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Characterization of a Factor with Oocyte Maturation Inducing Activity in *Spisula*

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Abstract. A substance that induces germinal vesicle breakdown (GVBD) of *Spisula* oocytes *in vitro* was isolated from *Spisula* body fluid (SBF) and ganglion cells by ultrafiltration through PM-10 membrane filter, charcoal extraction, and reversed phase HPLC. Its retention time on HPLC elution is coincident with that of reference serotonin (5-hydroxytryptamine, 5-HT) creatinine sulfate. The HPLC-purified substance from SBF and nerve ganglion induced GVBD in *Spisula* oocytes in a dose-dependent manner. 5-HT was identified in *Spisula* body fluid and ganglion cells by HPLC-electrochemical detection. The present results show that the factor in *Spisula* body fluid and ganglion cells that induces GVBD in *Spisula* oocytes *in vitro* is 5-HT or a closely related compound.

Introduction

Full-grown, naturally shed *Spisula* oocytes are arrested at the dictyate stage of meiosis and retain intact germinal vesicles (Allen, 1953). Upon fertilization meiosis is resumed, leading to germinal vesicle breakdown (GVBD). Oocyte maturation can be induced by chemical agents including KCl and ionophore A23187 (Allen, 1953; Schuetz, 1975).

Serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter, induces spawning in marine bivalves (Matsutani and Nomura, 1982). Many species of bivalves contain 5-HT in the nervous system (Welsh and Moorhead, 1960; Malanga *et al.*, 1972; Hiripi and Osborne, 1976; Stefano and Catapane, 1977; Smith, 1982). 5-HT has been detected in the central nervous system and gonads of the scallop, *Patinopecten yessoensis* (Matsutani and Nomura, 1984). Hirai *et al.* (1988) demonstrated that 5-HT injected into *Spisula* gonads induced spawning. When 5-HT is added to the suspending medium of *Spisula* oocytes, GVBD is induced. Recently, Toraya *et al.* (1987) reported that *Spisula* body fluid contains a substance that potentiates 5-HT action in inducing oocyte maturation.

The present study was undertaken to purify and characterize the substance with oocyte maturation inducing (OMI) activity from *Spisula* body fluid and ganglion cells. Evidence shows that the OMI activity in *Spisula* body fluid and nerve ganglion cells is structurally related to 5-HT.

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Materials and Methods

Chemicals

5-HT creatinine sulfate and trypsin were purchased from Sigma Co. (St. Louis, Missouri); Norit A (activated charcoal) from Fisher Chemical Co. USA; methanol, acetone, acetic acid, and hydrochloric acid (all HPLC grade) from T. J. Baker Chemical Co. (New Jersey). Artificial seawater (ASW) (Cavanaugh, 1974) was obtained from the Chemical Room, Marine Biological Laboratory (Woods Hole, Massachusetts).

Preparation of oocytes

Individuals of *Spisula solidissima* were kept in a laboratory aquarium with cold running seawater at the Marine Biological Laboratory (Woods Hole, MA) until used in the study. Ovaries from *Spisula* females were excised, minced in ASW, and strained through a pad of cheesecloth into a large beaker containing ASW. The oocytes were settled by gravity and the supernatant aspirated off. The washing procedure was repeated at least three times. The washed oocytes were used in the assay.

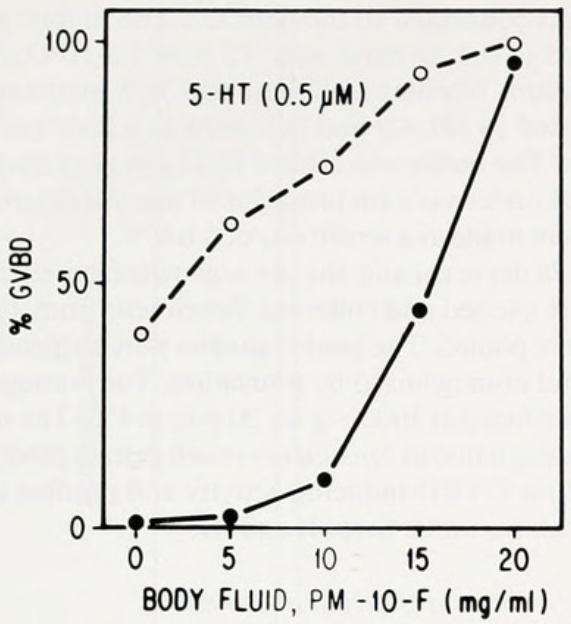

Figure 1. Dose-dependent GVBD-inducing activity of *Spisula* body fluid. The ultrafiltrate (SBF-PM-10F) was tested alone (● —— ●) and with serotonin (○ --- ○). Assay was performed as described under Materials and Methods.

Assay for maturation inducing activity

Samples to be assayed for OMI activity were dissolved in 1.0 ml of ASW and placed in a Falcon tissue culture dish (35 mm). One drop of *Spisula* oocytes (about 2000) was added and incubated at room temperature. After 30–40 min, oocytes were examined under a light microscope to determine the presence or absence of germinal vesicles. More than 100 oocytes were scored for each determination. The percentage of oocytes showing GVBD was calculated from the total number of oocytes scored. The values shown in the figures are the mean of three different experiments.

Purification of the factor with OMI activity

Spisula body fluid (SBF): body fluid was obtained by cutting the adductor muscles and the mantle and collecting the mantle fluid. The pooled body fluid was filtered through cheesecloth and centrifuged at 3000 × *g* for 20 min. The supernatant fluid was lyophilized, assayed for OMI activity, and purified.

OMI activity was purified by the following steps:

Step I. The lyophilized SBF was resuspended in distilled water (half the original volume) and centrifuged. The supernatant (200 ml) was filtered through a PM-10 membrane (Amicon Corp.) fixed in a 200 ml Amicon pressure cell under N_2 pressure. The filtration was performed in a cold room for approximately 24 h. The filtrate (175 ml) was placed in dialysis tubing with mol. wt. cut off of 1000 daltons and dialyzed against distilled water. The PM-10 filtrate (PM-10-F) and the retentate (DR) were assayed for OMI activity using *Spisula* oocytes.

Step II. Activated charcoal (30 mg/ml) was added to the PM-10-F sample and the mixture stirred slowly at 4°C for 30 min. The mixture was centrifuged at 800 × *g* for 10 min at 4°C. The sediment was resuspended in an equal volume of 0.1 *N* HCl, stirred for 30 min, and centrifuged at 3000 × *g* for 20 min at 4°C. The supernatant was filtered through a Whatman No. 1 filter paper. The filtrate was lyophilized and further purified on HPLC.

Step III. The test samples (100 μg/200 μl) were injected into a reverse-phase partisil-10 ODS-3 column (4.6 × 250 mm) (Whatman Inc., Clifton, New Jersey). A short precolumn (5 × 60 mm) packed with an octadecyl-coated pellicular support (Co. Pell ODS, Whatman) was used as the guard column. The column was eluted in isocratic run with 0.1 *M* acetic acid at a flow rate of 1 ml/min. Each pooled fraction was lyophilized and assayed for the OMI activity using *Spisula* oocytes.

High-performance liquid chromatography with electrochemical detection (HPLC-ECD)

Spisula body fluid (SBF) extract was prepared as described above. The nerve cell extract (SME) was prepared by the method of Smith (1982). The sample extract

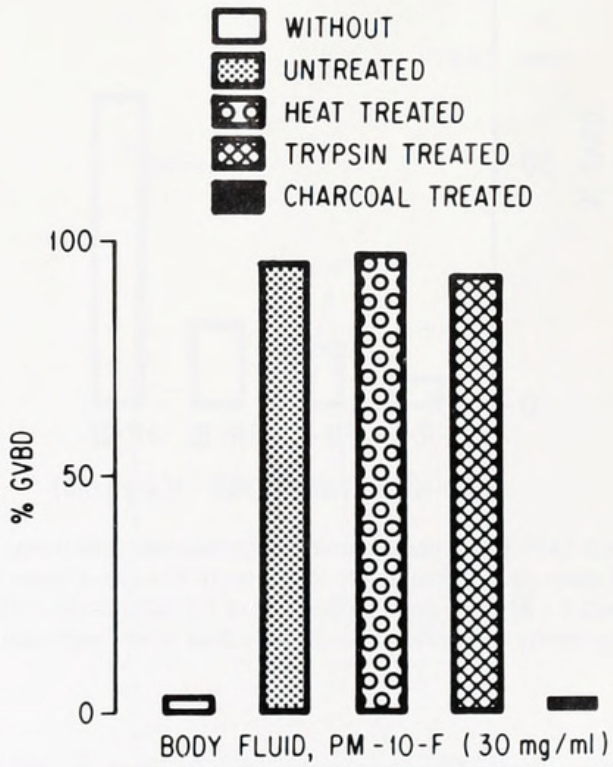

Figure 2. GVBD inducing activity of *Spisula* body fluid after treatment by heating, with trypsin and charcoal extracted. Fraction SBF-PM-10F was used. The procedures for the heat and trypsin treatments, charcoal extraction, and the bioassay are described under Materials and Methods.

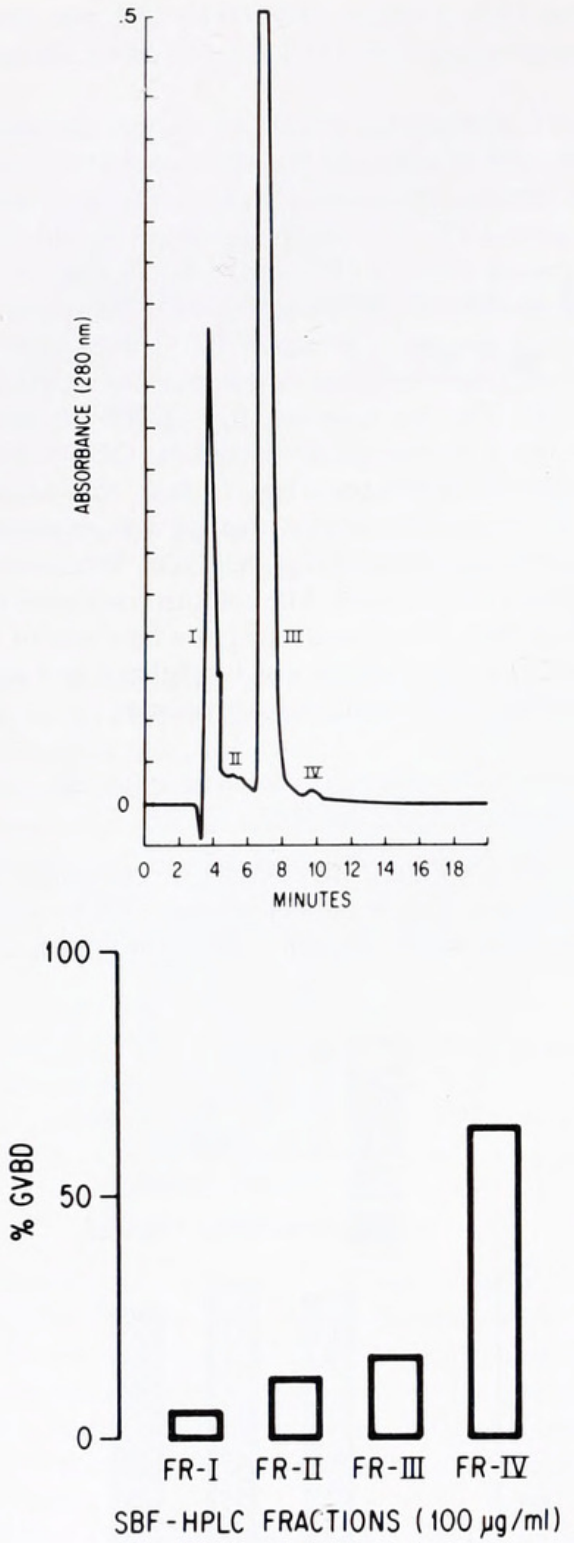

Figure 3. (A) HPLC elution profile of *Spisula* body fluid extract on reversed phase ODS-3 column (46 × 250 mm). The active factor was eluted with 0.1 *M* acetic acid at a flow rate of 1.0 ml/min. (B) GVBD-inducing activity of purified *Spisula* body fluid (SBF) separated by HPLC.

and reference 5-HT were analyzed separately by HPLC using a Bondapak C_{18} reverse-phase column (3.9 mm × 50). The HPLC was performed using an electrochemical detector system (Waters 460). A glassy carbon working electrode set at 0.650 V against the reference electrode was connected to the detector. The mobile phase consisted of 0.1 *M* citric acid, 75 m*M* Na_2HPO_4, 0.75 m*M* sodium 1-heptanesulfonate and 10% methanol (v/v) adjusted to pH 4.0 and delivered at a flow rate of 1 ml/min. The buffer was filtered (0.22 µm pore size) and degassed under vacuum just prior to use. All determinations were made at a sensitivity of 5 nA/V.

Spisula nerve ganglia: the nerve ganglia from each animal were excised and collected. Specimens from 10 animals were pooled. The pooled ganglia were suspended in ASW and homogenized by sonication. The homogenate was centrifuged at 3000 × *g* for 20 min at 4°C. The supernatant designated as *Spisula* nerve cell extract (SNE) was assayed for GVBD-inducing activity and purified as described above under Steps II and III.

Heat, trypsin, and charcoal treatment

Heat treatment was performed by placing the sample in boiling water at 100°C for 1 h. Digestion with trypsin was carried out by adding the protease (10 µg/ml) and incubating the mixture at 30°C for 1 h. Treatment with activated charcoal (30 mg/ml) was performed at room temperature for 1 h. Each procedure was performed separately and the treated samples assayed for maturation inducing activity.

Results

Spisula body fluid factor (SBF)

Crude SBF induced GVBD in 60% of the oocytes. The GVBD-inducing activity was potentiated in the presence of 5-HT. When SBF was subjected to ultrafiltration, the

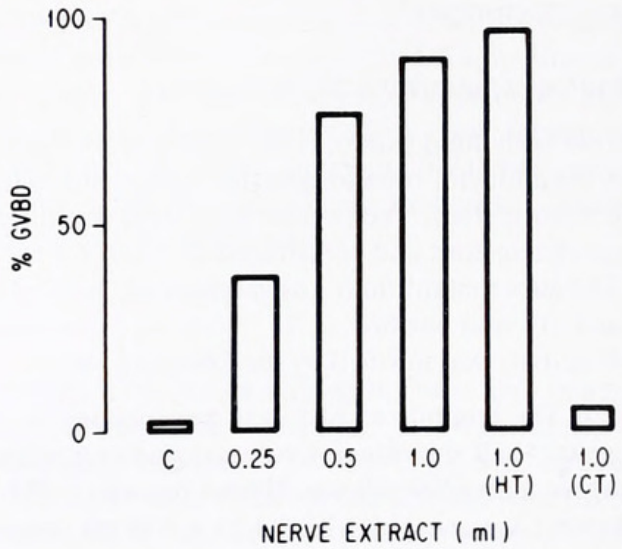

Figure 4. Dose-dependent induction of GVBD by *Spisula* nerve cell extract (SNE). After heat treatment (HT) and charcoal extraction (CT) SNE was assayed for GVBD-inducing activity as described under Materials and Methods.

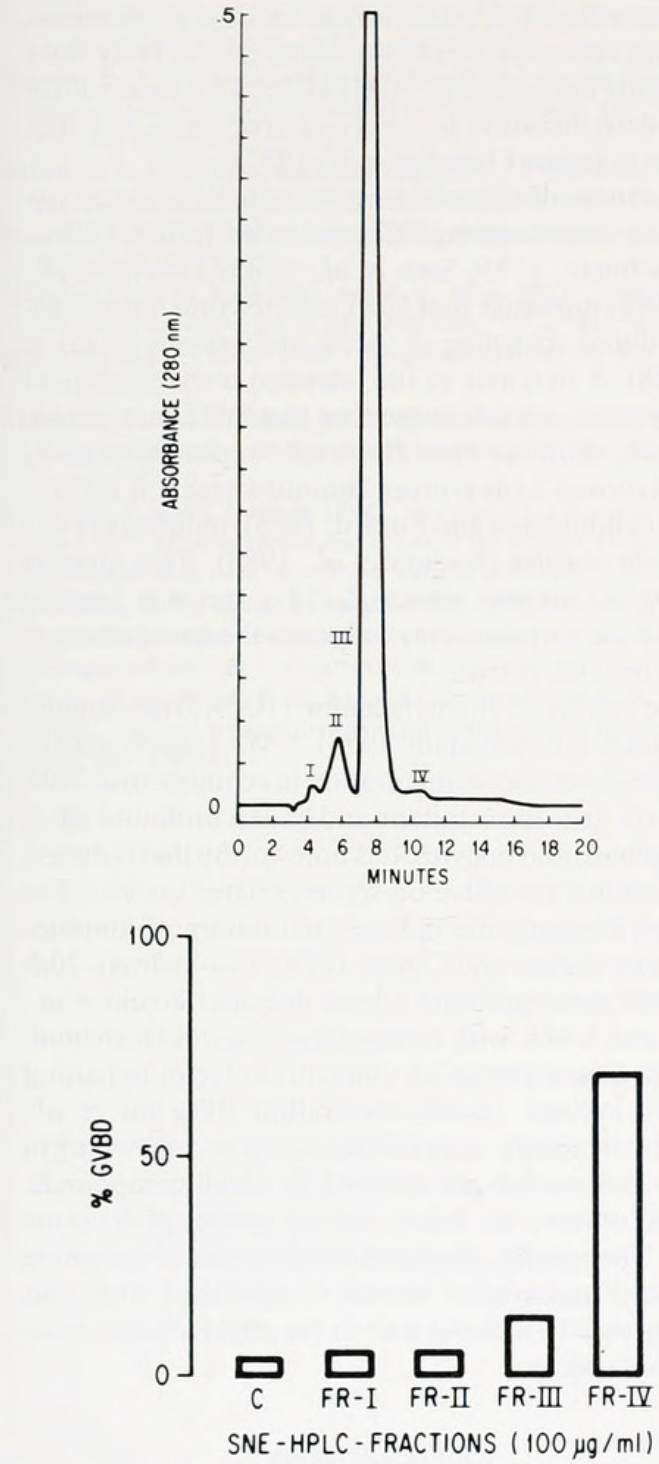

Figure 5. (A) Reversed phase HPLC elution profile of *Spisula* nerve cell extract (SNE). Eluting conditions were the same as described in Figure 3A. (B) GVBD inducing activity of HPLC-purified fractions of *Spisula* nerve cell extract (SNE). The four fractions separated by HPLC were assayed.

activity was located in the filtrate (PM-10-F). The filtrate alone or in the presence of 5-HT showed a dose-dependent GVBD-inducing activity (Fig. 1). When the filtrate (30 mg by weight per ml) was heated or treated with trypsin, the GVBD-inducing activity was retained. The activity in the filtrate was extracted by charcoal (Fig. 2) and lost on dialysis.

SBF extract was purified by HPLC. Four fractions were collected according to the elution profile (Fig. 3A). Fraction IV (FR-IV) possessed the greatest GVBD-inducing activity (Fig. 3B).

Spisula nerve ganglia factor (SNE)

SNE possessed potent, dose-dependent GVBD-inducing activity (Fig. 4). The activity was stable to heat and trypsin treatment. However, it was completely removed after charcoal treatment (Fig. 4). SNE was purified by HPLC and separated into four fractions (Fig. 5A). Only

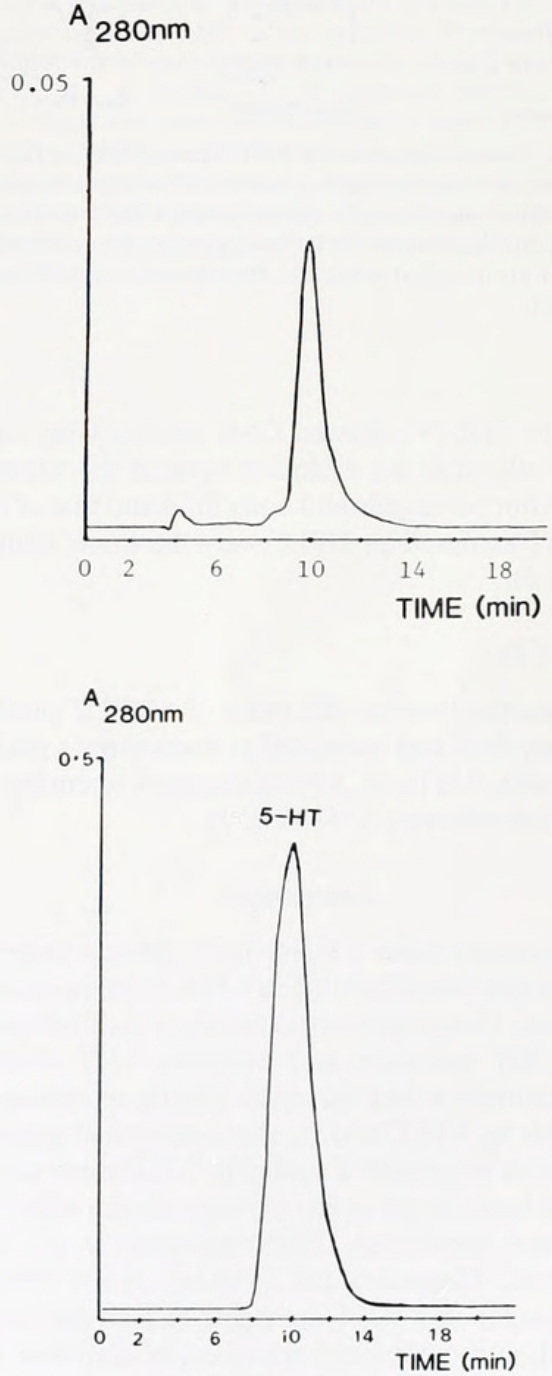

Figure 6. Reversed phase HPLC elution profile of the biologically active fraction (HPLC-FR-IV) of *Spisula* body fluid [A] and reference 5-HT [B]. Eluting condition was the same as described in Figure 5.

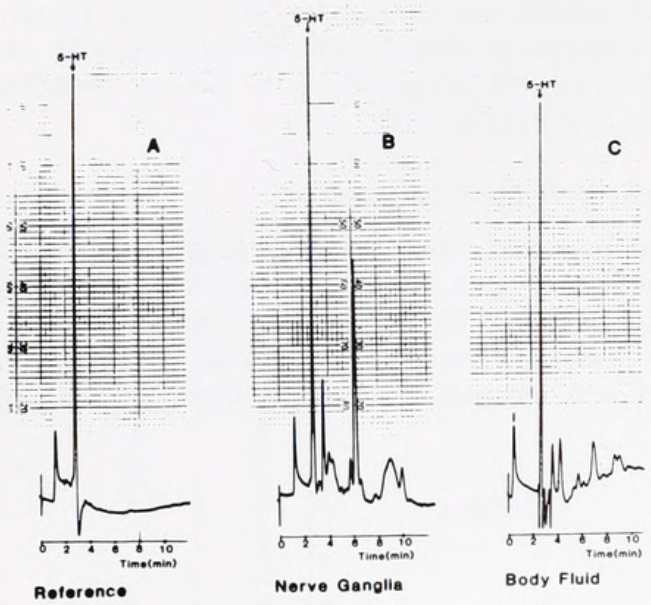

Figure 7. Isocratic separation of 5-HT utilizing HPLC with electrochemical detection. Representative elution profiles for (A) the reference 5-HT; (B) *Spisula* nerve ganglia extract (SNE); and (C) *Spisula* body fluid (SBF). The methods for HPLC and parameters for electrochemical detection are described in the text. Note detection of 5-HT peak in SNE and SBF.

fraction IV (FR-IV) showed OMI activity (Fig. 5B). It is noteworthy that the retention time of the substance isolated from nerve cells and body fluid and that of reference 5-HT analyzed by HPLC were the same, about 10 min (Fig. 6).

HPLC-ECD

With electrochemical detection, the HPLC profile of crude body fluid and nerve cell extracts show a peak coincident with 5-HT, *i.e.*, having the same retention time (2.5 min) as reference 5-HT (Fig. 7).

Discussion

In the present study a factor from *Spisula* body fluid and nerve cells was identified as 5-HT or a closely related compound. This conclusion is based on the findings that both the SBF substance and reference 5-HT which induced maturation had the same eluting retention time on analysis by HPLC and by electrochemical detection. The purified substance is probably 5-HT since the hormone has been found in the nervous system of bivalves (Welsh and Moorhead, 1960; Malanga *et al.*, 1972; Smith, 1982; Matsutani and Nomura, 1984). Both the SBF substance and 5-HT are stable to heat and trypsin treatment, and completely adsorbed by activated charcoal. They are not retained on dialysis in a tubing with a mol. wt. cut-off of less than 1000 daltons. Thus the SBF substance and 5-HT appear to be identical or closely related compounds. Also by HPLC-ECD, a peak coincident with reference 5-HT was found in the body fluid and ganglia extracts (Fig. 7). HPLC-ECD is used widely for the determination of 5-HT, its precursors, and metabolites in tissues (Trouvin *et al.*, 1987).

Maturation of *Spisula* oocytes can be induced by sperm on insemination, KCl, and other factors (Allen, 1953; Schuetz, 1975; Sato *et al.*, 1985; Toraya *et al.*, 1987). We emphasize that 5-HT injected into *Spisula* gonads induced spawning of sperm and oocytes (Hirai *et al.*, 1988). A majority of the spawned oocytes retained their germinal vesicles, suggesting that 5-HT may not induce maturation *in vivo*. However, we found recently that 8-hydroxy-2-(di-n-propylamino)-tetralin, a 5-HT_{1A} agonist (Middlemiss and Fozard, 1983), induced GVBD in *Spisula* oocytes (Kadam *et al.*, 1988). This suggests that *Spisula* oocytes possess 5-HT_{1A} receptor binding sites and the hormone may influence the metabolism of *Spisula* oocytes *in vivo*.

In the starfish, 1-methyladenine (1-MA) functionally resembles 5-HT (Kanatani, 1973). 1-MA triggers spawning and induces oocyte maturation in echinoderms. Both factors are neurotransmitters and have a profound effect on the gonads and oocytes. It is noteworthy that inducers of maturation are active on species-related oocytes. For example, progesterone induces maturation of amphibian oocytes (Masui and Clarke, 1979), 17α-hydroxy-20β-dihydroprogesterone with teleost oocytes (Suzuki *et al.*, 1981), and 1-MA with echinoderm oocytes (Kanatani, 1973). In *Chaetopterus*, an unidentified factor in natural seawater induces oocyte maturation (Ikegami *et al.*, 1976). Interestingly, oocyte maturation and spawning in *Spisula* and starfish are induced by small compounds. These substances are active only on species of the same family. The specific physiological inducers of spawning and oocyte maturation should be identified since this property may be a useful trait in the phylogenetic classification of species.

Acknowledgments

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