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PURIFICATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE PEPTIDES FROM THE SEACUCUMBER *HOLOTHURIA ATRA*

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Introduction

Peptides function in various cellular systems to organize the biological responses. Neuropeptides, hormones, and antimicrobial peptides are of particular importance for Eumetazoa. Better understanding of the structure-function relationships of these biologically active peptides is an absolute requisite to elucidate the molecular mechanisms of biosystems. A number of bioactive peptides have been isolated from the Vertebata, and their target molecules have been also identified in some cases. Those include, for example, the receptors, enzymes, and phospholipid biomembranes. Along with the structure-activity studies of these peptides, structurally similar peptides have been isolated from other animals of the Arthropoda and Mollusca and utilized as useful tools to elucidate the molecular interaction between peptides and acceptors.

Biologically active peptides in animals of the other phyla have been only poorly explored. The seacucumbers of the Echinodermata possess their unique body feature and are expected to contain various kinds of bioactive peptides. In the present study, the seacucumber *Holothuria atra* collected at the atoll area of Pohnpei, Micronesia, was provided as materials to make attempts to isolate the peptides which may function as neuropeptides. We here describe the purification and characterization of peptides from the body wall of the seacucumber *Holothuria atra*.

Materials and Methods

Purification—The whole body walls (7 kg) of the seacucumber *Holothuria atra* were cut into small fragments, which were frozen in liquid nitrogen to crumble. The pulverized material (1.6 kg) was boiled in water (6,500 ml) for 10 min. The mixture was crushed with a blender to form a mass of bubbles, and the solution was then homogenized with a Polytoron homogenizer. The resulting homogenate was centrifuged (15,000 g) for 40 min at 4℃. The supernatant was treated with activated charcoal at 100℃. The filtrate was evaporated at room temperature, and the concentrated solution (ca. 200 ml) was treated with 1 M HCl (20 ml). The precipitate (ca. 35 g) was removed by centrifugation and the brown supernatant (300 ml) was used for further purification.

The solution (150 ml) was applied to a column (3 × 25 cm) of silica-based C18 gel (Wakogel LP-60C18, Wako, Osaka). Fractionation was carried out stepwise with aqueous methanol, and the eluate with 60% MeOH was collected and evaporated (503 mg). The residue was further purified on a preparative reversed-phase HPLC column (Lichrosorb RP-18, 1×25 cm, Cica-Merck) and each peak emerged was collected and freeze-dried. For amino acid

analysis, hydrolysis was carried out in constant-boiling hydrochloric acid (110℃, 24 h). The analyses were carried out on a Hitachi model 835 amino acid analyzer.

Biological activity — Assays using guinea-pig ileum was performed essentially as reported previously (Matsumoto et al., 1991). Briefly, the strips of longitudinal muscle removed from the ileum of guinea-pig (350-450 g) were hung under 1 g resting tension in the organ bath (5 ml) filled with Krebs-Ringer hydrogen carbonate buffer (pH 7.4) (composition in mM: NaCl, 127; KCl, 2.5; $CaCl_2$, 1.8; $NaHCO_3$, 25; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; and glucose, 10) gassed with the 95% O_2/5% CO_2 mixture at 37℃. Prior to administration of peptides to be tested, the strips were allowed to stand for 1 hr to change the buffer every 15 min, and then stimulated several times by injecting 10 nM carbachol. The tension was recorded isotonically using an isometric force transducer (NEC San-Ei Instrument Co., Tokyo). The reference contractile activity was obtained by 10 μM carbachol at the end of experiment.

Results and Discussion

The fractionation of extract from body walls of the seacucumber *Holothuria atra* on C18 open column (Fig. 1) afforded the materials of mixtures, and the one eluted with 60% MeOH was found to contain peptides as indicated with the peptide reagent. The reversed-phase HPLC (Fig. 2) showed dozens of peaks and some of these fractions were shown to contain peptides. When the peptide fractions (fraction number **1-6**) were assayed for amino acid analysis, it was found that the size of peptides, namely the number of amino acids, was not so big (the average number = ca. 15). Although some of these peptides were negative in the ninhydrin test for N-terminus amino group, the examination to test whether their N-termini were blocked or they were in the cyclic forms was not carried out in the present study.

When the amino acid compositions of six major peptidic peaks were compared, it is clear that they are rich in glutamic acid and/or glutamine (Table 1). In particular, the content of these amino acids was calculated to be more than 40% for peaks **1**, **2** and **3**. This indicates

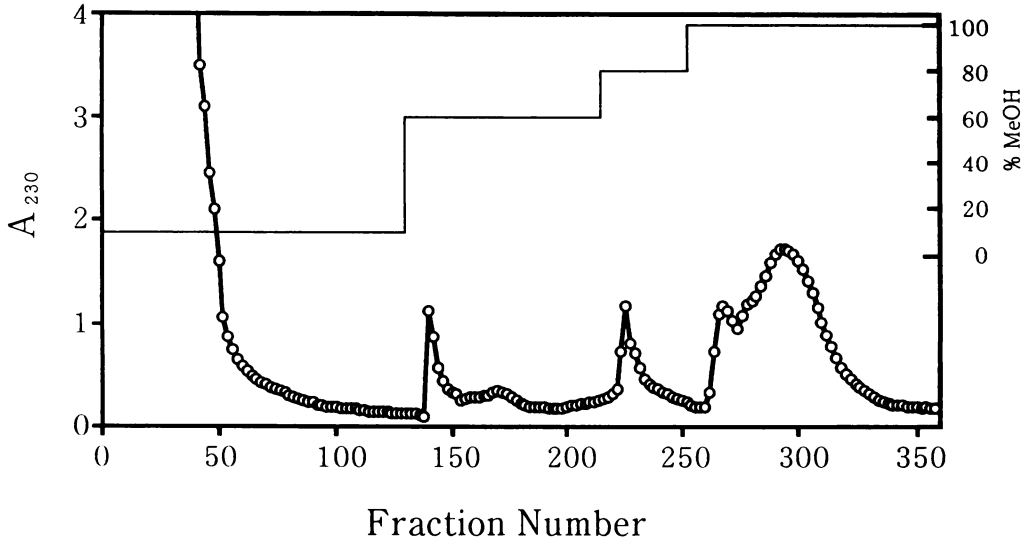

Fig. 1. Reversed-phase C18 silica-gel chromatography of the extract from body walls of the seacucumber *Holothuria atra*.

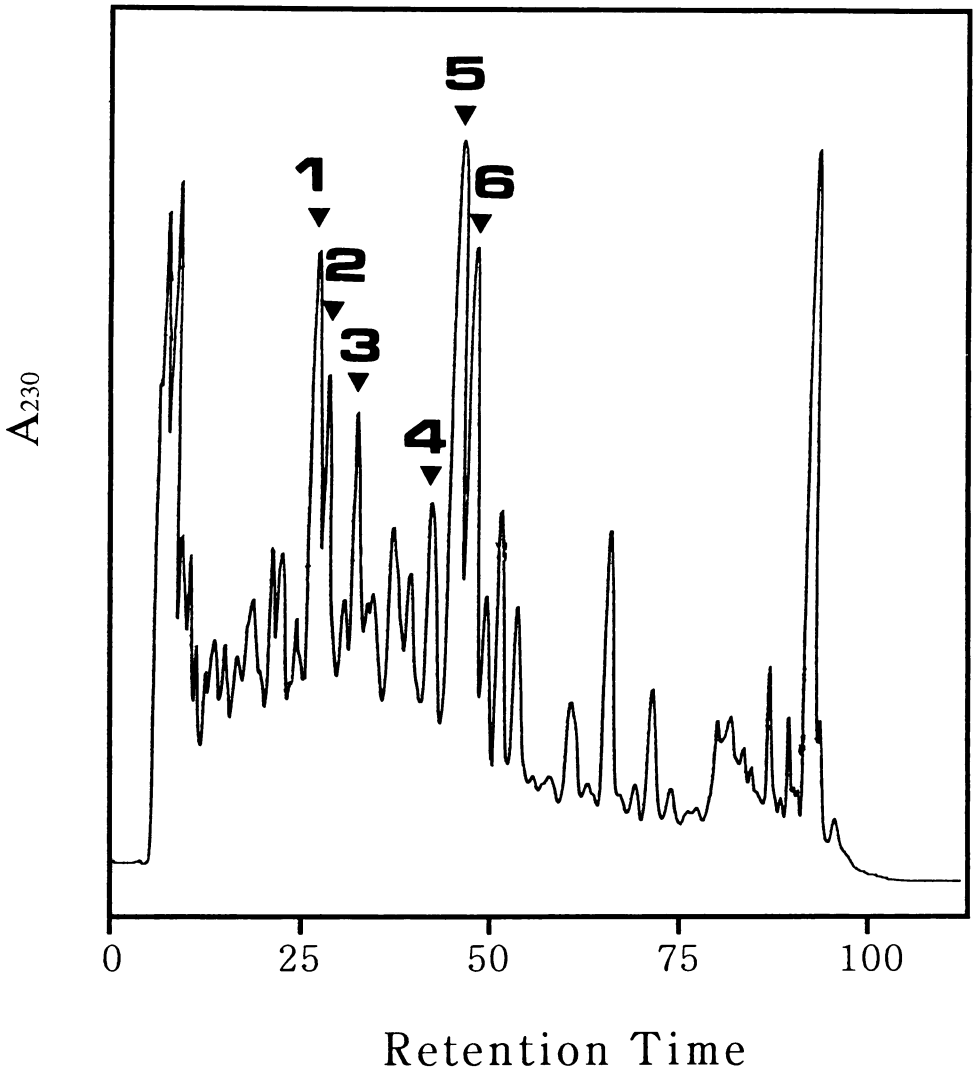

Fig. 2. Reversed-phase HPLC elution profile of the 60% MeOH eluate from C18 silica-gel chromatography.
Solvents were 0.1% trifluoroacetic acid (A) and acetonitrile containing 20% A solution (B). Elution was performed with a linear gradient of B solution from 45-70% for 60 min after initial elution of equilibrium for 5 min. Flow rate was 2.2 ml/min through the whole elution.

that the peptides contained in these peaks are probably very acidic. The constituents of other amino acids have no characteristic in common.

When the peptides isolated were examined for their ability to contract the guinea pig ileum, the only peptide from peak 4 exhibited a strong contraction (Fig. 3). This peptide might interact directly with the receptor which transmits the signal to contract the ileum or might mediate indirectly the signal by stimulating the release of some neurotransmitter. At this moment it is not clear whether this peptide is a neuropeptide or not.

Table 1. Amino acid compositions of the peptides isolated from the body walls of the seacucumber *Holothuria atra*.

Sample Number	1	2	3	4	5	6
Asx	2	2	2	1	2	1
Thr	1	1	1	1	1	1
Ser	2	2	2	2	2	2
Glx	10	12	8	4	6	2
Gly	3	3	3	2	3	2
Ala	1	1	1	1	1	1
Val	1	1	1	1	1	1
His	1	1	1	1	1	1
Total	21	23	19	13	17	11

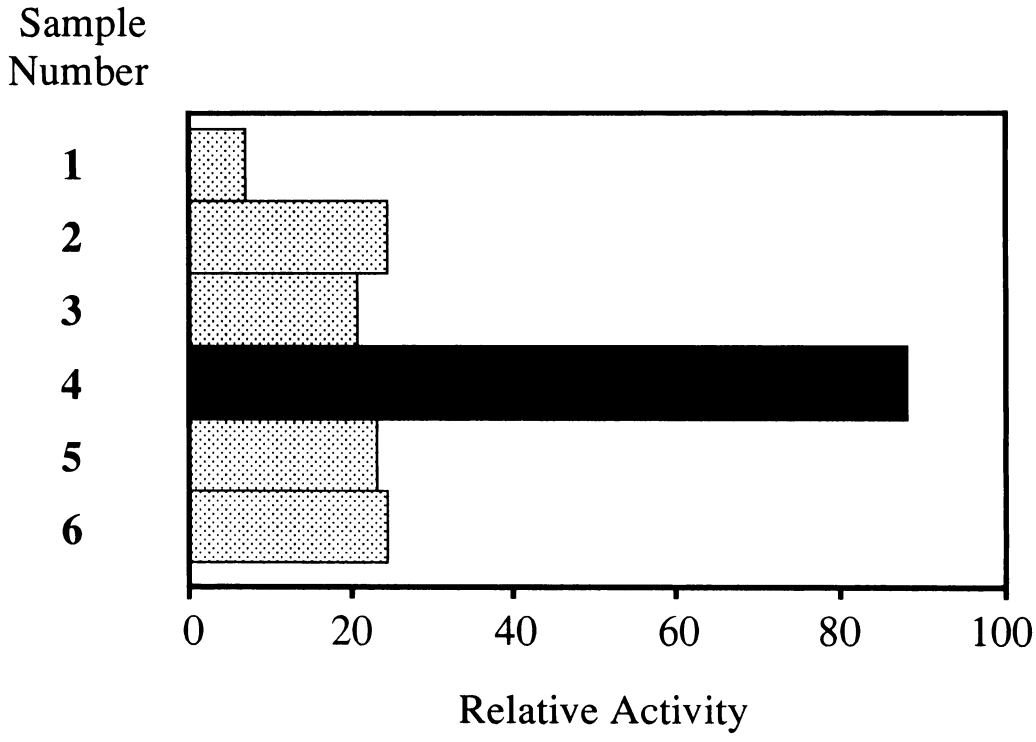

Fig. 3. Relative contractile activity of peptides isolated from the body walls of the seacucumber *Holothuria atra* in the guinea-pig ileum.

Since the peptides isolated in this study were assayed only for the contraction of guinea-pig ileum, it is a requisite to test them for many other pharmacological examinations. Especially the screening for receptor binding assays would clarify their potential affinity to a certain receptor and provide a useful ligand which competes with the ordinary ligand. Such studies are in progress in our laboratory.

References

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