Journal of Insect Biotechnology and Sericology 74, 1-4 (2005)

Optimization of an Enzyme-Linked Immunosorbent Assay for Ecdysteroids

Takahiro Shiotsuki[1, 2, *], Yue-Jin Hua[2, 4], Taneaki Tsugane[2, 5], Shirley Gee[3] and Bruce D. Hammock[3]

[1] *Developmental Biology Department, and* [2] *Insect Gene Function Research Center, National Institute of Agrobiological Science, Tsukuba, Ibaraki 305, Japan, and* [3] *Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616, U.S.A.*

(Received June 7, 2004; Accepted August 26, 2004)

The practical conditions of an enzyme-linked immunosorbent assay (ELISA) for ecdysteroids were optimized. A complex of ecdysone and horseradish peroxidase (HRP) was prepared as an enzyme tracer, and a competition between the tracer and free ecdysteroids was employed for the ELISA system. The binding of rabbit antiserum against 20-hydroxyecdysone (20E) was better with the direct method than indirect method that used anti-rabbit IgG. Optimal blocking using 1% casein before the competition assay had lower background compared to no blocking or blocking using 1% BSA. The optimized method showed a determination range of 0.06 - 6 ng equivalent of 20E. The system made it possible to determine ecdysteroids in both *in vitro* and *in vivo* samples. Changes in ecdysteroid titer in hemolymph of *Agrius convolvuli* between 4th molting and eclosion determined by the ELISA optimized in this study was similar to that reported concentrations in *Manduca sexta*.

Key words: enzyme-linked immunosorbent assay (ELISA), ecdysteroid titer, *Bombyx mori, Agrius convolvuli*

INTRODUCTION

For insect endocrinological studies, it is critical to determine two major non-peptide hormones, that is, juvenile hormones and ecdysteroids. The titration of the latter is more well-established with radioimmunoassay (RIA), which is widely employed in studies of this field. Recently, the development of enzyme-linked immunosorbent assays (ELISA) for the ecdysteroid titration have been reported (Kingan, 1989; von Gliscynski *et al.*, 1995; Pascual *et al.*, 1995). In principle, the ELISA is more advantageous in terms of time-saving and lack of requirement for radioactive tracers. However, RIA is still used, largely because the high degree of repeatability and accuracy. For this reason, ELISA system was optimized utilizing different conditions for practical use in this study.

MATERIALS AND METHODS

Chemicals

Ecdysone (E) and 20-hydroxyecdysone (20E) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) for both preparations of enzyme tracer and standard for ELISA. The 2-hemisuccinate of E or 20E was prepared using a published method (Soumoff *et al.*, 1981) with slight modifications. That is, E or 20E (10 mg) and 2.6 mg of succinic anhydride was placed into 1 ml of dry tetrahydrofuran (THF), filled with nitrogen gas, then a catalytic amount of 4-(dimethylamino)pyridine and a drop of pyridine was added to the mixture and stirred in a water bath at approximately 50°C for 10 h, and then let stand overnight at room temperature. The solvent was evaporated, then applied to a silica gel column and eluted with chloroform/ethanol (7:3 v/v) to give the ecdysone 2-hemisuccinate (E-2hs) in approximately 40% yield. Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim GmbH, Germany for the preparation of the enzyme tracer. Other reagents and solvents were of reagent grade.

Antiserum and enzyme tracer

Female New Zealand white rabbits were immunized with a human serum albumin (HSA) conjugate of the hemisuccinate of 20E as a hapten, and antiserum was prepared for the previous work for radioimmunoassay (RIA) (Takeda *et al.*, 1986). A conjugate of E-2hs and HRP was prepared as follows: 3.3 mg of E-2hs was dissolved in 150 µl of ice-cold pyridine/water (1:1 v/v), this solution was moved into cooled vial containing 26 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), rinsed with 30 µl of pyridine/water (1:3 v/v), then 5 mg of the HRP in 125 µl of distilled water was added after EDC was dissolved, rotated gently occasionally, then allowed to stand overnight at 20°C. The reaction mixture was applied to Sephadex G-25 (10 × 180 mm) to separate the E-HRP conjugate, unreacted E-2hs and pyridine with 0.1 M phosphate buffer, pH 7.5.

Condition of reaction in enzyme assays

Polystyrene microtiter plates (Maxisorp by Nunc, Denmark) and a microplate reader (SPECTRAmax by Molecular Devices, Menlo Park, CA, U.S.A.) were used in this

*To whom correspondence should be addressed:
Fax: +81 29 838 6028. Tel: +81 29 838 6073.
Email: shiotsuk@affrc.go.jp

[4] Present address: Institute of Nuclear-Agricultural Science (INAS), Zhejiang University, Hangshou, China.

[5] Present address: Chiba prefectural agriculture research center, Chiba 266-0006 Japan.

study. Five buffers were used, namely a coating buffer (0.5 M carbonate buffer, pH 9.5), PBS (0.2 M phosphate buffer plus 8% saline, pH 7.8), PBST (PBS supplemented with 0.1% Tween 20), washing buffer (1/10 PBS supplemented with 0.1% Tween 20) and substrate buffer (0.1 M sodium acetate buffer, pH 5.5). The plates were coated overnight with 100 µl of antiserum in the coating buffer at 4°C. The dilutions of the antiserum providing highest sensitivity and sufficient color development were found to be in a dilution range from 1:8,000 to 1:16,000. After coating, the plates were washed three times with the washing buffer, and treated with 200 µl of 1% bovine serum albumin (BSA) or 1% casein sodium salt in the PBS for 2 h at room temperature as blocking. The plates were washed three times with the washing buffer, and E-HRP conjugate in PBST and standard or sample solution in distilled water were added and incubated for 2 h at room temperature. In a routine protocol after optimization, 42.5 µl of the PBST was added first to all wells to prevent drying, then 42.5 µl of the tracer diluted by 1:20,000 in PBST and 15 µl of ecdysteroids in distilled water were added simultaneously for every replicates. Then, the plates were washed three times with the washing buffer again, the remained enzyme activity was detected with chromogenic substrate, 0.5% 3,3',5,5'-tetramethylbenzidine (TMB) solution with 0.01% H_2O_2 in the substrate buffer. The color was developed by incubation for 30 to 45 min at room temperature. The enzyme reaction was terminated with an addition of 50 µl of 1 M H_2SO_4, and absorbance at 450 nm was monitored. The standard curve was calculated by non-linear regression analysis fit to a theoretical sigmoid curve with 4-parameters by an analysis software SOFTmax PRO (Molecular Devices).

Analyses of intact samples

Ecdysteroids in both *in vitro* and *in vivo* samples were determined with the optimized ELISA system, that is, tissue culture media of prothoracic gland (PG) from silkworm, *Bombyx mori*, and hemolymph of sweet potato hornworm, *Agrius convolvuli*. The sweet potato hornworm was reared on artificial diet as reported by Kiguchi and Shimoda (1994). PGs were removed from *B. mori* larva at a stage of wandering (day 9 of 5th instar larvae), and cultured at 25°C in 20 µl Grace's medium on microplate for 3 h. After incubation, 180 µl of methanol was added to the cultured media and mixed vigorously in the well to extract ecdysones. Aliquots from same media were transferred to assay glass tubes, and the solvent was removed by reduced pressure, subsequently distilled water was added and aliquot of the solution was taken for ELISA or RIA, or both.

RESULTS AND DISCUSSION

Comparison between direct and indirect methods for immobilizing antibody on the microplate

Antiserum against 20E previously obtained for RIA of ecdysteroid was used. For binding of the antiserum on the solid phase, incubation of the antiserum in the coating buffer (as direct method) and incubation of anti-rabbit IgG antibody in the coating buffer prior to incubation of antiserum in PBS (as indirect method) were compared. The background in the indirect method was higher than that of the direct method as shown in Fig. 1, probably because of any interference with anti-rabbit IgG antibody. The direct immobilization was adopted for further study since lower background and more changes in absorbance was observed in comparison with the indirect immobilization.

Effect of blocking on ELISA standard curve

It has been reported that more consistent ELISA result is observed by a treatment of blocking (Harlow and Lane, 1988), thus the use of a blocking agent was investigated. After coating the plates with antiserum and washing, the plate was incubated with PBS, 1% BSA in PBS, or 1% casein in PBS for 2 h at room temperature. Comparison among the different conditions were performed to obtain several parameters in each standard curve, that is, slope at point of inflection (larger value is more precise in estimation), IC_{50} as concentration at the point of inflection (smaller value is higher sensitivity), and R^2 (regression parameter, larger value is better regression). Those parameters of the standard curves under different blocking conditions are listed in Table 1. When no BSA was supplemented in tracer solution, blocking with casein gave the lowest IC_{50} values, however, BSA, and no blocking the IC_{50} value was the highest. In addition, it has been reported (Harlow and Lane, 1988) that a supplement of

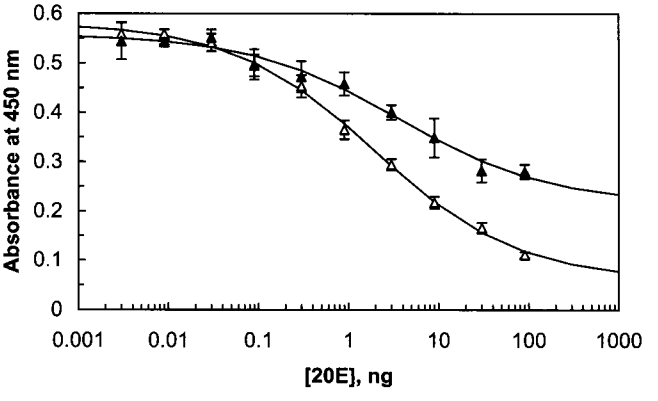

Fig. 1. ELISA standard curves of ecdysone by direct (open triangle) and indirect (closed triangle) binding on solid phase as relationship between concentration of 20E and absorbance at 450 nm. Mean values and standard errors were calculated in quadruplicate assay on 96-well plate.

Table 1. Effect of different conditions on parameters of the ELISA standard curve.

blocking*	supplement in tracer solution**	slope	IC_{50} pg in 50 µl	R^2
No	no	0.380	11.2	0.998
	0.1% BSA	0.556	11.5	0.997
1% BSA	no	0.239	4.2	0.996
	0.1% BSA	0.401	13.1	0.999
1% casein	no	0.555	3.5	0.992
	0.1% BSA	0.591	8.0	0.993

*Supplement in × 1 PBS solution.
**Supplement in × 1 PBST solution.

BSA in the tracer solution can result in a better assay. However, in this case the IC_{50} values became larger when supplementing with BSA. According to the results obtained, it was concluded that the best condition among tested was a combination of blocking with 1% casein sodium, and no BSA supplement in tracer solution.

Comparison of standard curves between E and 20E

Under the optimized conditions, standard curves for the two major ecdysteroids, E and 20E, were compared. As shown in Fig. 2, the curve for 20E showed a wider range and lower IC_{50} value than that for E. The observed IC_{50} values were 1.16 and 0.81 ng for E and 20E, respectively. The greater sensitivity for 20E is consistent with the fact that the antiserum was prepared with 20E as the hapten. If necessary, it is possible to separate E and 20E by reverse-phase HPLC prior to ELISA analysis.

Comparison of estimation between ELISA and RIA

In order to apply the optimized system for titration of practical samples, culture media of prothoracic gland (PG) from *Bombyx mori* was used as *in vitro* test samples first.

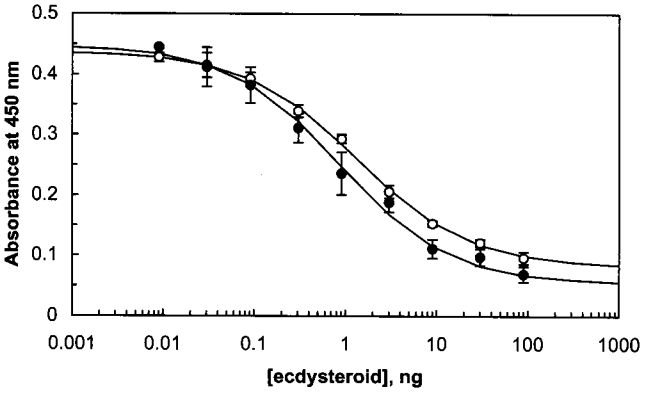

Fig. 2. ELISA standard curves of ecdysone (open circle) and 20-hydroxyecdysone (closed circle) as relationship between concentration of ecdysteroid and absorbance at 450 nm. Mean values and standard errors were calculated in quadruplicate assay on 96-well plate.

A same extract from the cultured media was applied for both RIA and ELISA. As shown in Fig. 3. the correlation was good between the values estimated by RIA and ELISA; the observed slope and correlation coefficient (R) were 0.931 and 0.925, respectively.

Determination of hemolymph ecdysteroids in *Agrius convolvuli*

In contrast to *in vitro* cultured samples, intact samples such as hemolymph contain many components including steroids and non-ecdysteroids. Hemolymph of sweet potato hornworm at stages from ultimate larval molting to adult emerging was used as test samples. According to the published protocol for the RIA, a small amount (10-50 µl) of hemolymph was extracted with methanol, and applied to the ELISA. When the estimated concentration was out of range of standard curve, an appropriate dilution of the hemolymph was made and assayed repeatedly. The hemolymph was collected individually and the ecdysteroid titer was calculated statistically in each group of male and female. There were no significant differences between those (Fig. 4.). *A. convolvuli* is known to be a closely related species to tobacco horn worm, *Manduca sexta*, and the obtained changes in ecdysteroid titer in *A. convolvuli* here was very similar to that reported concentrations in *M. sexta* (Bollenbacher *et al.*, 1981).

Advantage of the optimized ELISA for ecdysteroids

As described above, the ELISA for ecdysteroids was optimized for several conditions, and the strategy is appli-

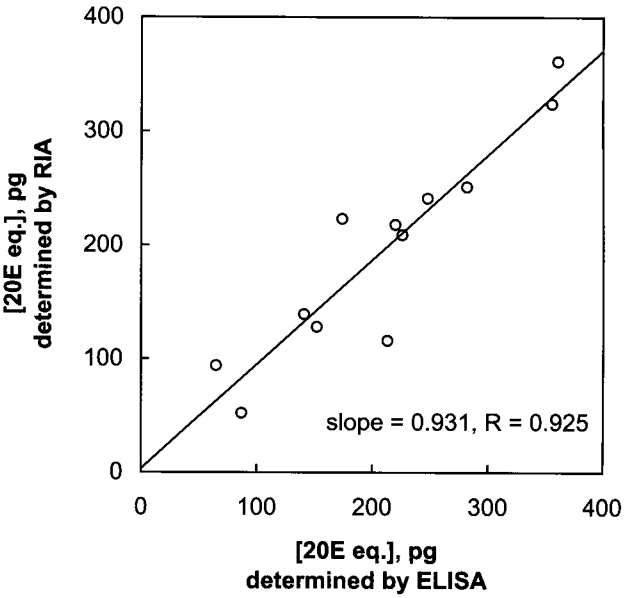

Fig. 3. Correlation of ecdysteroid titers (20E equivalent) between determinations by RIA and ELISA. Aliquot from same cultured media with *B. mori* prothoracic glands was applied for both the assays.

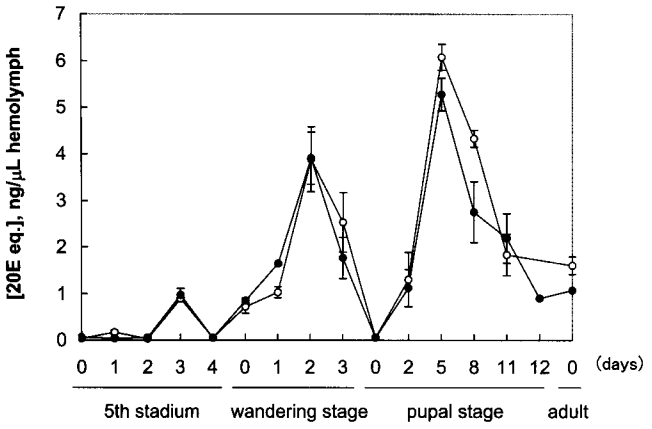

Fig. 4. Changes of ecdysteroid titer in hemolymph of *Agrius convolvuli* (open circle : male, and closed circle : female) at stages from ultimate larval (5th) instar to eclosion. Mean values and standard errors were calculated in triplicate.

cable to other ELISA systems. The estimation of ecdysteroid titer by the optimized system correlated well with that by RIA, which is widely used in this research field. The system could be used for both samples *in vitro* (organ cultured medium) and *in vivo* (intact insect hemolymph). Whereas it takes several hours in each two days for the RIA protocol, the ELISA can be run in a single day. The time consuming step in RIA is separation of free tritiated ecdysone and antibody-bound ligand with ammonium sulfate or activated charcoal, but no such step is required in ELISA and all reactions are performed on microplate. Furthermore, it takes 5 min for one RIA sample without replicates for measurement of radioactivity by the scintillation counter. However, the measurement in ELISA is spectrophotometrically achieved by microplate reader, and takes just 1 min for one plate (blank, reference, 9 points of standard curve and 13 samples in quadruplicate). Thus the optimized ELISA has many advantages over the RIA and should be applied in order to obtain results for insect endocrinology.

ACKNOWLEDGMENTS

We thank Dr. Masami Shimoda for the collecting hemolymph from *Agrius convolvuli*, and to Drs. Marvin Goodrow and Don Stautamier for suggestion in the preparations of tracer. This work was supported in part by grants-in-aid to T.S. for the "Insect Technology Project" from the Ministry of Agriculture, Forestry, and Fisheries.

REFERENCES

Bollenbacher, W.E., Smith, S.L., Goodman, W., and Gilbert, L.I. (1981) Ecdysteroid titer during larval-pupal-adult development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* **44**, 302-306.

Harlow, E., and Lane, D. (1988) "Antibodies: A laboratory handbook", Cold Spring Harbor Laboratory, New York, pp. 553-612.

Kiguchi, K., and Shimoda, M. (1994) The sweet potato hornworm, *Agrius convolvuli*, as a new experimental insect: continuous rearing using artificial diets. *Zool. Sci.* **11**, 143-147.

Kingan, T.G. (1989) A competitive enzyme-linked immunosorbent assay: Applications in the assay of peptides, steroids, and cyclic nucleotides. *Anal. Biochem.* **183**, 283-289.

Pascual, N., Belles, X., Delbecque, J.P., Hua Y-J., and Koolman, J. (1995) Quantification of Ecdysteroids by immunoassay: Comparison of enzyme immunoassay and radioimmunoassay. *Z. Naturforsch.* [*C*] **50**, 862-867.

Soumoff, C., Horn, D.H.S., and O'Connor, J.D. (1981) Production of a new antiserum to arthropod molting hormone and comparison with two other antisera. *J. Steroid Biochem.* **14**, 429-435.

Takeda, S., Kiuchi, M., and Ueda, S. (1986) Preparation of anti-20-hydroxyecdysone serum and its application for radioimmunoassay of ecdysteroids in silkworm hemolymph. (in Japanese with English summary) *Bull. Sericultural Exp. Sta.* **30**, 361-374.

Von Gliscynski, U., Delbecque, J.P., Bocking, D., Sedlmeier, D., Dircksen, H., and Lafont, R. (1995) Three new antisera with high sensitivity to ecdysone, 3-dehydroxy- ecdysone and other A-ring derivatives: Production and characterization. *Eur. J. Entomol.* **92**, 75-79.