunoreactivity is obtained at pH 8.5 with the steroid/enzyme molar ratio of higher than 30 and 10, respectively, in the AP and GOD labelings by the *p*-NP ester method. The binding abilities obtained were comparable to those with the NS ester method (pH 7.3). It should be noted that, with the *p*-NP ester method, the use of a higher pH value than 9 is not effective in increasing immunoreactivity.

In the NS ester method, we have reported that the use of molar ratios of 20–40 at pH 7.3 in the AP labeling⁸⁾ and 4–8 in the GOD labeling¹⁾ provide satisfactory immunoassay sensitivity. These findings were obtained in the testosterone assay system using the enzyme-labeled

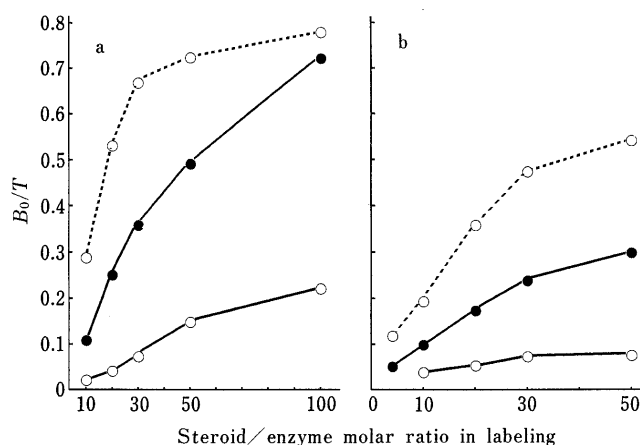


Fig. 2. Binding Abilities of the AP- (a) and GOD-Labeled (b) T·CMT Prepared at pH 8.5 (●) or 7.3 (○) by the Practical *p*-NP (—) and NS (---) Ester Methods Using Various Steroid/Enzyme Molar Ratios

4-hydroxytestosterone 4-hemiglutarate, but it has been found that the present antigen T·CMT exhibits a similar immunoreactivity.³⁾ Hence, we recommend molar ratios of 40–100 and 10–20 at pH 8.5, respectively, in the AP and GOD labelings by the *p*-NP ester method. In fact, dose–response curves for testosterone with a high sensitivity could be obtained, when the labels thus prepared were used.

The present information should be helpful in the further development of enzyme immunoassay for steroid hormones and other haptenic compounds. The *p*-NP ester method appears to be useful for the enzyme labeling of highly polar carboxylated haptens. Of course, the NS ester method is convenient and efficient in most cases. Application of the *p*-NP ester method to other immunoassay systems including enzyme immunoassays for steroid glucuronides is in progress.

Acknowledgement This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

References

- 1) H. Hosoda, R. Tsukamoto, M. Shishido, W. Takasaki, and T. Nambara, *Chem. Pharm. Bull.*, **35**, 4856 (1987), and the references cited therein.
- 2) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
- 3) H. Hosoda, K. Fukuda, and Y. Gotoh, *Chem. Pharm. Bull.*, **39**, 2373 (1991).
- 4) H. Hosoda, K. Tadano, S. Miyairi, and T. Nambara, *J. Steroid Biochem.*, **10**, 513 (1979).
- 5) P. Portmann, A. Jörg, K. Furrer, H. Walker, P. Leuthard, J. Sudan, F. Perriard, J. Comment, G. Leva, and J. Nell, *Helv. Chim. Acta*, **65**, 2668 (1982).
- 6) H. Tsuge, O. Natsuaki, and K. Ohashi, *J. Biochem. (Tokyo)*, **78**, 835 (1975).
- 7) H. Hosoda, R. Tsukamoto, and T. Nambara, *Chem. Pharm. Bull.*, **37**, 1834 (1989).
- 8) H. Hosoda, W. Takasaki, S. Aihara, and T. Nambara, *Chem. Pharm. Bull.*, **33**, 5393 (1985).