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[SHORT COMMUNICATION]

Measurement of Molt-inhibiting Hormone Titer in Hemolymph of the American Crayfish, *Procambarus clarkii*, by Time-Resolved Fluoroimmunoassay

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ABSTRACT—In order to determine the titer of molt-inhibiting hormone (Prc-MIH) in the hemolymph of the American crayfish *Procambarus clarkii*, a time-resolved fluoroimmunoassay (TR-FIA) was established using specific antibodies against N-terminal and C-terminal segments of Prc-MIH. The lowest limit of detection of Prc-MIH in TR-FIA was 10 amol/assay. The Prc-MIH titers in the hemolymph were 6.53 fmol/ml at the intermolt stage and 1.25 fmol/ml at the early premolt stage. This result is consistent with the long-known hypothesis that the Y-organ is inhibited by MIH during the intermolt stage, whereas the Y-organ is activated by being freed from the inhibitory regulation of MIH.

Key words: molt-inhibiting hormone, ecdysteroid, crustacea, molting, time-resolved fluoroimmunoassay

INTRODUCTION

Ecdysteroid, which triggers molting in crustaceans, is secreted from a pair of Y-organs (Spindler *et al*., 1980; Skinner, 1985; Jegla, 1989; Sonobe *et al*., 1991). The ecdysteroid titer in the hemolymph is low during the intermolt stage, rises to the maximum level during the premolt stage, then declines rapidly just prior to molting (Stevenson *et al*., 1979; Soumoff and Skinner, 1983; Nakatsuji *et al*., 2000). Ecdysteroid secretion from the Y-organs is presumed to be negatively regulated by molt-inhibiting hormone (MIH), which is a neuropeptide secreted from the X-organ-sinus gland system located in the eyestalk (Skinner, 1985; Jegla, 1989; Webster, 1998). Recently, it has been demonstrated that the level of MIH mRNA expression in the X-organ (Lee *et al*., 1998) and the MIH content in the sinus gland (Nakatsuji *et al*., 2000) change in a molt-stage-specific manner. These results have suggested the possibility that MIH titer in the hemolymph fluctuates during the molt cycle. However, changes in the hemolymph MIH titer during the molt cycle have not yet been determined in any crustaceans. The aim of the present study is to determine the titer of MIH (Prc-MIH) in the hemolymph of the American crayfish *Procambarus clarkii* using a time-resolved fluoroimmunoassay (TR-FIA).

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MATERIALS AND METHODS

Animals

Adult males of the American crayfish, *P. clarkii*, were used in our experiments. They were reared at 25°C and fed a pellet diet every other day in the laboratory. The molt stages of the crayfish were determined based on the changes in the volume of gastrolith in the stomach and ecdysteroid titers in the hemolymph (Nakatsuji *et al*., 2000). The gastrolith volume was monitored by X-ray photography (Nakatsuji *et al*., 2000). The ecdysteroid titer in the hemolymph was determined by radioimmunoassay (RIA) according to the method described previously (Sonobe *et al*., 1991).

Preparation of samples for TR-FIA

The hemolymph (250–500 μl) was withdrawn from the arthrodial membrane between the coxa and the base of cheliped using a 1-ml syringe with a 23-gauge needle and immediately mixed with two volumes of 60% acetonitrile on ice. The mixture was centrifuged (20,000*g*, 20 min), and the supernatant was evaporated under reduced pressure. The dried residue was suspended in the sample buffer for TR-FIA (see below), then centrifuged (20,000*g*, 5 min), and the supernatant was subjected to TR-FIA. Samples (50 μl) were assayed in duplicate.

In order to evaluate the recovery of Prc-MIH from the hemolymph in the extraction procedure, 0.5, 5 and 50 fmol of authentic Prc-MIH (Kawakami *et al*., 2000; Sonobe *et al*., 2001) were added to 500 μl of the hemolymph, which was collected from the crayfish whose eyestalks were removed 7 days before hemolymph sampling in order to eliminate endogenous Prc-MIH in the hemolymph. Authentic Prc-MIH added to the hemolymph was extracted with acetonitrile as described above and quantified by TR-FIA.

TR-FIA for Prc-MIH

Antibodies were raised against Prc-MIH(1-7) and Prc-MIH(55-75)-NH_2 fragments conjugated with bovine serum albumin, and purified using affinity columns conjugated with Prc-MIH(1-7) and Prc-MIH(55-75)-NH_2 fragments, respectively (Nakatsuji *et al*., 2000). The amino acid sequences of these fragments show no similarity with any segment of the hyperglycemic hormone in *P. clarkii* (Yasuda *et al*., 1994), which belongs to the same family as Prc-MIH (Van Herp, 1998). Anti-Prc-MIH(55-75)-NH_2 IgG was biotinylated (Bayer and Wilchek, 1990) and used as the secondary antibody in the TR-FIA.

Wells of a polystyrene microtiter plate (Costar, USA) were filled with 80 μl of a solution of the primary antibody, anti-MIH(1-7) IgG (4 μg/ml of 0.1 M phosphate buffer, pH 7.5, containing 0.1% NaN_3) and incubated for 16 hr at 4°C. Subsequently, the wells were washed five times with the washing buffer (0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.01 M $MgCl_2$ and 0.05% Tween-20) with shaking, then incubated with 250 μl of the blocking buffer (0.01 M phosphate buffer, pH 7.0, containing 0.05% casein, 0.1 M NaCl, 0.01 M $MgCl_2$ and 0.1% NaN_3) for 1 hr at 25°C or overnight at 4°C. Authentic Prc-MIH was dissolved in the sample buffer (0.01 M phosphate buffer, pH 7.0, containing 0.05% casein, 0.4 M NaCl, 0.01 M $MgCl_2$ and 0.1% NaN_3) and assayed in triplicate. The test solutions (50 μl) were added to the wells coated with the primary antibody and incubated for 6 hr at 25°C with shaking. The wells were washed five times with the washing buffer, filled with 50 μl of the biotinylated secondary antibody solution (200 ng/well) diluted with the blocking buffer, and incubated for 1.5 hr at 25°C with shaking. After the wells were washed five times with the washing buffer, 50 μl of europium-labeled streptavidin (Wallac, Finland) solution diluted to 0.1 μg/ml with DELFIA assay buffer (Wallac) was added, and the wells were incubated for 30 min at 25°C with shaking. After washing five times with the washing buffer, DELFIA enhancement solution (Wallac) was added and the mixture was allowed to react for 5 min with shaking. The fluorescence intensity of europium chelates that developed was measured with a time-resolved fluorometer (Wallac).

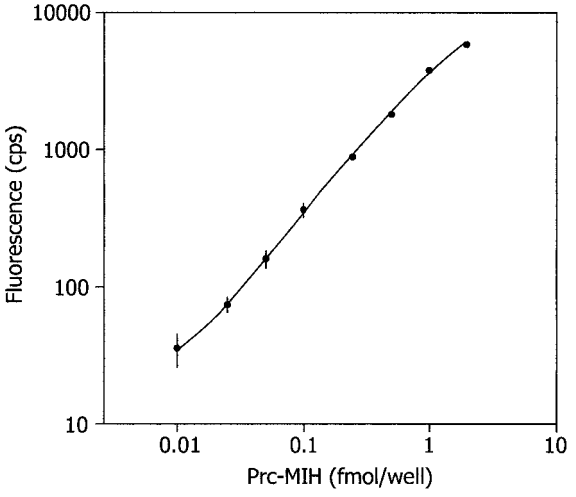

Fig. 1. Prc-MIH standard curve for TR-FIA. Synthetic Prc-MIH (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1 and 2 fmol/well) was used as the standard. Results are expressed as means ± SE (n=3).

RESULTS AND DISCUSSION

Fig. 1 shows a standard curve for TR-FIA using authentic Prc-MIH. The lowest limit of detection of Prc-MIH was 10 amol/well, suggesting that our TR-FIA is 50-fold as sensitive as the enzyme immunoassay (0.5 fmol/tube) for Prc-MIH that we have established previously (Nakatsuji *et al*., 2000). Moreover, TR-FIA was more sensitive than competitive RIA (1-2 fmol/tube) and enzyme-linked immunosorbent assay (0.5 fmol/well) for MIH of *Carcinus maenas* established by Webster (1993). Recoveries of Prc-MIH from the hemolymph are summarized in Table 1. No Prc-MIH was detected in the hemolymph in which authentic Prc-MIH was not added, while about 70% of exogenous Prc-MIH was

Table 1. Recovery of authentic Prc-MIH added to the hemolymph.

Added Prc-MIH (fmol/ 500 μl)	Recovered Prc-MIH (fmol/ 500 μl)	Recovery (%)
0	0	
0.5	0.34 ±0.09	68.0 ±18.0
5.0	3.69 ±0.05	73.8 ±1.0
50.0	37.63 ±1.77	75.3 ±3.5

Authentic Prc-MIH (0.5, 5 and 50 fmol) was added to 500 μl of the hemolymph, which was collected from the crayfish whose eyestalks were removed previously. Prc-MIH was extracted from the hemolymph with acetonitrile. Recovered Prc-MIH was quantified by TR-FIA. The values are expressed as means ± SE (n=4).

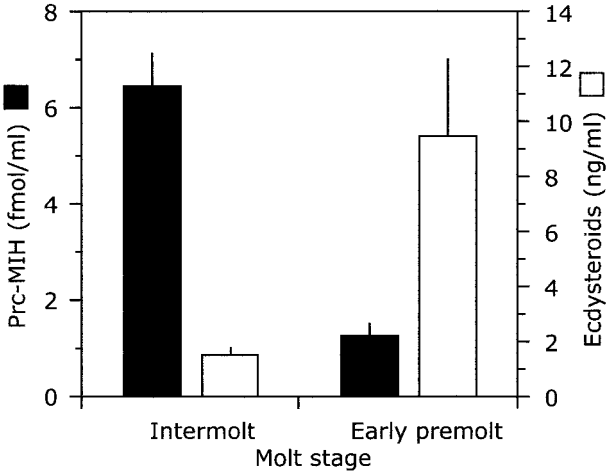

Fig. 2. Correlation between the titers of Prc-MIH and ecdysteroids in the hemolymph during the intermolt and early premolt stages. The hemolymph was individually collected from 34 animals at the intermolt stage and 30 animals at the early premolt stage. The titers of Prc-MIH and ecdysteroids in the hemolymph were quantified by TR-FIA and RIA, respectively. Results are expressed as means ± SE. The titers of Prc-MIH and ecdysteroids in the hemolymph at the early premolt stage were significantly different from those at the intermolt stage at $P<0.05$ on t-test.

consistently recovered from the hemolymph samples when authentic Prc-MIH was added at various concentrations.

The Prc-MIH titers in the hemolymph at the intermolt and early premolt stages were determined by TR-FIA, and compared with the ecdysteroid titers in the hemolymph (Fig. 2). The Prc-MIH titer in the hemolymph at the intermolt stage (6.53 fmol/ml) was about five times higher than that at the early premolt stage (1.28 fmol/ml). On the contrary, the ecdysteroid titer in the hemolymph was about one-sixth lower at the intermolt stage (1.50 ng/ml) than at the early premolt stage (9.42 ng/ml). To our knowledge, this is the first demonstration of changes in hemolymph MIH titer. The results obtained are consistent with the hypothesis that the decrease in the titer of hemolymph MIH at the premolt stage may trigger the initiation of ecdysteroid secretion from the Y-organs (Skinner, 1985; Jegla, 1989; Webster, 1998). Our present results, furthermore, indicate that TR-FIA is useful for investigating the changes in MIH titer in the hemolymph during the molt cycle. Detailed analysis of these changes is now in progress.

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