Responses of ligated larvae of the flesh fly, *Boettcherisca peregrina* (Diptera: Sarcophagidae), to injected ecdysteroids: a possible usage for a simple *in vivo* assay for ecdysteroid measurement

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Abstract

Mature larvae of the flesh fly, *Boettcherisca peregrina*, ligated at the tenth abdominal segment in the last larval stage showed a disturbance of puparium formation (pupariation) in the anterior portion. The anterior portion of the larval body was injected with samples tested. The disturbance of pupariation in the anterior portion was dose-dependently rescued by a single injection of ecdysteroids. The minimum effective doses of exogenous ecdysteroids to induce puparial tanning at the anterior portion were 125, 64, 64, 32, 16 and 8 ng for inokosterone, ecdysone, 20-hydroxyecdysone-22acetate, ponasterone A, 20-hydroxyecdysone and polypodine B, respectively. This assay showed a similar ecdysteroid-sensitivity to a conventional bioassay but produced results approximately 2 days earlier than the conventional assay that employed the isolated abdomen of *B. peregrina* larvae ligated at the sixth body segment.

Key words: *In vivo* assay, ecdysteroids, ligation, flesh fly, *Boettcherisca peregrina*

INTRODUCTION

Several *in vivo* assay methods for ecdysteroid activity (reviewed by Cymborowski, 1989) have been established by using the isolated abdomen of dipteran larvae such as blowflies, *Calliphora erythrophala* (Karlson and Hanser, 1953), *C. stygia* (Thomson et al., 1970), the housefly, *Musca domestica* (Kaplanis et al., 1966) and the flesh fly, *Boettcherisca peregrina* (Ohtaki et al., 1967). At present, radioimmunoassay (RIA) is widely used for measuring ecdysteroid titers in insect hemolymph, because of its ease in obtaining quick and accurate results (Reum and Koolman, 1989). However, RIA has some limitations. It requires the radio-labeled ecdysone as a ligand and samples must possess an epitope to react with the antibody.

In general, a bioassay is methodologically more tedious in preparation of appropriate test insects and the effectiveness must be judged on an arbitrary criterion. However, the bioassay can be applicable to screen a wide range of ecdysteroid activities even in sample materials where no hormonal activities were detected by RIA.

Previous ligation experiments using *B. peregrina*

larvae showed that mature larvae tightly ligated at the middle of the body failed to pupariate both at anterior and posterior portions of ligated bodies under dry conditions, while intact larvae usually pupariated after 17 h at 25°C (Ohtaki, 1966). The disturbance of pupariation by ligation was caused by the interruption of ecdysone secretion from the ring gland (Moribayashi et al., 1992). Furthermore, the disturbance in pupariation at the anterior portion could be rescued by the injection of an appropriate small dose of ecdysteroids (Ohtaki, 1972; Moribayashi et al., 1992). These results prompted us to test the possibility that this phenomenon may be used to establish a new *in vivo* assay method for measuring ecdysteroid activity. We here report a simple and quick assay for ecdysteroids by using mature larvae of *B. peregrina* ligated at the tenth segment.

MATERIALS AND METHODS

Rearing and preparation of larvae for assay. *B. peregrina* collected in Tokyo in 1990 and thereafter maintained in our laboratory, the *Bp* (Tokyo) strain, was used. Adult flies were reared under LD

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Fig. 1. Flow chart of the process for quick assay of *Boettcherisca peregrina* larvae. See text for detail.

Fig. 2. Scoring of ecdysteroid responsiveness at the anterior portion of the ligated *B. peregrina* larvae. See text for detail.

16:8 at 27°C. Mature larvae at wandering stage were collected and immersed in water for 2 days and then transferred to dry conditions. The dry larvae were subjected to an ecdysteroid assay within 2 h after the dry treatment.

Ecdysteroid treatment and scoring for responsiveness. Figure 1 shows procedures and timing of the bioassay. One microliter of ecdysteroid solution in ethanol was injected to the anterior portion through the hind spiracle with a micro-syringe (Hamilton syringe 1710 SN 12MM, Hamilton Co., Reno, Nevada, USA) under a binocular microscope ($\times 10$). Just before injection, the larvae were ligated loosely at the tenth abdominal segment with

cotton thread (gauge 40), so that the loose ligation allowed insertion of the syringe needle through the hind spiracle. The loop of thread at the tenth segment was tightened immediately after the injection of a test sample. Each segment of mature larvae was identified according to Ohtaki (1972). Ten larvae were used for each concentration of ecdysteroid. The larvae injected were kept under LD 16:8 at 27°C for 24 h and then the ecdysteroid activity was determined by morphological changes induced by the injection, i.e., different degrees of tanning and/or brownish dark coloration of larval cuticle in the anterior body portion (Fig. 2).

The anterior portions of larvae that were injected

with the test samples were graded to give arbitrary scores (+4, +3, +2, +1 and 0) 24 h after injection. The grades depended on the intensity of responses: whole body tanned, over 50% of the body tanned, less than 50% and only small areas tanned were scored +4, +3, +2, +1, respectively. No sign of pupal development was scored 0.

Ecdysteroid molecules. Six different standard ecdysteroids were used: ecdysone, 20-hydroxyecdysone, 20-hydroxyecdysone-22acetate, inokosterone, ponasterone A and polypodine B to confirm the sensitivity of this *in vivo* assay. Ecdysone, ponasterone A, polypodine B and 20-hydroxyecdysone-22acetate were purchased from Sigma, St. Louis, USA and 20-hydroxyecdysone and inokosterone (25-deoxy-20,26-dihydroxyecdysone) were purchased from Rhoto Pharmaceutical Co., Ltd., Japan.

RESULTS

Effect of the ligation *per se* on pupal development

Mature larvae ligated at the tenth segment within 2 h after dryness did not show any signs of pupariation at either anterior or posterior portions of the ligated bodies until 72 h after ligation (Table 1), even though the ring gland was in the anterior portion. Ninety to 95% of the larvae ligated at the eleventh and twelfth segments underwent pupariation at the anterior portions in 72 h after ligation (Table 1). Further 25, 15 and 10% of the larvae ligated at the sixth, eighth and ninth segments, respectively, underwent pupariation at the anterior portions 72 h after ligation (Table 1). No pupariation occurred at any posterior portions by ligation

within 2 h after dryness.

Detection limits and sensitivity relative to the conventional method

Ligation at the tenth segment showed no sign of pupariation until 72 h after ligation (Table 1). From these results, the ligation at the tenth segment was used for determination of ecdysteroid activity. Table 2 shows the hormonal activities of inokosterone injected into the larvae ligated at the tenth segment at doses of 16–1,000 ng/larva. The hormonal activities were determined 24 h after injection and the response was dose-dependent. The detection limit of this assay method for inokosterone was 125 ng per larva (Table 2). The detection limits for five other ecdysteroids (the detailed assay data against each ecdysteroid are not shown here) are shown in Table 3. The detection limits against six ecdysteroids were as follows:

inokosterone (125 ng) < ecdysone (64 ng) = 20-hydroxyecdysone-22acetate (64 ng) < ponasterone A (32 ng) < 20-hydroxyecdysone (16 ng) < polypodine B (8 ng). No change in the ligated posterior portion was detected at any dose examined for polypodine B.

DISCUSSION

Table 3 shows the detection limits of ecdysteroid activity in two assay methods using *B. peregrina* larvae ligated either at the tenth (the present results) or the sixth segment (Ohtaki et al., 1967). The sensitivity of the present assay relative to that of Ohtaki et al.'s assay which was established by the induction of the pupariation at posterior portions of larvae ligated at the sixth segment, ranged

Table 1. Incidence of pupariation at the anterior or posterior portions of *B. peregrina* 24 h, 48 h and 72 h after ligation at each segment

Segment ligated	Number of larvae tested	% pupariation <24 h		% pupariation <48 h		% pupariation <72 h	
		Anterior portion	Posterior portion	Anterior portion	Posterior portion	Anterior portion	Posterior portion
6	20	15	0	25	0	25	0
8	20	10	0	15	0	15	0
9	20	5	0	10	0	10	0
10	20	0	0	0	0	0	0
11	20	60	0	90	0	90	0
12	20	80	0	90	0	95	0

Table 2. Hormonal activities of inokosterone assayed by the new method

Inokosterone dose (ng/larva)	No. of larvae tested	No. of larvae showing each score					Total score
		(+4)	(+3)	(+2)	(+1)	0	
1,000	10	3	1	1	0	5	17
500	10	0	1	5	0	6	13
250	10	0	1	2	2	5	9
125	10	0	0	1	2	7	4
64	10	0	0	0	0	10	0
32	10	0	0	0	0	10	0
16	10	0	0	0	0	10	0

These scores were determined 24 h after injection through the hind spiracle and ligation at the 10th segment. See the Materials and Methods for detail in the score of hormonal activity.

Table 3. Comparison of detection limits of hormonal activity for ecdysteroids between the two assay methods

Ecdysteroids	Response of anterior portion		Response of posterior portion		A/B
	ng/insect (A) ^a	ratio/ecdysones	ng/insect (B) ^b	ratio/ecdysones	
Inokosterone	125	2.0	64	1.8	2.0
Ecdysone	64	1.0	35	1.0	1.8
20-Hydroxyecdysone-22acetate	64	1.0	—	—	—
Ponasterone A	32	0.5	11	0.3	2.9
20-Hydroxyecdysone	16	0.3	18	0.5	0.9
Polypodine B	8	0.1	—	—	—

^a From the present results using the anterior portion.

^b From Ohtaki et al. (1967). Ohtaki et al. used the posterior portion of ligated larvae.

from 0.9 to 2.9 times less for four ecdysteroids tested. The detection limit for 20-hydroxyecdysone, the main and most active molecule of the insect authentic-ecdysteroids, was similar in the two assay methods, i.e., the present result: 16 ng/insect vs. Ohtaki et al.'s result: 18 ng/insect (Table 3). Other dipteran insects, the housefly, *M. domestica* (Kaplanis et al., 1966), the blowflies, *C. erythrocephala* (Karlson and Hanser, 1953), and *C. stygia* (Thomson et al., 1970) showed 5 ng, 7.5 ng and 50 ng per insect for the detection limits for 20-hydroxyecdysone, respectively.

The simplicity and short time required for preparing test insects are of user's advantage in this bioassay. In the Ohtaki et al.'s method, extra steps were required for preparing the test larvae before ligation and injection of ecdysteroids into the larvae (Ohtaki et al., 1967). The mature larvae kept under wet conditions for 4 days must be transferred to dry conditions and ligated at the sixth segment 6 h after drying. Then, the larvae that showed pupal

development only in the anterior portion 24 h after ligation at the sixth segment were collected and used for bioassay. In our method, the test larvae are kept for 2 days under wet conditions and then subjected to dry conditions just before the injection of test-sample. No additional time was required once larvae become dry. The new assay takes approximately 3 days less time than Ohtaki's method for preparing test larvae before sample injection. Additionally, just ten larvae per test sample are required in the new assay method, while Ohtaki et al.'s method requires a large number of larvae (2–3 times of the new assay).

The present study, as well as Ohtaki (1972), showed that *B. peregrina* larvae ligated at the tenth segment were inhibited from pupariation tanning in the portions both anterior and posterior to the ligation. The inhibition of tanning at the anterior portion by ligation at the tenth segment is an unexpected phenomenon, since the brain-ring gland complex responsible for releasing the neurosecre-

tory factors and ecdysteroids for pupariation are located anterior to the ligation. Ligation on the tenth segment inhibited ecdysone secretion from the larval ring gland, whereas ligation at the eleventh segment of the larval body did not inhibit pupariation and ecdysone secretion (Moribayashi et al., 1992).

At present, no clear interpretations are available for why the ligation at the tenth segment inhibited pupariation in the anterior portion. Further studies are required to elucidate the roles of ligation at the tenth segment on the endocrine and/or neuroendocrine systems and to find the localization of responsible organ(s) or tissues in the tenth segment and posterior part.

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