[Chem. Pharm. Bull. 27(10)2345—2350(1979)]

UDC 577.158.08 : 543.426.06

Fluorescence Enzyme Immunoassay for Insulin using Peroxidase-Tyramine-Hydrogen Peroxide

KAORU MATSUOKA, MASAKO MAEDA, and AKIO TSUJI

School of Pharmaceutical Sciences, Showa University[1]

(Received March 29, 1979)

A method for the enzyme immunoassay of insulin is described. Insulin was conjugated with horseradish peroxidase by the periodate oxidation method. Separation of the bound and free fractions was accomplished by a double-antibody solid phase method using Sepharose 4B-anti-rabbit IgG goat IgG. The amount of bound enzyme-labelled insulin was determined by measuring the fluorescence developed after incubation with tyramine and hydrogen peroxide. Tyramine was a better fluorogenic substrate for the determination of peroxidase activity homovanillic acid. Insulin levels in serum could be measured over the range of 2.5 to 160 μU/ml, which is similar to that of insulin radioimmunoassay. Insulin values in 34 serum samples were determined using this method and RIA. A good correlation ($r=0.89$) was obtained. The coefficient of variation was 0.6—2.6%. The method is roughly equivalent to RIA with respect to sensitivity. EIA largely overcomes the problems of RIA because there is no radiation hazard or disposal difficulties. Moreover, the laboratory equipment required is relatively inexpensive and readily available, while the label enzyme, HRP, is reasonably price and has a long shelf life. The enzyme immunoassay of insulin described in this paper should make it possible to perform routine assays even in laboratories with limited facilities.

Keywords——fluorescence; enzyme immunoassay; insulin; tyramine; hydrogen peroxide; fluorescence enzymeimmunoassay

Radioimmunoassay (RIA) is the most widely used technique for measuring hormones and drugs in plasma at relatively low concentrations. RIA, however, has various disadvantages, such as health hazards, high cost and problems of disposal, resulting from the use of radioisotopes. Enzyme immunoassay (EIA) using enzymes as labels has been developed as an alternative to RIA.[2,3] This method, however, is less sensitive than comparable RIA procedures. Several attempts have been made to establish a highly sensitive EIA method using fluorescence reactions for the determination of enzyme activity. Most of them use β-D-galactosidase as the label enzyme.[4–8] Peroxidase activity in EIA procedures is usually measured by colorimetric methods. Recently, Numazawa *et al.*[9] reported a fluorimetric EIA method for estradiol using peroxidase as a label enzyme. They established a highly sensitive EIA method at picogram levels by using homovanillic acid as a substrate. As part of a series of studies aimed at developing highly sensitive EIA procedures for hormones and drugs, we also used homovanillic acid for the fluorimetric EIA of insulin in a preliminary report,[10] but the fluorescence was not stable. Therefore, we sought a more stable and sensitive

1) Location: *1-5-8, Hatanodai, Shinagawa-ku, Tokyo.*

2) G.B. Wisdom, *Clin. Chem.*, **22**, 1243 (1976).
3) A.H.W.M. Schuurs and B.K. Van Weemen, *Clin. Chim. Acta*, **81**, 1 (1977).
4) K. Kato, Y. Hamaguchi, H. Fukui, and E. Ishikawa, *J. Biochem.*, **78**, 235 (1976).
5) K. Kato, Y. Hamaguchi, H. Fukui, and E. Ishikawa, *J. Biochem.*, **78**, 423 (1975).
6) K. Kato, Y. Hamaguchi, Y. Fukui, and E. Ishikawa, *Eur. J. Biochem.*, **62**, 285 (1976).
7) T. Kitagawa and T. Aikawa, *J. Biochem.*, **79**, 233 (1976).
8) M. Kikutani, N. Ishiguro, T. Kitagawa, S. Imamura, and S. Miura, *J. Clin. Endocrinol. Met.*, **47**, 980 (1978).
9) M. Numazawa, A. Haryu, K. Kurosaka, and T. Nambara, *FEBS Letters*, **79**, 396 (1977).
10) A. Tsuji, M. Maeda, H. Arakawa, and K. Matsuoka, *Proceeding of the Symposium on Chemical Physiology and Pathology*, **16**, 47 (1976).

substrate for peroxidase than homovanillic acid. This paper describes the development of a fluorimetric EIA for insulin in serum samples by a double-antibody solid phase method (DASP) with tyramine as a substrate for the determination of peroxidase activity. In addition, the EIA and RIA methods are compared.

Materials and Methods

Reagents——Tyramine was purchased from Tokyo Kasei Co. and purified by recrystallization from ethanol. Homovanillic acid was supplied by Sigma Chemical Co. and used without further purification. Horseradish peroxidase (type IV, 110 U/mg), used as the lable enzyme, was purchased from Sigma Chemical Co., and Sepharose 4 B from Pharmacia Fine Chemicals. All other chemicals and solvents used were of analytical grade. Anti-insulin (guinea pig) and anti-guinea pig IgG serum (rabbit) were prepared by immunization according to the usual procedure. The IgG fraction of anti-guinea pig IgG serum (rabbit) was obtained by DEAE-cellulose column chromatography after ammonium sulfate precipitation.

Insulin-free Serum——Forty ml of pool serum was mixed well with 5 g of charcoal for 2 hr at room temperature, then centrifuged at 10000 *g*. The supernatant was used as insulin-free serum.

Double-antibody Solid Phase (DASP)——DASP was prepared by the method of Axen *et al.*[11] Sepharose 4B (20 ml) was activated at pH 10.5 by addition of 5% cyanogen bromide solution (20 ml) and an aliquot of 0.5 M sodium hydroxide solution for 4—5 min. After washing with water and 0.1 M sodium hydrogen carbonate solution, the activated product was mixed with 36 mg of purified anti-guinea pig IgG rabbit IgG fraction in 3 ml of 0.1 M sodium hydrogen carbonate solution. The reaction was run overnight at room temperature with slow stirring. The coupled product was washed twice with 0.1 M sodium hydrogen carbonate solution and once with 0.2 M acetate buffer (pH 4.0). The washed product was stirred in 0.2 M acetate buffer (pH 4.0) overnight. After washing twice with 0.05 M phosphate buffer containing 0.9% sodium chloride and 0.1% BSA and once with 0.05 M phosphate buffer, the product was stored in 50 ml of this buffer.

Insulin-HRP Conjugate——Insulin-HRP conjugate was prepared by Nakane's method.[12] The carbohydrate moieties were oxidized with preiodate, and after 30 min, excess periodate was destroyed by the addition of ethylene glycol. After one hr at room temperature, the reaction mixture was dialyzed against 0.01 M sodium carbonate buffer (pH 9.5) at 4°. Insulin was added and the reaction was continued for 3 hr at room temperature. The reaction mixture was reduced with sodium borohydride. After standing for 3 hr at 4°, the reaction mixture was dialyzed against 0.05 M phosphate buffer (pH 7.0) containing 0.9% sodium chloride. The conjugate was purified from unreacted antigen by gel chromatography (Bio Gel P-60, 1×45 cm). The absorbance at 280 nm and the peroxidase activity of each fraction were measured. The immunoreactivity was also assayed by the EIA procedure described below. Though the binding ratio of insulin to HRP in the conjugate could not be determined, the total enzyme activity of fractions that could be used in the assay was approximately 18—26% of the total initial activity. The excluded peak fraction was stabilized by adding BSA and stored at 4°. This stock solution could be used for at least 6 months with little loss of activity.

Enzyme Immunoassay Procedure——All solutions were diluted with 0.05 M phosphate buffer (pH 7.0) containing 0.1% BSA. The insulin-HRP conjugate was diluted with the assay buffer to such an extent that a fluorescence intensity of about 100 against the reagent blank intensity (about 10) was obtained for the bound enzyme activity using the diluted anti-insulin serum (the dilution was estimated by RIA). The antiserum dilution was examined again by EIA and was so chosen that it gave a fluorescence intensity of about 80 for the bound enzyme activity when using the amount of insulin-HRP conjugate stated above. The assay protocol was as follows: 20 μl of insulin standard solution containing 0 to 32 μU with 20 μl of insulin-free serum or 20 μl of serum sample with 20 μl of the assay buffer were transferred into polystyrene tubes. 100 μl of the diluted anti-serum and 500 μl of the assay buffer were added and the tubes were incubated for 1 hr at room temperature. 100 μl of insulin-HRP conjugate solution and 500 μl of DASP suspension were added and the tubes were incubated overnight at 4° on an immunorotor. The tubes were centrifuged for 5 min at 3000 rpm. The precipitated DASP was washed three times with 2 ml of saline. 800 μl of the assay buffer, 50 μl of 1% tyramine solution and 50 μl of 0.007% H_2O_2 solution were added serially and the tubes were incubated for 30—60 min at 37°. The enzyme reaction was stopped by adding 50 μl of 1.25% KCN solution and 50 μl of 0.5 M NaOH solution. The tubes were centrifuged at 3000 rpm for 5 min and the fluorescence intensity of the supernatant was measured at 405 nm (excitation at 320 nm) with a Hitachi MPF 2A spectrofluorophotometer. The results were calculated in the same way as for RIA.

11) R. Axen, J. Porath, and S. Ernback, *Nature*, (London), **214**, 1302 (1967).
12) P K. Nakane and A. Kawaoi, *J. Histochem. Cytochem.*, **22**, 1084 (1974).

Results and Discussion

Several studies on EIA methods for insulin, in which alkaline phosphatase,[13] glucoamylase,[14] β-D-galactosidase[4-8] and peroxidase[15] were used as label enzymes, have been reported. Recently, commercial insulin assay kits have become available.[16] In this paper, horseradish peroxidase was used as a label enzyme, because it is one of the best-studied enzymes, it is cheap, and it is very stable under assay and storage conditions. Peroxidase activity in EIA is usually measured by colorimetric methods. In order to develop more sensitive EIA methods for hormones and drugs, we have used the chemiluminescence reaction with luminol-H_2O_2 as a substrate for peroxidase activity assay.[17] Though this method could be applied to cortisol,[18] a preliminary experiment showed that the chemiluminescence method could not be used for insulin, because of the presence of interfering substances in human serum. Therefore, a fluorimetric method was chosen for the EIA of insulin.

Fluorimetric methods have been used in highly sensitive EIA procedures for insulin using glxcoamylase[14] and β-D-galactosidase.[4-8] Numazawa *et al.*[9] used homovanillic acid as a substrate for peroxidase according to Guilbault's report,[19] and we also used this substrate in a preliminary study.[10] However the fluorescence produced from homovanillic acid was unstable and it is expensive for a routine assay. Tyramine, recrystallized from ethanol, was found to be a excellent substrate for peroxidase activity assay among several compounds examined. In order to establish optimum conditions for the assay, various factors were examined. As shown in Fig. 1, the fluorescence intensity became constant above 2.0% tyramine, while the blank fluorescence intensity gave twice the intensity of 1% tyramine solution. The fluorescence intens ity of the reaction mixture reached a plateau between 0.006% and 0.008% H_2O_2 solution. Therefore, 1.0% tyramine and 0.007% H_2O_2 were selected. As shown in Fig. 2, the fluorescence development was linear for approx. 30 min at 0.05 U/ml and 60 min at 0.01 U/ml, then the curves of fluorescence development began to flatten. Therefore, an appropriate reaction time was selected according to the concentration of peroxidase or the enzyme activity of insulin-HRP conjugate bound to DASP. In order to compare the sensitivity of this method with that of the procedure using homovanillic acid and H_2O_2 according to Guilbault,[19] the peroxidase activity was measured by both methods. As shown in Fig. 3, the fluorimetric method using tyramine was approx. 5 times more sensitive than that with homovanillic acid.

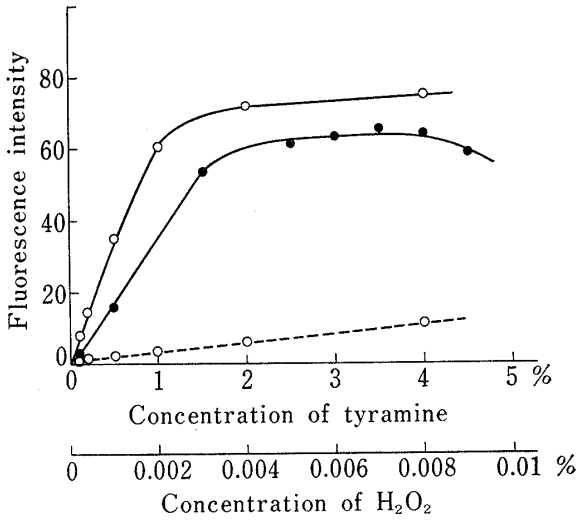

Fig. 1. Effects of the Concentration of Tyramine and H_2O_2 on the Fluorescence Intensity

—○—: Curve obtained with various concentrations of tyramine (0.05 U/ml of peroxidase).
--○--: The blank values obtained with various concentrations of tyramine.
—●—: Curves obtained with various concentrations of H_2O_2 (0.05 U/ml of peroxidase).

13) K. Miedema, J. Boelhouwer, and J.W. Otten, *Clin. Chim. Acta*, **40**, 187 (1972).
14) E. Ishikawa, *J. Biochem.*, **73**, 1319 (1973).
15) G. Kleinhammer, H. Lenz, R. Linke, and W. Gruber, *Z. Anal. Chem.*, **279**, 145 (1976).
16) T. Yoshida, M. Yoshida, and H. Yoshida, *J. Kyoto Pref. Univ. Med.*, **86**, 421 (1977).
17) H. Arakawa, M. Maeda, and A. Tsuji, *Bunsekikagaku*, **26**, 322 (1976).
18) H. Arakawa, M. Maeda, and A. Tsuji, *Anal. Biochem.*, in press.
19) G.G. Guilbault, P. Brignac, Jr., and M. Zimmer, *Anal. Chem.*, **40**, 190 (1968).

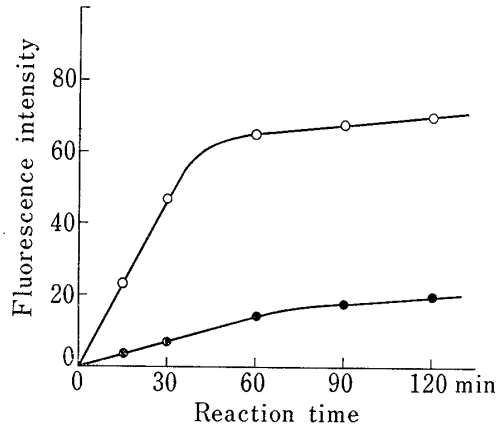

Fig. 2. Relationship between the Fluorescence Intensity and Enzyme Reaction Time

—○—: 0.05 U/ml of peroxidase.
—●—: 0.01 U/ml of peroxidase.

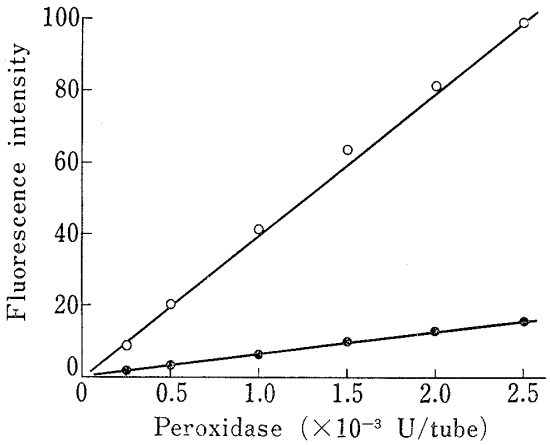

Fig. 3. Standard Curves for Peroxidase using Tyramine and Homovanillic Acid as Substrates

—○—: tyramine.
—●—: homovanillic acid.

In preliminary studies the conjugation method using glutaraldehyde as described by Avrameas[20] was examined, but the yield of conjugate was less than that obtained by Nakane's method.[12] The sensitivity of EIA depends on various factors, such as the quality of antiserum, the immunoreactivity of enzyme–antigen conjugate, the order of addition of reagents and the incubation time. The procedure used to separate bound and free fractions is most important in relation to the sensitivity of EIA. Though the insolubilized first antibody (anti–insulin antibody) prepared by coupling the purified antibody to Sepharose 4B beads was superior to the use of antibody–coated polystyrene tubes, the DASP method described here was more sensitive. The insulin-HRP conjugate and DASP suspension were added at the same time after the reaction of anti–insulin serum and insulin for 60 min; this proved to be more sensitive than the competitive equilibrium method.

A typical standard curve obtained by the standard procedure is shown in Fig. 4. The lowest amount of insulin that could be detected under the optimal conditions with this standard curve was 0.05 μU per assay tube, corresponding to 2.5 μU/ml of serum. This concentration corresponds to the 95% point of the standard curve in Fig. 4. The coefficients of variation at each concentration of insulin were 1.5, 2.6, 2.6, 1.6, 0.6 and 1.9%, respectively.

It is well known, particularly from RIA experience,[21] that serum and plasma at low dilutions may interfer with the immune reaction. An additional problem in EIA is the possibility of their affecting the enzyme reaction.[3,22] Some substances such as peroxidase–like substances and hemolyzed samples exhibit high blank values in the peroxidase assay when HRP is used as a label. Though little is known about disturbing factors their influence on the enzymatic part of the assay could be minimized by measuring the enzyme activity of the bound fraction, attached to the DASP which was washed thoroughly prior to the enzyme reaction. This is a major advantage of the DASP method. The influence of interfering factors was investigated by measuring standard insulin solutions to which various amounts of insulin-free serum were added. As shown in Fig. 5, no interference was observed upon addition of 20 μl of serum, while 50 μl and 100 μl of serum reduced the fluorescence intensity between 0 μU/ml and 120 μU/ml. However, the results show that it is possible

20) S. Avrameas, *Immunochemistry*, **6**, 43 (1969).
21) J.M.H. Buckler, In Radioimmunoassay Methods ed. by K.E. Kirkham and W.M. Hunter, Churchill Livingstone, Edinburgh, 1971, p. 273.
22) H.M. Barbour, *J. Immunol. Methods*, **11**, 15 (1976).

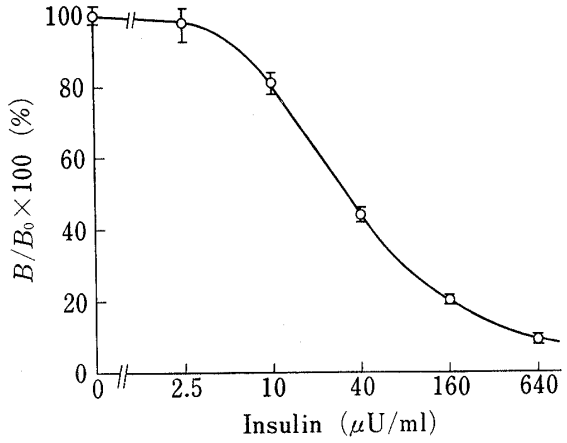

Fig. 4. Standard Curve for Insulin

$\overline{\phi}$ mean ± 2 s.

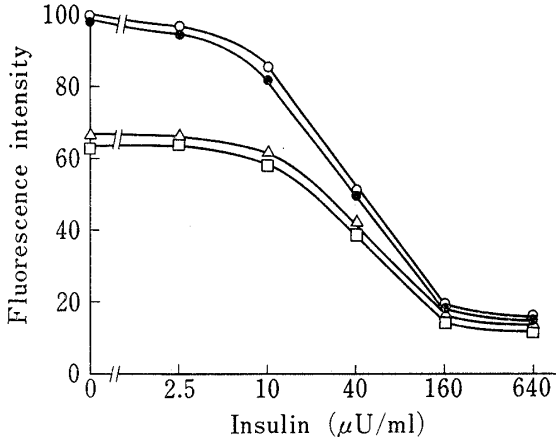

Fig. 5. Effect of Serum on the Standard Curve

—○—: 0 μl of serum, —●—: 20 μl of serum,
—△—: 50 μl of serum, —□—: 100 μl of serum.

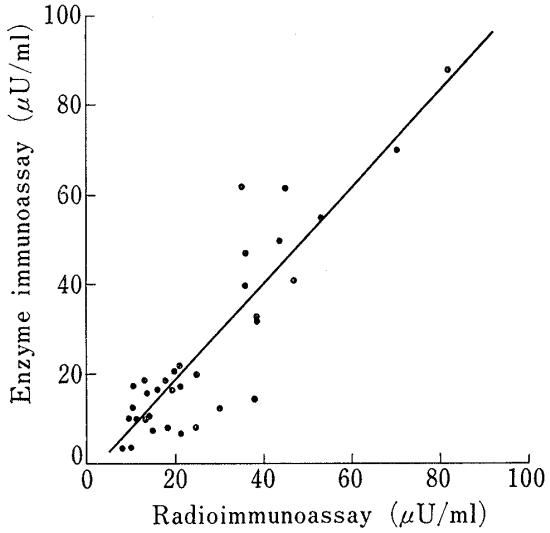

Fig. 6. Comparison of Insulin Serum Levels determined by EIA and RIA

$Y=1.08\,X-2.93$
$r=0.89$, $n=34$

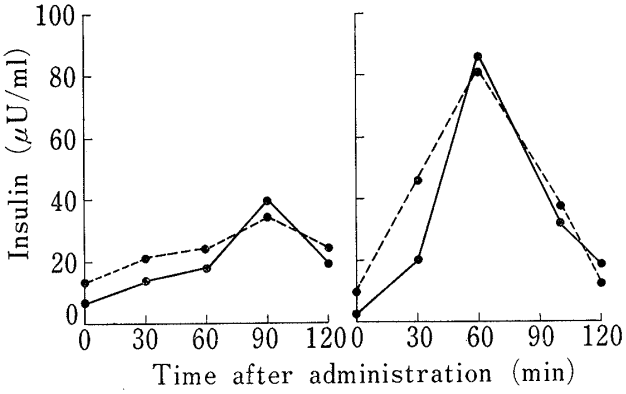

Fig. 7. Individual Insulin Levels during 50 g Oral Glucose Tolerance Tests as determined by EIA and RIA

—●—: EIA, —●—: RIA.

to obtain a useful standard curve even in the case of addition of 100 μl of insulin–free serum. Based on these results, 20 μl of insulin–free serum was added to the standard insulin solution to obtain sufficient sensitivity with a tolerable level of interference. The enzyme activity of the bound fraction was measured after thorough washing prior to the enzyme reaction in order to eliminate possible interference by plasma or serum.

The reliability of the present method was then evaluated by comparison with the results obtained by RIA. As shown in Fig. 6, there is a good correlation ($r=0.89$) between the insulin values as measured by these two methods. The regression line is $Y=1.08\,X-2.83$, where X equals the values determined by RIA. The insulin levels in the oral glucose tolerance tests were also determined by EIA and RIA. As shown in Fig. 7, there was good agreement.

Many RIA procedures have been established and used in clinical diagnosis for up to 20 years. Though their usefulness is beyond doubt, there are some serious problems, such as radiation hazard, disposal difficulties, and high costs of the reagents and equipment. The

present enzyme immunosasay is as sensitive as RIA and essentially avoids the above problems; it requires only inexpensive equipment and laboratory facilities, and the label enzyme, HRP, used in this work is relatively cheap and has a long shelf life for routine assays. The EIA procedure for insulin presented here should make it possible to perform routine assays even in laboratories with limited facilities.

Acknowledgement The authors are indebted to Dr. T. Hishida and Mr. K. Oikawa, Showa University School of Medicine, for suplying serum samples. This work supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.