Optimization of an Enzyme-Linked Immunosorbent Assay for Ecdysteroids

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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 Giisczynski et al., 1995; Pascal 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
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% H₂O₂ 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 H₂SO₄, 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, Agria consolvoluli. 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), IC₅₀ as concentration at the point of inflection (smaller value is higher sensitivity), and R² (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 IC₅₀ values, however, BSA, and no blocking the IC₅₀ 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.](image-url)
Table 1. Effect of different conditions on parameters of the ELISA standard curve.

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

*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.

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. 2. ELISA standard curves of ecysyne (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.](image)

![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.](image)
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.

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REFERENCES


