Isolation and Characterization of Spawning Inhibitors in Ovary of the Starfish, *Asterias amurensis*

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Biologically active substances which inhibit spawning of mature starfish, *Asterias amurensis*, have been isolated from ovaries of the same organisms and identified as asterosaponins A and B.

It has been established that spawning of starfish is induced by injection of water extract of their radial nerves into the body cavity.^{1~3)} The active principle causative of the induction is a polypeptide called "gonad-stimulating substance" (GSS), which stimulates gonads to produce a "maturation-inducing substance".⁴⁾ The latter component has been elucidated as 1-methyladenine, and it acts directly on the surface of oocytes to cause maturation.^{5,6)} Then, discharge of the eggs from gonophores takes place through dissolution of intercellular cementing substances among the eggs followed by contraction of gonadal cell walls.⁷⁾

On the other hand, it has been known that gonads fail to react to GSS at the earlier stage of breeding season but they become more sensitive toward the end of the season. This suggests that some mechanism may operate inside gonads to suppress spawning. Recently, we showed the presence in gonads of *Asterias amurensis* and *Asterina pectinifera* of spawning inhibitors, which counteract the action of GSS to prevent unfavorable spawning early in the breeding season.⁸⁾ As for the *Asterina*, the active component has been isolated and identified as L-glutamic acid.^{8,9)} However, the action of this acid was specific to the *Asterina*, showing little effect on another

species, the *Asterias*. Accordingly, existence of inhibitors other than L-glutamic acid was expected in the latter starfish, and their isolation from ovaries was attempted.

In the previous communication, the isolation and identification of the spawning inhibitors from the *Asterias* have been preliminary reported.¹⁰⁾ Here we wish to describe details of the experimental results.

EXPERIMENTAL AND DISCUSSION

I. Collection of organs

The common Japanese starfish, *Asterias amurensis*, were collected early in the breeding season of 1967 and 1970 at Tokyo Bay and kept in crawls in the sea or in aquaria supplied with running sea water at Misaki Marine Biological Station. Then, ovaries were isolated from the starfish, immediately frozen and lyophilized. By this treatment, the ovaries could be stored in a desiccator without any appreciable loss of biological activity during more than two years.

II. Bioassay of spawning inhibitors

The lyophilized ovaries were homogenized in sea water and centrifuged for 50 min at 30,000×g and 2°C. The supernatant was serially diluted, at concentrations of 2.5~40

mg dry weight per ml, with sea water in which lyophilized nerve tissue prepared according to the method of Kanatani *et al.*³⁾ had been dissolved at a dose of 10 μ g/ml.* Into each solution thus prepared was placed an ovarian fragment isolated from the starfish at the height of the breeding season, and the degree of spawning was observed.

As the result, the dried ovaries at a concentration of 10 mg/ml were found to counteract the action of GSS contained in 10 μ g of the nerve tissue (Fig. 1). Lyophilized testes prepared in the way similar to that for ovaries showed counteraction at the same concentration. Observation of sperm-shedding *in vitro* is difficult to assess and ovarian

fragments were employed exclusively throughout the experiment.

Furthermore, *in vivo* assays were carried out by injecting a solution containing the dry ovary or testis at 10 mg/ml together with nerve extract (10 μ g lyophilized nerve per ml) into the coelomic cavity of either intact male or female starfish. Both ovary and testis extracts were effective to inhibit spawning in females as well as males.

III. Inhibitory activity of several amino acids for spawning

Since the spawning inhibitor contained in gonads of the *Asterias* had been elucidated to be L-glutamic acid,^{8,9)} efficacy of L-glutamic acid as well as of the following L-amino acids was examined for the *Asterias*: glycine, alanine, serine, threonine, valine, methionine, leucine, isoleucine, phenylalanine, tyrosine, proline, hydroxyproline, tryptophane, aspartic acid, arginine, lysine and histidine.

An ovarian fragment from the *Asterias* was placed in sea water containing 10^{-2} M of an amino acid together with 20 μ g/ml of lyophilized nerve tissue, and the degree of spawning was observed.

None of the amino acids tested showed inhibitory activity. Thus, the presence of spawning inhibitors other than these in the gonad of the *Asterias* has been proved.

IV. Purification of spawning inhibitors from ovary extract

Extraction. The spawning inhibitors were isolated from ovaries of *Asterias amurensis* by the procedure illustrated in Fig. 2.*

Acetone powder (184 g) prepared from the lyophilized ovaries (200 g) was extracted with cold 20% aqueous acetone and filtered. To the filtrate was added cold acetone to make

FIG. 1. Inhibition of Spawning by Extracts of Ovary (O) and Testis (T) in *Asterias amurensis*.

Ovarian fragments were placed in sea water containing various concentrations of gonad extract together with nerve extract (10 μ g of lyophilized nerve per ml). Shaded columns show incomplete inhibition. Percentage inhibition was calculated from the results of 18 tests for ovary extract and 8 tests for testis extract.

* The minimum dose of the nerve tissue required to bring appreciable discharge of eggs from cut surface of an ovarian fragment was determined, prior to the assay, to be 10~20 μ g per ml of sea water.

* The same inhibitors were obtained from lyophilized testes by the similar process. In this paper, however, only the isolation from ovaries is described.

FIG. 2. Procedure for Purifying Spawning Inhibitors from Ovary of *Asterias amurens*.

the final concentration 80%. After standing overnight at 3°C, precipitates were removed by filtration, and the filtrate was concentrated with a rotary evaporator being kept below 40°C. The aqueous residue was lyophilized and again suspended in distilled water, which was then adjusted to pH 8.0 with dilute sodium carbonate solution and extracted with its equal volume of *n*-butanol. Almost all the biological activity remained in the aqueous phase. After acidification to pH 3.6 with dilute sulfuric acid, the aqueous phase was re-extracted with its equal volume of *n*-butanol. Biological activity was observed in the butanol phase, which was then evaporated to dryness.

Gel-filtration on Sephadex G-50. The solid was dissolved in 10 ml of 0.05 M sodium chlo-

ride containing 2% *n*-butanol and applied onto a Sephadex G-50 column (7.0 x 127 cm), which was equilibrated and eluted with the same solvent system (fraction size, 17.5 ml; flow rate, 100 ml/hr; 3°C). For the assay of spawning inhibition a portion (0.2 ml) was taken from each fraction and diluted with 3.8 ml of sea water containing GSS. Fractions showing activity were combined and concentrated to 5 ml.

Gel-filtration on Sephadex G-25. The residual solution was subjected to gel-filtration by use of a Sephadex G-25 column (1.32 x 140 cm) and 0.1 M pyridine-acetate buffer of pH 6.0 (fraction size, 7.5 ml; flow rate, 70 ml/hr; 3°C). Active fractions were combined and evaporated to dryness.

Chromatography on DEAE-Sephadex A-25. The solid was dissolved in 150 ml of 0.02 M sodium phosphate buffer (pH 7.5) containing 2% *n*-butanol. The solution was applied onto a DEAE-Sephadex A-25 column (1.5 × 10 cm) equilibrated with 0.02 M sodium phosphate buffer (pH 7.2) containing 0.05 M sodium chloride and 2% *n*-butanol. Linear gradient elution was performed with 0.05~0.5 M sodium chloride (fraction size, 5 ml; flow rate, 40 ml/hr; 6°C). Active fractions eluted with 0.12~0.33 M sodium chloride were combined and concentrated to 15 ml. To the aqueous residue was added 100 ml of cold acetone, and the mixture was centrifuged for 40 min at $6,000 \times g$. Then the supernatant was evaporated to dryness.

Gel-filtration on Sephadex G-25. The white amorphous powder was dissolved in 5 ml of distilled water and again subjected to gel-filtration by use of a Sephadex G-25 column (1.5 × 106 cm) and 0.1 M pyridine-acetate buffer of pH 6.0 (fraction size, 2 ml; flow rate, 5 ml/hr; 6°C). The active fractions containing little inorganic phosphate and sodium chloride were combined and evaporated to dryness.

Silica gel column chromatography. The solid residue was washed with 4 ml of ethyl acetate to afford a colorless amorphous powder (191.2 mg). The powder (190 mg) was dissolved in 5 ml of chloroform-methanol-acetic acid (6:2:1, v/v) and was applied onto a column (1.3 × 13 cm) of silica gel (Merck), which was eluted with the same solvent system (fraction size, 10 ml; flow rate, 20 ml/hr). Each fraction was subjected to thin layer chromatography (TLC) on a Kieselgel GF₂₅₄ plate of 0.375 mm thickness and was developed with chloroform-methanol-acetic acid-water (30:10:5:2, v/v) (Solvent A). Spots were detected under a UV lamp at 2537Å or by spraying 10% (w/v) phosphotungstic acid in

ethanol. Fractions No. 11~20 (Fraction A) showed a spot at *R_f* 0.15 and fractions No. 26~40 (Fraction B) at *R_f* 0.07. On evaporation fractions A and B afforded 68.9 mg and 41.1 mg of solids, respectively.

Purity of spawning inhibitors isolated. Purity of each sample thus obtained was examined in the following ways. With each sample, TLC [Kieselgel GF₂₅₄ with 70% aqueous *n*-propanol or *n*-butanol-acetic acid-water (12:3:5, v/v); Avicel with *n*-butanol-pyridine-water (12:4:5, v/v) or *n*-butanol-acetic acid-water (12:3:5, v/v)] as well as paper electrophoresis [0.1 M pyridine-acetate buffer (pH 6.0)] gave a single spot under a UV lamp or with phosphotungstic acid spray.

Biological activity of inhibitors. Both samples were assayed to determine minimum effective doses against the action of 10 µg of the lyophilized nerve. The inhibitor from Fraction A showed inhibition at 12.5~125 µg/ml and that from Fraction B at 63~125 µg/ml.

V. Identification of spawning inhibitors

Both samples from Fractions A and B caused hemolysis and easily foamed in water indicating themselves as saponine-like substances.

Identification of inhibitor from Fraction A. The UV spectrum of Fraction A in water showed an absorption maximum at 244 mµ on a Cary-14 spectrophotometer. The IR spectrum (Fig. 3) run on a JASCO IR-S spectrometer in Nujol mull was similar to that of asterosaponin A, which had been isolated by Yasumoto *et al.*¹¹⁾ from the same organism.

Further, TLC by use of a Kieselgel GF₂₅₄ plate and solvent A revealed identical *R_f* values at 0.15 for asterosaponin A and Fraction A.

FIG. 3. IR Spectrum of the Inhibitor from Fraction A.

Then, the inhibitor (10 mg) from Fraction A was hydrolyzed with 2 ml of 2 N hydrochloric acid in a boiling water bath for 2 hr. The hydrolyzate was extracted with chloroform, which was washed successively with saturated sodium bicarbonate solution and water. A brown gum (4.1 mg) obtained after evaporation of the solvent showed the presence of at least six components on a Kieselgel GF₂₅₄ plate which was developed with benzene-acetone (3:2, v/v). The chromatogram was identical with that of a crude mixture of aglycones separated from asterosaponin A by the same procedure. On each chromatogram, two components with *R_f* values of 0.29 (Fraction 2) and 0.39 (Fraction 1) were dominant. The chemical structure of Fraction 2 has been established recently as 3 β ,6 α -dihydroxy-5 α -pregn-9(11)-en-20-one by the present authors.¹²⁾ Fraction 1 was further fractionated into several components, whose isolation and characterization will be reported in the near future.

Yasumoto *et al.*¹¹⁾ reported that asterosaponin A contains one mole of sulfuric acid and two moles each of 6-deoxy-D-glucose and 6-deoxy-D-galactose. In the present study, the aqueous layer remaining after chloroform extraction of the acid hydrolyzate of the inhibitor was subjected to analysis for sulfuric acid content by the method of Dodgson¹³⁾ as well as for hexose content with the phenol-

sulfuric acid reagent.¹⁴⁾ Sixty μ g of sulfuric acid and 450 μ g of hexose were found to be present in the hydrolyzate from 1 mg of the inhibitor.

Further, a small portion of the aqueous layer was evaporated to dryness, and the remaining solid was subjected to trimethylsilylation.¹⁵⁾ A mixture of *O*-trimethylsilyl (TMS) derivatives was subjected to gas-liquid chromatography (GLC) by use of a Hitachi gas chromatograph, Model K-53, equipped with a hydrogen flame ionization detector. A glass column (0.3 $\times$ 200 cm) was packed with 3% OV-1 on Chromosorb W (80~100 mesh) and was maintained at 150°C. The flow rate of the carrier gas, nitrogen, was 40 ml per min. Three peaks with retention times corresponding to the TMS derivative of 6-deoxy-D-galactose were detected at 4.8, 5.6 and 6.7 min, and two peaks responsible for the TMS derivative of 6-deoxy-D-glucose at 7.8 and 10.7 min (Fig. 4). On the basis of calculation for peak areas, 6-deoxy-D-galactose and 6-deoxy-D-glucose

FIG. 4. Gas Chromatogram of TMS Derivatives of Hydrolyzate of Fraction A.

(a), (b) and (c): TMS of 6-deoxygalactose, (d) and (e): TMS of 6-deoxyglucose.

were found to exist in an equimolar ratio. Thus, the inhibitor from Fraction A has been established to be identical with asterosaponin A.

Identification of inhibitor from Fraction B. The inhibitor in water showed a UV maximum at 244 m μ . The IR bands ($\nu_{\text{max}}^{\text{NaJol}}$ cm $^{-1}$: 3500, 1700, 1670, 1650, 1180, 1050 and 960) were similar to those of asterosaponin B reported by Yasumoto *et al.*¹⁶⁾ When subjected to TLC by use of Kieselgel GF₂₅₄ and Solvent A, the inhibitor and asterosaponin B showed identical spots at *Rf* 0.07.

The GLC pattern of TMS derivatives obtained from an acid hydrolyzate of the inhibitor is shown in Fig. 5. In addition to

FIG. 5. Gas Chromatogram of TMS Derivatives of Hydrolyzate of Fraction B.

(a), (b) and (c): TMS of 6-deoxygalactose; (d) and (e): TMS of 6-deoxyglucose; (f) and (g): TMS of xylose; (h), (i) and (j): TMS of galactose.

the peaks responsible for the TMS derivatives of 6-deoxy-D-galactose and 6-deoxy-D-glucose, two peaks ascribable to D-xylose (retention times; 6.8 and 8.8 min), and three peaks to

D-galactose (retention times; 13.2, 15.3 and 18.9 min) were observed. The molar ratio of 6-deoxy-D-glucose, 6-deoxy-D-galactose, D-xylose and D-galactose was established as 2:1:1:1. These data were consistent with those assigned for asterosaponin B by Yasumoto *et al.*¹⁶⁾

Inhibitory activity of asterosaponins A and B. When the authentic asterosaponins A and B were subjected to bioassay, the saponin A at the concentrations of 12.5~125 μ g/ml effectively inhibited the action of lyophilized nerve at a dose of 10 μ g/ml and the saponin B did at 63~125 μ g/ml. These values correspond well with those of the inhibitors mentioned above.

Yasumoto *et al.* described that asterosaponins distribute in every part of the starfish in high concentrations during summer whereas contents of the saponins are extremely low and found only in gonads during winter when the animals are at the breeding season at Tokyo Bay.¹⁷⁾ Further, GSS is present almost at the constant concentration in the radial nerves throughout the year,^{2,3)} but only induces spawning during a short period each year. This annual change might correspond to the aforementioned physiological action of asterosaponins.

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