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Research paper

Enzyme-linked immunosorbent assay of relaxin-like gonad-stimulating peptide in the starfish *Patiria* (*Asterina*) *pectinifera*

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ABSTRACT

A relaxin-like gonad-stimulating peptide (RGP) from starfish *Patiria* (*Asterina*) *pectinifera* is the first identified invertebrate gonadotropin for final gamete maturation. Recently, we succeeded in obtaining specific antibodies against *P. pectinifera* RGP (PpeRGP). In this study, the antibodies were used for the development of a specific and sensitive enzyme-linked immunosorbent assay (ELISA) for the measurement of PpeRGP. A biotin-conjugated peptide that binds to peroxidase-conjugated streptavidin is specifically detectable using 3,3′,5,5′-tetramethylbenzidine (TMB)/hydrogen peroxide as a substrate; therefore, biotin-conjugated RGP (biotin-PpeRGP) was synthesized chemically. Similarly to PpeRGP, synthetic biotin-PpeRGP bound to the antibody against PpeRGP. In binding experiments with biotin-PpeRGP using wells coated with the antibody, a displacement curve was obtained using serial concentrations of PpeRGP. The ELISA system showed that PpeRGP could be measured in the range 0.01–10 pmol per 50 μl assay buffer. On the contrary, the B-chains of PpeRGP, *Asterias amurensis* RGP, *Aphelasterias japonica* RGP, and human relaxin showed minimal cross-reactivity in the ELISA, except that the A-chain of PpeRGP affected it slightly. These results strongly suggest that this ELISA system is highly specific and sensitive with respect to PpeRGP.

1. Introduction

Relaxin-like gonad-stimulating peptide (RGP) of starfish *Patiria* (=*Asterina*) *pectinifera* is the first identified invertebrate gonadotropic hormone responsible for final gamete maturation (Mita et al., 2009, Mita, 2013). *P. pectinifera* RGP (PpeRGP) is a heterodimeric peptide with a molecular weight of 4740, comprising an A-chain of 24 amino acids (aa) and a B-chain of 19 aa. Although RGP, originally named gonad-stimulating substance (GSS) (Kanatani and Shirai, 1967; Kanatani, 1969), is the primary mediator of oocyte maturation and ovulation in starfish, its effect on oocyte maturation is indirect. Resumption of meiosis in immature oocytes and release from the ovary are induced by a second mediator, maturation-inducing hormone (MIH), identified as 1-methyladenine (1-MeAde) in starfish (Kanatani et al., 1969; Kanatani, 1985). Thus, RGP plays an important role in 1-MeAde production in ovarian follicle cells (Hirai and Kanatani, 1971; Hirai et al., 1973). In this sense, RGP is functionally analogous to vertebrate luteinizing hormone (LH), especially piscine and amphibian LHs, acting on the ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of the oocyte (Nagahama et al., 1995).

For a long time since the initial finding of gonadotropic activity in an aqueous extract of radial nerve cords by Chaet and McConnaughy (1959), a biological assay using intact ovaries has been the only means to measure RGP content. The bioassay shows high sensitivity to RGP (Shirai, 1986). However, it is necessary that sexually mature females are first inspected for their sensitivity to 1-MeAde treatment using ovarian fragments. Therefore, the biological assay can be performed only during the breeding season.

Recently, using a sulfanyl-polyethylene glycol (PEG) derivative of PpeRGP, we succeeded in obtaining specific antibodies against PpeRGP (Katayama and Mita, 2016). The antibodies were also used for a radioimmunoassay (RIA) as a quantitative method for PpeRGP (Yamamoto et al., 2017). However, measuring PpeRGP content using an RIA system is suboptimal, because radioiodinated PpeRGP must be prepared for each experiment. In addition, expensive gamma counter equipment is required for quantifying the radioactivity. In this study, therefore, we tried to develop an enzyme-linked immunosorbent assay (ELISA) system as a novel quantitative method for PpeRGP using the antibodies prepared previously.

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Furthermore, chemical structures of RGP are identified in several species of starfish. Previous studies have shown that the chemical structure of PpeRGP is mostly conserved among starfish of the Order Valvatida (Ikeda et al., 2015; Mita et al., 2015b; Mita, 2016; Smith et al., 2017). In contrast, the chemical structures of RGP identified in *Asterias amurensis* (AamRGP) and *Aphelasterias japonica* (AjaRGP) of the Order Forcipulatida were quite different from that of PpeRGP (Mita et al., 2015a; Mita and Katayama, 2016). Neither AamRGP nor AjaRGP induces spawning in the ovarian fragments of *P. pectinifera* (Mita et al., 2015a; Mita and Katayama, 2016), although PpeRGP is active in *A. amurensis* and *A. japonica* ovaries (Mita and Katayama, 2016). This suggests that PpeRGP acts non-species-specifically *in vivo*. Thus, this study also examined whether the ELISA system is species-specific for RGP from *P. pectinifera*.

2. Materials and methods

2.1. Animals

Starfish, *P. pectinifera*, were collected from Yokosuka (Kanagawa, Japan), Choshi (Chiba, Japan), Ushimado (Okayama, Japan), and Asamushi (Aomori, Japan). Animals were kept in circulating artificial seawater at 15 °C and used within 2 months of collection.

2.2. Peptide synthesis

RGP peptides of *P. pectinifera* (PpeRGP) and *A. japonica* (AjaRGP) were synthesized essentially in accordance with the method for synthesizing insulin-like peptide from the prawn *Marsupenaeus japonicus* as described previously (Katayama et al., 2014). *A. amurensis* RGP (AamRGP) was purchased as a custom order from the Peptide Institute (Osaka, Japan). Peptide copies of the A- and B-chains of PpeRGP were also synthesized commercially (Torai Research Center, Kanagawa, Japan). Biotin-PpeRGP was synthesized by conjugation of the PEG derivative of PpeRGP with biotin essentially in accordance with the method described previously (Katayama and Mita, 2016). Human relaxin-3 (hRLN3) was obtained from Phoenix Pharmaceutical Inc. (Burlingame, CA, USA).

2.3. Preparation of anti-PpeRGP antibodies

Conjugation of the sulfanyl-PEG derivative of PpeRGP to keyhole limpet hemocyanin (KLH) and immunization of two rabbits was carried out by Eurofins Genomics (Tokyo, Japan). The sera were collected at 4 weeks after immunization. The antibodies were purified from the antisera using a Dojindo IgG Purification Kit-A (Cosmo Bio Co., Ltd, Tokyo, Japan), in accordance with the manufacturer's instructions.

2.4. Evaluation of antibodies by ELISA

To obtain titer curves of anti-PpeRGP antibody with respect to PpeRGP and biotin-PpeRGP, ELISA was conducted. The well of an Iwaki ELISA plate (96 wells) (AGC Techno Glass Co., Tokyo, Japan) was coated with peptide (1 μM in 10 mM phosphate buffered saline, PBS) and incubated overnight at 4 °C. After washing and blocking with PBS containing 1% bovine serum albumin (BSA) and 0.05% NaN_3, the plate was dried at 4 °C. The antibody (100 μl) diluted in PBS containing 0.05% Tween 20 (PBST) was added to wells and incubated for overnight at 4 °C. After washing, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc., CA, USA) diluted in PBST was added to the wells and incubated for 2 h at room temperature. After washing, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB)/hydrogen peroxide solution (SureBlue™ TMB microwell peroxidase substrate (1-component), KPL, Gaithersburg, MA, USA) was added to the wells and incubated for 30 min at room temperature. Reaction was stopped by adding 100 μl of 1 M sulfuric acid, and then absorbance at 450 nm was measured by a microplate reader Model 680 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.5. Induction of oocyte maturation and ovulation

Bioactivities of synthetic PpeRGP and biotin-PpeRGP were assayed using ovarian fragments from *P. pectinifera*, as described previously (Shirai, 1986). Modified van't Hoff's artificial seawater (ASW) adjusted to pH 8.2 with 0.02 M borate buffer was prepared (Kanatani and Shirai, 1970) and the ovaries of mature female starfish were excised and cut using scissors into small fragments containing only a few lobes. The ovarian fragments were then incubated in ASW containing PpeRGP or biotin-PpeRGP at a range of concentrations (5×10^{-8}–4×10^{-10} M) for 1 h. The samples were examined to determine whether or not spawning had occurred and were scored (Shirai, 1986) as follows: (+++) spawning occurred and most oocytes had matured; (++) about 50% of oocytes had matured, (+) a few oocytes had matured and (−): no spawning occurred. The scores were converted to numerical values (+++ = 100; ++ = 67; + = 33; − = 0) so that the effective dose for inducing gamete spawning in 50% of ovarian fragments could be determined graphically. Means ± SEM were determined from four separate assays using ovaries from different animals.

2.6. Preparation of organs

In order to measure the PpeRGP content, various organs such as radial nerve cords, circumoral nerve-rings, pyloric caeca, pyloric stomach, cardiac stomach, tube-feet, ovaries, and testes were excised from *P. pectinifera*. Gametes were squeezed from the ovaries and testes with forceps. These organs were homogenized in four volumes of 0.6 M perchloric acid using a Physicotron NS-360D homogenizer (Microtech Co., Ltd. Chiba, Japan). After centrifugation at 10,000*g* for 10 min at 4 °C, the supernatant was neutralized with 2 M KOH to pH 6.5–7.0 and stood for 1–2 h on ice. After centrifugation at 10,000*g* for 30 min at 4 °C, the obtained supernatant was lyophilized as a preservation step. Samples were dissolved in adequate amounts of milli Q water before use in either the ELISA or bioassay for PpeRGP.

2.7. Procedure of ELISA for RGP measurement

PpeRGP content in organ extracts was measured using an ELISA system based on competition between PpeRGP and a fixed quantity of biotin-PpeRGP for a limited number of binding sites on the PpeRGP specific antibodies. The reference standard and test samples were serially diluted with PBST containing 0.1% BSA. The ELISA procedure was as follows: rabbit anti-PpeRGP antibodies diluted 1000 times in bicarbonate buffer at pH 9.5 were precoated on the wells of an ELISA plate by incubating at 4 °C overnight. After washing the wells with PBS twice and blocking with PBS containing 1% BSA and 0.05% NaN_3, the plates were dried in air and stored at 4 °C before use. After washing the wells with PBST three times, 50 μl of the organ extracts and 50 μl of 1 nM biotin-PpeRGP in PBST containing 0.1% BSA were incubated in the PpeRGP antibody pre-coated wells for 1 day at 4 °C. After washing the wells with PBST three times, 100 μl of HRP-conjugated streptavidin (Thermo Fisher Scientific, Waltham, MA, USA) in PBST containing 0.1% BSA was dispensed into each well and incubated on a plate shaker for 3 h at 37 °C. After washing the wells with PBST three times, 100 μl of TMB/hydrogen peroxide solution was dispensed into each well and incubated on a plate shaker for 15–30 min at

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