Communications to the editor

ENZYME-COUPLED IMMUNOASSAY OF VIOMYCIN*

Sir:

Most quantitative analyses of antibiotics have been performed by bioassay using their antimicrobial activities. The analyses, however, do not give high sensitivities. Recent progress in enzyme immunoassay made possible to give higher sensitivities for assay of several biologically active compounds rather than radioimmunoassay[1~4]. We have been studying novel highly sensitive assay of antibiotics and in this paper, we wish to report an enzyme immunoassay of viomycin which can detect 100 pg to 4 ng of the antibiotic.

Preparation of rabbit antiserum to viomycin:

Viomycin was converted to N_β-monoacetylviomycin by three steps according to the methods reported[5]. N_β-Monoacetylviomycin (100 mg) was treated with succinic anhydride (12 mg) and the desired N_β-acetyl-N_ϵ-hemisuccinylviomycin (56 mg) was isolated by Sephadex column chromatography. The carbonyl group of the resulting hemisuccinyl derivative (50 mg) was then coupled with free amino groups of bovine serum albumin (BSA) (Sigma Chemicals Co., St. Louis) (50 mg) by a mixed anhydride method[6]. The resulting BSA-N_β-acetylviomycin conjugate was separated and purified by Sephadex LH-20 column chromatography. The purified conjugate, thus obtained (43 mg), gave one band on its sodium dodecyl sulfate (SDS) disk electrophoresis and from its electrophoretic distance its molecular weight was estimated as 74,000 according to the method of DUNKER and RUECKERT[7]. Thus, 8.6 moles of viomycin are assumed to be introduced per molecule of BSA, calculating for the molecular weight of BSA as 67,000 and that of N_β-acetyl-N_ϵ-hemisuccinylviomycin residue as 810. The above obtained conjugate was emulsified with FREUND's complete adjuvant (Nakarai Chemicals, Kyoto) and injected intramuscularly at ten days interval for four times to female rabbits. The rabbits serum was kept at $-20°C$ until used for immunoassay.

Preparation of viomycin-β-D-galactosidase (EC 3.2.1.23) conjugate:

Viomycin sulfate (6 mg) in 0.05 M phosphate buffer pH 7.0 was incubated with *m*-maleimidobenzoyl N-hydroxysuccinimide ester (MBS)[4] (3.14 mg) at 30°C for 30 minutes with occasional stirring. The mixture was chromatographed on a column of Sephadex LH-20. The fraction No. 14 (2g/tube) contained MBS-acylated viomycin. The maleimide content of the fraction No. 14 was determined by measuring the decrease of the thiol content on its addition to mercaptoethanol (0.1 μmole) according to the method of SEDLACK and LINDSAY[8], to give 120 nmoles/ml. Twenty μl of the above obtained fraction (MBS-acylated viomycin content 2.4 nmoles) was added to the solution of β-D-galactosidase from *Escherichia coli* (Boehringer Mannheim, W. Germany) (93 pmoles) in phosphate buffer pH 7.0 and incubated at 4°C for overnight. The mixture was chromatographed on Sepharose 6B column with an eluent of 0.02 M saline phosphate buffer pH 7.0 and the peak enzyme active fraction was used for enzyme immunoassay. A measurement of β-D-galactosidase activity was performed using 10^{-4}M solution of 4-methylumbelliferyl-β-D-galactoside in the buffer A [0.02 M phosphate buffer pH 7.0 containing 0.1% egg white albumin (ICN Pharmaceuticals, Inc., Cleveland, Ohio), 1mM $MgCl_2$, 0.1% NaN_3] as a substrate solution according to the method of KATO *et al.*[1]

Dilution study of rabbit antiserum to viomycin for the immunoassay:

An aliquot of the conjugate (5 μl of a 10-fold diluted solution of the above obtained fraction in the buffer A) with and without 50 ng of viomycin were incubated with 10 μl of several-fold diluted solutions of the rabbit antiserum to viomycin in the buffer A by 250, 500, 1,000, 5,000 25,000 and 125,000 folds respectively, for 16 hours at 4°C. The bounded conjugate was precipitated by centrifugation and its enzyme activity was measured to give the results shown in Fig. 1.

Amount of the antiserum to give 50% inhibition of the enzyme activity of the bound conjugate for the 5 μl of a 10-fold diluted conjugate fraction was 10 μl of a 5.5×10^3-fold dilution of the antiserum. While, for 50 ng of viomycin 10 μl of a 8.4×10^2-fold dilution of the antiserum was consumed. Judging from the above obtained results and calculating the minimum detectable

* Part XI of the series "Studies on viomycin".

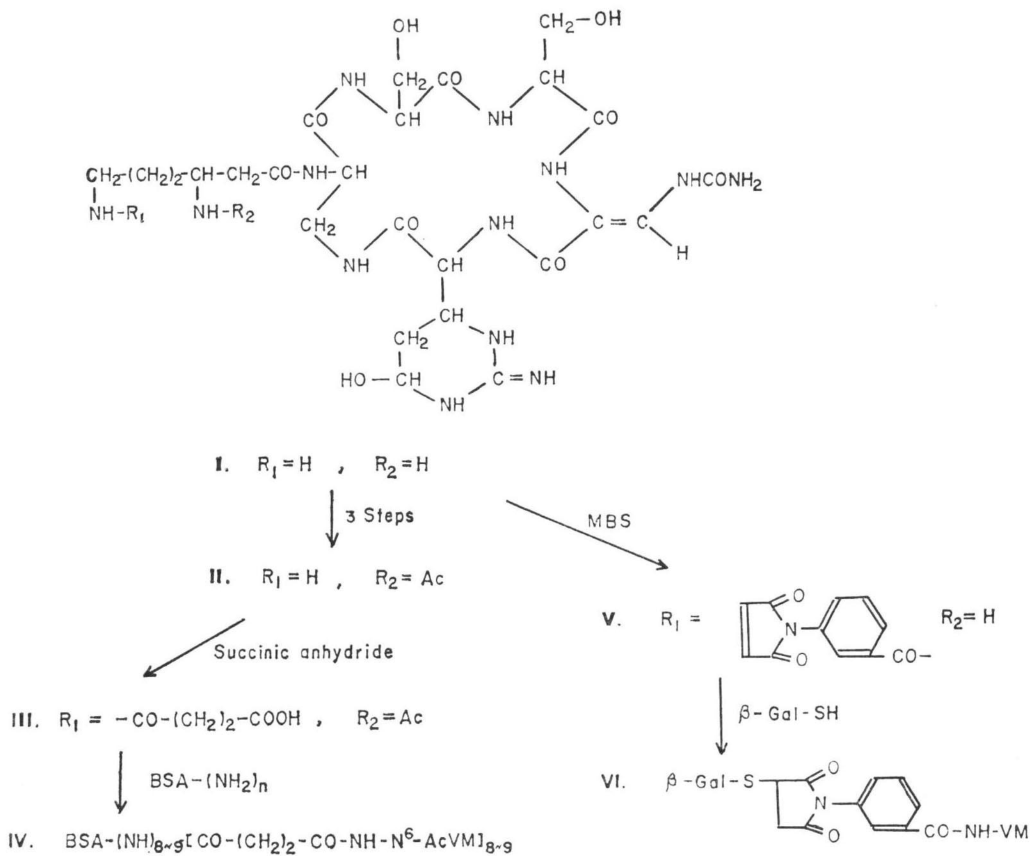


Fig. 1. Enzyme activities of the bound conjugates with (closed circle) or without (open circle) presences of 50 ng of viomycin using several-fold diluted rabbit antiserum to viomycin.

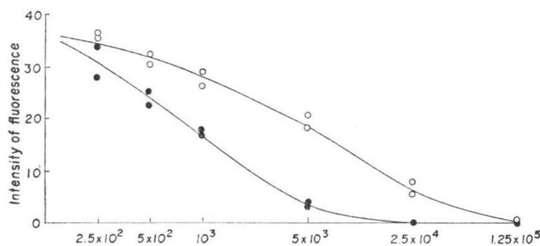
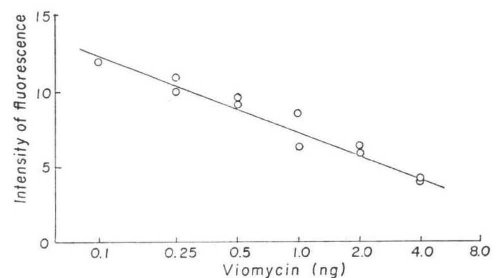


Fig. 2. Viomycin assay using β-D-galactosidase-MBS-viomycin conjugate.



amounts of the conjugate, enzyme immunoassay of viomycin was performed as follows.

Viomycin assay:

The possiblity of viomycin assay using β-D-galactosidase-MBS-N_{β}-acetylviomycin conjugate was tested by measuring the competitive bindings of viomycin and the conjugate to the rabbit antiserum to viomycin. For this, a mixture of 10 μl of a 100-fold dilution of the peak fraction from the Sepharose 6B column, an adequate amounts (0.05 to 4 ng) of viomycin and 10 μl of a 20,000-fold diluted solution of the antiserum in the buffer A in a final volume of 0.2 ml was incubated at 4°C for 16 hours. Then, 10 μl of a 100-fold diluted solution of normal rabbit serum in the buffer A was added. After further incubation for 8 hours, the mixture was centrifuged at $800 \times g$ for 15 minutes and the β-D-galactosidase activi-

ties were measured. Typical results are shown in Fig. 2.

For enzyme immunoassay of viomycin a specific antiserum to viomycin had to be obtained. For this, a strategy of preparing the hapten-protein conjugate was first, protection of N_β-amino function of viomycin by an acetyl group by the method reported[5]. Then, only one of the remaining N-terminal amino function was bonded with free amino groups of bovine serum albumin by hemisuccinylation followed by amido bond formations of the resulting carboxyl group and free amino groups of the protein. The desired BSA-N_β-acetylviomycin conjugate was obtained in a fairly good yield and it gave a single band on its SDS disk electrophoresis. Very recently, we have introduced a novel coupling reagent MBS and applied the reagent for enzyme coupled immunoassay of insulin.[4] Using the reagent viomycin was easily coupled with the enzyme. The position of the MBS acylation was assumed at N_ε-amino group of viomycin judging from the results of previous experiments[5,9]. Utilizing the antiserum and the viomycin-enzyme conjugate, viomycin was detected in the range 100 pg to 4 ng. Applications of the present assay for several studies are in progress.

TSUNEHIRO KITAGAWA
TAKAFUMI FUJITAKE
HYOZO TANIYAMA
Faculty of Pharmaceutical Sciences,
Nagasaki University,
Bunkyo-machi, Nagasaki 852, Japan
TADAOMI AIKAWA
Department of Physiology,
School of Medicine,
Nagasaki University,
Sakamoto-machi, Nagasaki 852,
Japan

(Received September 20, 1976)

References

1) KATO, K; Y. HAMAGUCHI, H. FUKUI & E. ISHIKAWA: Enzyme-linked immunoassay. I. Novel method for synthesis of the insulin-β-D-galactosidase conjugate and its applicability for insulin assay. J. Biochem. (Tokyo) 78: 235~237, 1975
2) KATO, K.; H. FUKUI, Y. HAMAGUCHI & E. ISHIKAWA: Enzyme-linked immunoassay: Conjugation of the FAB' fragment of rabbit IgG with β-D-galactosidase from *E. coli* and its use for immunoassay. J. Immunol. 116: 1554~1560, 1976
3) DRAY, F., J. M. ANDRIEU & F. RENAUD: Enzyme immunoassay of progesterone at the picogram level using β-galactosidase as label. Biochim. Biophys. Acta 403: 131~138, 1975
4) KITAGAWA, T. & T. AIKAWA: Enzyme-coupled immunoassay of insulin using a novel coupling reagent. J. Biochem. (Tokyo) 79: 233~236, 1976
5) KITAGAWA, T.; T. MIURA, M. TAKAISHI & H. TANIYAMA: Studies on viomycin. VIII. Selective modifications of the terminal amino groups of viomycin. Chem. Pharm. Bull. (Tokyo) 23: 2123~2127, 1975
6) BODANSZKY, M. & M. A. ONDETTI: "Peptide synthesis". Interscience Publishers, New York, pp. 84~98, 1966
7) DUNKER, A. K. & R. R. RUECKERT: Observations on molecular weight determinations on polyacrylamide gel. J. Biol. Chem. 244: 5074~5080, 1969
8) SEDLACK, J. & R. H. LINDSAY: Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with ELLMAN's reagent. Anal. Biochem. 25: 192~205, 1968
9) KITAGAWA, T.; T. MIURA, C. TAKAISHI & H. TANIYAMA: Studies on viomycin. IX. Amino acid derivatives of viomycin. Chem. Pharm. Bull. (Tokyo) 24: 1324~1330, 1976