Ph. D. Thesis

COMPARATIVE ASPECTS OF STRUCTURE AND ACTION
OF BIOACTIVE SUBSTANCES IN THE VENTRAL
NERVE CORD OF URECHIS UNICINCTUS

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CHAPTER 1

GENERAL INTRODUCTION

In the last two decades, a good many of neuropeptides were isolated from vertebrates and invertebrates and their structures were determined. Those peptides can be classified by numbers of groups, i.e. peptide families, according to their structures. In general, all the members of each family have an identical structure portion in their amino acid sequences which is important for their actions, while they diversify in the other structure portions which are less important. Thus, it is suspected that an ancestral neuropeptide has evolved into diverse members of a peptide family conserving an important portion and changing the other structures. To obtain further evidence for this notion, it is required to search for neuropeptides in various animal species. Because, it is believed that a great number of neuropeptides in both vertebrates and invertebrates still remain unidentified. Especially, a large part of the neuropeptides in invertebrates except molluscs and arthropods are not yet identified.

A search for neuropeptides in various animals is also necessary for physiological studies. If one attempts to clarify a neural mechanism of an animal, he is required to identify the neuropeptides involved in the mechanism. Because, each animal group is supposed to have a number of neuropeptides specific to the group, though most of them would be the members of known peptide families.

In contrast to neuropeptides, non-peptidic neurotransmitters, classical neurotransmitters such as biogenic amines and amino acids, are commonly utilized in the nervous systems of both vertebrates and invertebrates, and their pharmacological and

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biochemical natures have been well documented. However, it cannot be ruled out the possibility that there exist unidentified non-peptidic neurotransmitters in the nervous systems of animals, especially in those of invertebrates. Furthermore, physiological roles of the classical neurotransmitters and pharmacological properties of their receptors in invertebrates are not yet well clarified. That is, it is necessary to study the classical neurotransmitters in invertebrates from a comparative point of view.

In this study, I attempted to search for both peptidic and non-peptidic bioactive substances in the nervous system of an echiuroid worm, Urechis unicinctus, by using its inner circular body-wall muscle as the bioassay system (Fig. 1.1). Assay system is generally most important to search for unknown bioactive substances. Muscles have a sensitivity for many substances which play various roles as neurotransmitters not only at peripheral system but also in central system. Therefore, muscle is one of the most useful assay system for isolation of novel neuroactive substances.

Urechis is a member of the Echiura which is closely related to the Annelida. Both of the animal groups have similar closed circulatory systems, nephridia and digestive systems. Both of them utilize hemoglobin as an oxygen carrier. Echiuroids also seem to be related to molluscs, since the eggs of these animals, as well as annelids, hatch into trochophore larvae (Margulis and Schwartz, 1982). Annelids have internally segmented bodies, though echiuroids and molluscs do not have such a body structure. In this respect, annelids is also related to arthropods, and these animals are grouped as the Articulata. That is, echiuroids, annelids, molluscs and arthropods are more or less related to each other. Thus, it is interesting to study the structures and actions of bioactive substances of echiuroids and to compare them with those of molluscs, annelids and arthropods.

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Urechis unicinctus dwells in a U-shaped tube or a rock crevice of shallow sea bottom. Contraction and relaxation of body-wall muscle at alternating regions along the body are always observed in the animal. The structure of the body wall is basically identical with that observed in Urechis caupo by Lawry (1966a). The body-wall muscle consists of three layers, outer circular muscle, longitudinal muscle and inner circular muscle layers. A number of chemoreceptors are distributed on the surface of the body. The nerve cord is extended running on the inside of the ventral body-wall from the nerve loop around the proboscis to the anus. The peripheral nervous system consists of several hundred pairs of nerve branches which innervate the body wall. The ventral nerve cord can be easily excised, and therefore, the animal is suitable for neurotransmitter search which requires a large amount of isolated neural tissue.

Biogenic Amines

Occurrence of acetylcholine (ACh), choline acetyltransferase and acetylcholinesterase (AChE) are shown not only in vertebrates but also in invertebrates except coelenterates. Good evidence for ACh as a neurotransmitter in nervous system has been obtained from annelids, molluscs and arthropods (see review; Leake and Walker, 1980). The other biogenic amines, catecholamines, octopamine (OA) and serotonin (5-HT), are shown to be distributed throughout the animal kingdom. However, the levels of noradrenaline (NA) are generally lower than OA in protostomes including annelids (Juorio and Robertson, 1977; David and Coulon, 1985). Since OA resembles NA in biochemical and pharmacological properties (Atkinson et al., 1977; Kobayashi and Muneoka, 1980), it seems that noradrenergic system and octopaminergic system have been selected by deuterostomes and protostomes, respectively, in the processes of evolution of the animals.

In contrast to considerable physiological and pharmacologi-

cal studies on neurotransmitters in annelids, molluscs and ar-
thropods only a few work has been carried out on those in echiu-
roids. Lawry (1966b) has shown that Urechis caupo responds to
ACh by accelerated peristalsis and tonic contraction of the body
wall. Muneoka et al. (1981) and Muneoka and Kamura (1982a)
examined the pharmacological properties of mechanical responses
of the body-wall muscles of Urechis unicinctus, and suggested
that ACh may be an excitatory transmitter while NA may be an
inhibitory transmitter at the neuromuscular junctions in the body
wall. The possibility of occurrence of abundant NA in the nerv-
ous tissue (Muneoka and Kamura, 1982a) is very interesting from a
phylogenic point of view.

The pharmacological properties of the clude extract from the
ventral nerve cords of Urechis unicinctus are described in Chap-
ter 2. Simultaneous determination of catecholamines, OA and 5-HT
is described in Chapter 3. Determination of ACh is described in
Chapter 4.

Neuropeptides

The peptides isolated from molluscs and arthropods have a
large share in neuropeptides identified from invertebrates.

In 1977, Price and Greenberg first isolated the tetrapeptide
FMRFamide from the ganglia of the bivalve mollusc, Macrocallista
nimbosa. The peptide have a cardioexcitatory activity on the
animal. The FMRFamide-related tetrapeptide, FLRFamide, was first
isolated from the ganglia of the mesogastropoda, Pomacea paludosa
(Price, 1986), and a group of heptapeptides with the common
sequence XDPFLRFamide (X = pGlu, Gly, Asn or Ser) was isolated
from some pulmonate molluscs (Price et al., 1985, 1987a; Ebberink
et al., 1987). Two FMRFamide-related decapeptides were isolated
from the anterior byssus retractor muscle (ABRM) of the bivalve
Mytilus edulis and the ganglia of the prosobranch Fusinus ferru-
gineus (Kanda et al., 1990; Fujisawa et al., 1992). The tetra-

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peptides, FMRFamide and FLRFamide, were shown to be present in all the classes of molluscs (Price, Davies et al., 1987). Furthermore, FMRFamide-related peptides were found in the nervous systems of animals of other phyla. In the immunohistochemical studies, many immunoreactive FMRFamide-like substances were observed in other phyla (Boer et al., 1980; Dockray et al., 1981; Grimmelikhuijzen et al., 1982; Watson et al., 1984; Kuhlman et al., 1985; Kubben et al., 1986). By using radioimmunoassay (RIA) or bioassay method, FMRFamide-related peptides were isolated from annelids (Krajniak and Price, 1990; Baratte et al., 1990), arthropods (Nachman et al., 1986a, 1986b; Trimmer et al., 1987; Robb et al., 1989) and a nematode (Cowden, et al., 1989). Furthermore, some neuropeptides isolated from coelenterates (Grimmelikhuijzen and Graff, 1986; Graff and Grimmelikhuijzen, 1988a, 1988b; Grimmelikhuijzen et al., 1988; Grimmelikhuijzen et al., 1990) and echinoderms (Elphick, Price et al., 1991; Elphick, Reeve et al., 1991) by using RIA were shown to be FMRFamide like, though these peptides are considered not to be the members of FMRFamide family.

Several other molluscan neuropeptide families have been reported (Kobayashi and Muneoka, 1990). These families include small cardioactive peptide (SCP) family (Morris et al., 1982; Lloyd et al., 1987; Price et al., 1990) , bag-cell peptide (BCP) family (Scheller et al., 1983; Nagle et al., 1989), buccalin family (Cropper et al., 1988; Miller et al., 1989), myomodulin-CARP family (Cropper et al., 1987, 1991; Hirata et al, 1987) and Mytilus inhibitory peptide (MIP) family (Hirata et al., 1988; Ikeda et al., 1991; Fujisawa, Kubota, Ikeda et al., 1991; Ikeda, Yasuda-Kamatani et al., 1992).

The red pigment concentrating hormone (RPCH), pQLNFSPGW-amide, was isolated from eyestalks of the crustacea, Pandulus borealus (Fernlund and Josefsson, 1972), and the peptide was shown to regulate color change of the animal by acting on epider-

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mal chromatophores. The peptide has a structure related to adipokinetic hormone (AKH). AKH, pQLNFTPNWGTamide, was isolated from the corpora cardiaca of the two kinds of locusts, Locusta migratoria and Schistocerca gregaria, by measuring its lipid-mobilising activity when injected into the adult male locusts (Stone and Mordue, 1976). In the past decade, about 10 AKH-related peptides were isolated from various insects (Penzlin, 1989, Gade and Rosinski, 1990). They regulate lipid mobilization, hyperglycemia and myotrophic function. All the RPCH-AKH family peptides have $pGlu^1$, Phe^4, Trp^8 and C-terminal amide, and they consist of 8-10 amino acid residues.

Holman and his colleagues isolated various neuropeptides from head extracts of the cockroach, Leucophaea maderae, by using the hind gut as the bioassay system. The peptides are leucokinin I - VIII (Holman et al., 1986a, 1986b, 1987a, 1987b), leucopyrokinin (Holman et al., 1986c), leucomypsuppressin (Holman et al 1986d) and leucosulfakinin I and II (Nachman et al., 1986a, 1986b). Furthermore, three neuropeptides, locustamyotropin and locustatachykinin I and II, were isolated from the nervous system of Locusta migratoria by using the same bioassay system (Schoofs, Holman, Hayes, Tips et al., 1990; Schoofs, Holman, Hayes, Nachman et al.,1990). Leucosulfakinins have -MRFaimde structure which is also observed in the molluscan neuropeptide FMRFamide. Leucopyrokinin and locustamyotropin have -FXPRLamide at their C-terminal portions. The C-terminal sequence, -FXPRLamide, of these peptides is identical to the C-terminal pentapeptide fragments of the pheromone biosynthesis activating neuropeptides, PBANs, which have been isolated from the moth, Heliothis zea (Raina et al., 1989), and the silkworm, Bombyx mori (Kitamura et al., 1989, 1990). The C-terminal fragment -PRLamide also resembles the C-terminal sequence, -PRMamide, of the molluscan neuropeptides, SCPs.

In contrast to numbers of reports on the neuropeptides of

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molluscs and arthropods, there is no report on neuropeptides of echiuroid worms. In annelids which are closely related to echiuroids, occurrence of substances immunoreactive to enkephalin, β-endorphin, substance P, vasopressin, oxytocin and FMRFamide (Alumets et al., 1979; Kaloustian and Edmands, 1986; Kinoshita and Kawashima, 1986; Kuhlman et al., 1985), has been reported. Recently, authentic FMRFamide was isolated from a polychaeta, Nereis virens (Price et al., 1990). However, other species of annelid neuropeptides are not yet identified.

In this study, I isolated almost 30 species of neuropeptides from the ventral nerve cords of Urechis unicinctus. The isolation procedures, structures and activities of the neuropeptides of Urechis unicinctus are described in Chapter 4, 5 and 6.
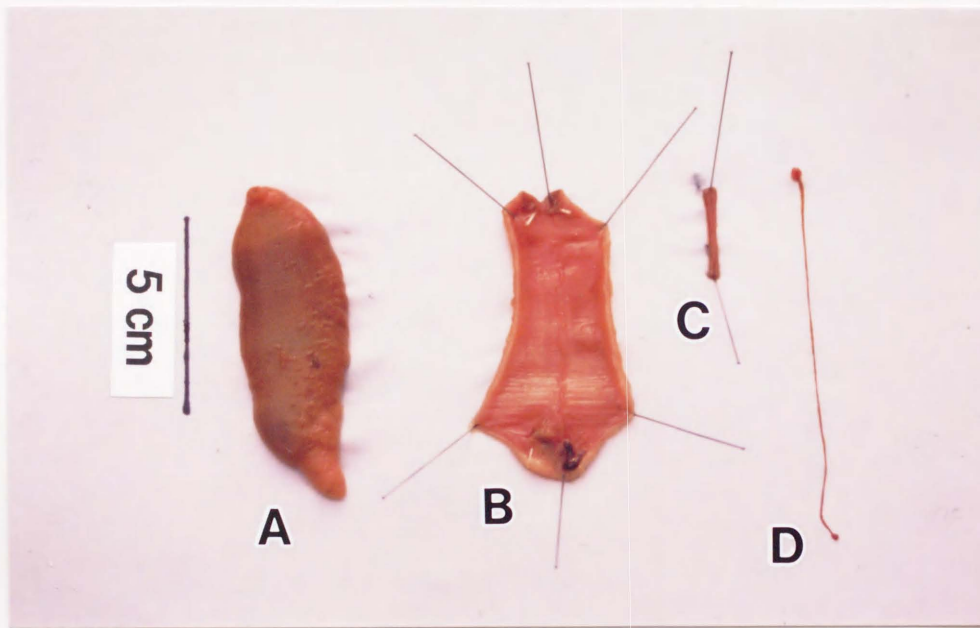
Fig. 1.1.   A: intact specimen of <u>Urechis unicinctus</u>.   B:ventral
nerve cord on the opened body wall.   C: strip of the inner circu-
lar body-wall muscle.   D: isolated ventral nerve cord.   The
muscle strip was used for bioassay of the nerve-cord extract.

CHAPTER 2

BIOACTIVITIES OF THE CLUDE EXTRACT

2-1 Introduction

Echiuroids are known to be one of the annelid-related phyla. There is an impressive body of evidence in favor of ACh as an excitatory transmitter in the body wall of annelids (Gardner and Waker, 1982). Therefore, it is reasonable to suppose that ACh is also an excitatory transmitter in the body wall of Urechis. On the other hand, it is very interesting that NA, not OA, may be a transmitter which inhibits contraction of the body-wall muscles of Urechis (Muneoka and Kamura, 1982a). Because, in protostome invertebrates including annelids, the levels of OA are generally higher than those of NA, and OA has been regarded as a more likely phenylethanolamine-type neurotransmitter than NA (Robertson, 1975; Robertson and Juorio, 1976; Juorio and Robertson, 1977; Webb and Orchard, 1980; Gardner and Waker, 1982).

In the present experiments, I examined the effects of the extract of the ventral nerve cords of Urechis on the body-wall muscle of the worm and on some molluscan muscles. The actions of the bioactive substances in the extract were pharmacologically characterized. The results suggested that several biogenic amines including NA were contained in the extract.

2-2 Materials and Methods

2-2-1 Animals

The following animals were used in the experiments: Urechis

unicinctus (Echiura), Mytilus edulis (Mollusca), and Meretrix
lusoria (Mollusca). Urechis and Meretrix were purchased from a
commercial source, and Mytilus were collected from Hiroshima Bay.
For the excision of the ventral nerve cords, Urechis were kept in
a cold box (4°C) and used within 6 hr of purchasing. For the
bioassay, the inner circular body-wall muscle of Urechis, the
anterior byssus retractor muscle (ABRM) of Mytilus and the heart
of Meretrix were used. These animals were stored in aerated
artificial seawater (ASW) at temperatures between 15 and 23°C and
used for experimentation within 5 days of purchasing or collec-
tion.

2-2-2 Fractionation of Nerve-cord Extract

The ventral nerve cords of Urechis were excised from 350
specimens and immediately frozen on dry ice. The frozen nerve
cords (total 15g) were immersed in 4% acetic acid (tissue concen-
tration of 0.25 g/ml), homogenized with a Polytoron on ice, and
centrifuged at 28,000g for 40 min at 4°C. The supernatant was
applied to five C-18 cartridges (Sep-Pak, Waters), in series.
The retained material in the cartridges was eluted with 15ml of
methanol, and thus the flowthrough and the retained material were
collected. One seventh of the flowthrough was lyophilized and
used for the examinations of its biological activities on the
inner circular body-wall muscle of Urechis and the ABRM of Myti-
lus. The remaining flowthrough was evaporated to dryness and
used for gel filtration. The eluate containing the retained
material was also evaporated and used for the examinations of its
biological activities on the muscles. By the same procedures I
obtained retained material from the nerve cords (71g) of 1,500
animals in order to subject to gel filtration. These procedures
of fractionation of the nerve cord extract are summarized in Fig
2.1.

The gel filtration was carried out by using a column (2.6 X

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40 cm) of Sephadex G-15. The dried flowthrough and retained material were taken up in 0.1 M acetic acid (0.5 ml), applied to the column and eluted with the same solvent, respectively. Fractions of 4 ml each (60 drops) were collected.

2-2-3 Bioassay

Small muscle bundles of the inner circular body-wall muscle of Urechis and the ABRM of Mytilus were dissected and mounted in an experimental chamber (2 ml), and tension changes in the muscles were recorded.

The methods of dissecting, stimulating and tension recording from the body-wall muscle were basically the same as those of Muneoka and Kamura (1982a). The specimen of Urechis was opened by cutting the body wall from the dorsal side and the visceral organs and the ventral nerve cord were removed. The preparation of the inner circular muscle was made by isolating the muscle layer from the lateral body-wall strips which were dissected by cutting the middle part of the body wall crosswise. The isolated muscle bundles were about 1.5 mm in diameter and 20 mm in length. One end of the strip was tied with a cotton thread to the experimental chamber and the other end was connected by a cotton thread to a force displacement transducer. Twitch contraction was elicited by applying an electrical pulse (20 V, 3 msec) of stimulation to the muscle at 10 min intervals. Each bioassay material or drug was applied to the bundle 8 min prior to the electrical stimulation. The methods used for the ABRM of Mytilus and the heart preparations of Meretrix were the same as those of Muneoka and Twarog (1977) and Welsh and Taub (1948), respectively. Their mechanical activities were recorded on a pen recorder through a force transducer and an amplifier.

An appropriate amount of each fraction was lyophylized and diluted in ASW. Stock solutions of most drugs (10^{-2} or 10^{-3} M in distilled water) were also diluted in ASW. Choline was directly

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dissolved in ASW. In the experiments of the body-wall muscle and the ABRM, the extract and drugs were applied by replacing the bath solution with ASW containing them. In the experiments of the clam heart, they were applied by injecting stock solutions into an aerated organ bath (10 ml).

The concentration of the extract was indicated as "unit": 1 unit corresponds to one-nerve-cord extract/1000 ml ASW. The experiments were carried out at room temperature (17-23°C).

2-2-4 Enzyme Treatments

An appropriate amount of stock solution of the peptidase subtilisin was added to the thawed fraction solution and pH was adjusted to 7.8 by Tris-HCl. The final concentration of the peptidase was 10^{-4}g/ml. Incubation was carried out at 36°C for 60 min. The peptidase was inactivated by boiling at 100°C for 30 min at the end of the incubation. The control fractions were only boiled. Incubation with aminopeptidase M was carried out by the same procedures. Incubation with AChE was carried out at 30°C for 30 min. The other procedures were the same as in the case of subtilisin.

2-2-5 Physiological Saline and Drugs

The physiological saline was ASW of the following composition: 445 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$ and 10 mM Tris-HCl; pH 7.8.

The following drugs were purchased from Sigma; acetylcholine bromide, choline chloride, dopamine hydrochloride, L-noradrenaline bitartrate, L-adrenaline bitartrate, DL-octopamine hydrochloride, propantheline bromide, eserine hemisulfate and mersalyl acid. Subtilisin, aminopeptidase M and AChE were purchased from Boehringer Mannheim. Phentolamine mesylate and butaclamol hydrochloride were purchased from Ciba and Research Biochemicals Inc., respectively. Benzoquinonium chloride was gifted from Sterling-

Winthrop Research Institute.

2-3 RESULTS

2-3-1 Effects of Biogenic Amines

The actions of biogenic amines on the isolated inner circular body-wall muscle of Urechis have not yet been systematically studied, though those on the lateral body-wall strip (strip simply dissected out by cutting the body wall crosswise) have been examined to some extent (Muneoka and Kamura, 1982a). In the present experiments, therefore, we examined the actions of several biogenic amines on the isolated muscle.

ACh induced a contraction of the muscle at 10^{-5} M or higher. Dopamine (DA) markedly potentiated twitch contraction of the muscle (Fig. 2.2A). The threshold concentration was about 10^{-9} M. At 10^{-6} M, DA increased peak tension of twitch contraction by 100% or more. In the case of the body-wall strip, such action of DA was not observed (Muneoka and Kamura, 1982a). NA dose-dependently inhibited twitch contraction at $10^{-8}-10^{-6}$ M, but at 10^{-5} M or higher it markedly potentiated the contraction (Fig. 2.2B). Adrenarine (AD) showed qualitatively similar actions with NA, though the former was slightly less potent than the latter. OA showed twitch-potentiating action at lower concentrations ($10^{-11}-10^{-7}$ M), but at higher concentrations (higher than 10^{-6} M) it showed twitch-inhibiting action(Fig. 2.2C). The maximum potentiating effect of OA was observed at 10^{-8} M. Although the threshold concentration of OA for the potentiation is very low, its maximum potentiating effect is not so marked as that of DA. 5-HT dose-dependently potentiated twitch contraction and increased the rate of relaxation of the contraction. The threshold concentration for the potentiation was about 10^{-9} M. These effects of 5-HT on the isolated muscle were almost identical with those on the body-wall strip (Muneoka and Kamura, 1982a).

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Potentiating action of DA was blocked by the α-blocker phentolamine (10^{-5} M) but that of 5-HT was not affected. Both inhibitory action of 10^{-7} M NA and potentiating action of 10^{-5} M NA were also blocked by phentolamine. Potentiating action of 10^{-8} M OA was not affected by phentolamine whereas inhibitory action of 10^{-5} M OA was blocked, and hence, in the presence of the blocker, 10^{-5} M OA did not show inhibitory action but showed potentiating action on twitch contraction of the body-wall muscle.

From these findings, it is supposed that the muscle has at least five classes of receptors for the biogenic amines; one is contractile receptors for ACh, another is inhibitory receptors for NA, and the other three are contraction-potentiating receptors for DA, OA and 5-HT, respectively. The inhibitory receptor for NA and the potentiating receptor for DA seem to have α-adrenoceptor-like pharmacological natures. NA at high concentrations (10^{-5} M or higher) might be able to activate the potentiating receptors for DA as an agonist. OA at high concentrations (10^{-5} M or higher) might be able to activate the inhibitory receptors for NA.

2-3-2 Bioactivities of The Flowthrough and The Retained Material

The flowthrough of the nerve-cord extract potentiated twitch contraction of the body-wall muscle (Fig. 2.3A). The threshold concentration for the potentiation was 1-3 units. After the flowthrough-containing ASW had been kept for 2hr or more at room temperature (20°C), it did not show twitch-potentiating activity but showed twitch-inhibiting activity (Fig. 2.3B). The flowthrough seems to contain at least two bioactive substances, potentiating and inhibitory substances. The former is considered to be quite unstable. This was also suggested by the fact that the potentiating activity was eliminated by boiling the flowthrough for 10 min at pH 7.8 (Fig. 2.4A). It is supposed that

the unstable potentiating substance is DA. The inhibitory activity was not destroyed by either boiling or treating the material with subtilisin (Fig. 2.4B).

The flowthrough also showed potentiating action on phasic contraction of the ABRM of Mytilus in response to repetitive electrical stimulation (Fig. 2.5A) and relaxing action on catch tension of the muscle (Fig. 2.5B). The potentiating action was nearly, but not completely, blocked by phentolamine (Fig. 2.6), suggesting that the flowthrough also contains a phenylethanol-amine-type substance such as NA.

The catch-relaxing action of the flowthrough was slightly depressed by mersalyl in most of the ABRMs examined (Fig. 2.7A), but in some other muscles the action was not affected by the mercurial (Fig. 2.7B). It has been shown that mersalyl powerfully blocks relaxing action of 5-HT and its analogues, such as tryptamine, on the ABRM but does not block those of catecholamines (Twalog et al., 1977; Muneoka et al., 1978). Therefore, it is suspected that the amount of 5-HT-like substance in the flowthrough is little, if any.

Relaxation of catch of the ABRM in response to the flowthrough was found to be depressed by 10^{-5} M butaclamol (Fig. 2.8A). It has been reported that relaxation of catch by DA is antagonized with butaclamol (Murakami et al., 1983), and hence submaximal DA-relaxation is completely blocked by 10^{-5} M butaclamol (Hirata et al., 1989b). In the present experiments, it was observed that relaxation of catch by NA was markedly depressed by 10-5 M butaclamol (Fig. 2.8B), but that by OA was not affected (Fig. 2.8C). These facts are consistent with that the flowthrough contains catecholamine-like substances such as DA and NA.

The material retained by C-18 cartridges showed contractile activity on the body-wall muscle at concentrations of 70 units or more. The activity was not affected by boiling the material but was destroyed by treating it with subtilisin (Fig. 2.9). The

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material seems to contain peptidic substances that have an excitatory action on the body-wall muscle. The material was also tested on the ABRM but any contractile activity was not observed even at 1,000 units.

2-3-3 Activities of Gel-filtrated Fractions of The Flowthrough

The flowthrough was evaporated and the residue was applied to the gel-filtration column. The biological activities of the filtrated fractions were examined on twitch contraction of the body-wall muscle. Three peaks (I_1, I_2 and I_3) of twitch-inhibiting activity were obtained, but twitch-potentiating activity was not observed in all of the fractions. The potentiating substance, which was considered to be unstable DA-like one, may be decomposed during evaporation. The maximal activity of peak I_1 was found at fraction 33 (Fig. 2.10). The maximal activities of peak I_2 and I_3 were found at fraction 57 and 75-77, respectively (Fig. 2.11).

The inhibitory activity of peak I_1 was not destroyed by boiling the peak substance. It was also not destroyed by treating the peak substance with subtilisin or aminopeptidase M (Fig. 2.12). The inhibitory activities of peak I_2 and I_3 were destroyed by boiling them for 60 min at pH 7.8.

The inhibitory action of peak I_1 was not antagonized with phentolamine (Fig. 2.13A). On the contrary, the actions of peak I_2 and I_3 were antagonized by the α-blocker (Fig. 13B and C). The inhibitory action of peak I_3 was recovered after washing out phentolamine (Fig. 2.13C) but that of peak I_2 was not. Further, after washing out phentolamine, twitch contraction of the muscle in the presence of peak I_2 was often followed by an irregular response (Fig. 2.13B).

Peak I_1 did not relax catch tension of the ABRM of Mytilus (Fig. 2.14A), but peak I_2 and I_3 relaxed it (Fig. 2.14B, C)

From the foregoing findings, it is suspected that the bioac-

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tive substances in peak I_2 and I_3 are NA-like ones. If this is the case, the substances would potentiate phasic contraction of the ABRM in response to repetitive electrical pulses of stimulation (Muneoka and Kamura, 1982b). As shown in Fig. 2.15, both of the peaks were found to potentiate the phasic contraction.

The substance in peak I_1 showed contractile action on the ABRM. The contraction was markedly enhanced in the presence of phentolamine (Fig. 2.16A). The potentiating actions of peak I_2 and I_3 on phasic contraction of ABRM were antagonized by phentolamine (Fig. 2.16B, C), again suggesting that the substances in the peaks are NA-like ones. After washing out phentolamine, the potentiating action of peak I_2 was not recovered, whereas that of peak I_3 was recovered.

It has been reported that phentolamine potentiates ACh contraction in the ABRM (Twarog, 1959). Therefore, the bioactive substance in peak I_1 might be ACh-like one. This notion was supported by the facts that peak I_1 inhibited cardiac activity of the clam Meretrix and the inhibitory action was completely blocked by the ACh-antagonist benzoquinonium (Fig. 2.17). Furthermore, the contractile action of peak I_1 on the ABRM, as well as that of the ACh, was depressed by the ACh-antagonist propantheline (Fig. 2.18A).

The above findings suggest that peak I_1 contains ACh-like substance. However, this substance seems not to be ACh. This is because of that ACh does not show any inhibitory action but shows an excitatory action on the body-wall muscle (Muneoka and Kamura, 1982a). The following findings also support the above notion. As shown in Fig. 2.18B, contraction of the ABRM in response to ACh was enhanced by the AChE inhibitor eserine (Twarog, 1954). After washing out eserine, further enhancement of the contraction was observed. In contrast, contraction in response to peak I_1 substance was not affected by eserine. Contractile action of ACh on the ABRM was completely destroyed by incubating the amine with

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AChE, whereas that of peak I_1 was not affected by AChE treatment (Fig. 2.19).

The bioactive substance in peak I_1 would be choline which is hydrolyzed product of ACh. However, contractile effect of choline on the ABRM was found to be vary weak even at high concentrations such as 10^{-2} M (Fig. 2.20A). Further, at concentrations 3×10^{-5} M or less choline showed little or no inhibitory action on twitch contraction of the body-wall muscle, and at concentrations 10^{-4} M or more it showed a contractile action (Fig. 2.20B), whereas peak I_1 showed dose-dependent inhibitory action on the twitch contraction, and hence, at high concentrations it markedly inhibited the contraction (Fig. 2.20C). That is, the bioactive substance in peak I_1 seems also not to be choline.

2-3-4 Activities of Gel-filtrated Fractions of The Retained Material

The retained material obtained from the second batch was evaporated and applied to the gel-filtration column. The biological activities of the fractions were examined on the isolated inner circular body-wall muscle. The tested concentration of each fraction was 3,000 units. As shown in Fig. 2.21, fractions 24-40 showed contractile activity on the muscle. The other fractions which did not show contractile activity were tested on twitch contraction of the body-wall muscle. In these experiments, I found two twitch-contraction-modulating peaks; one was of inhibition and the other was of potentiation. The maximal activities of the inhibitory and potentiating peaks were found at fractions 51 and 57, respectively (Fig. 2.22), though the two peaks seemed to be eluted partially overlapping with each other.

2-4 Discussion

2-4-1 Bioactive Substances in The Flowthrough

The results obtained in the present experiments suggest that the ventral nerve cord of <u>Urechis</u> <u>unicinctus</u> has at least four bioactive amine-like substances, one ACh-like and three catecholamine-like substances. I could not suggest the presence of 5-HT and OA in the nerve cord. However, by using an ion-pair liquid chromatography on a reversed-phase column coupled with an electrochemical detector, I detected several biogenic amines including 5-HT and OA in the nerve cord extract (see Chapter 3). Thus, it is supposed that, I stopped fractionation before eluting 5-HT and OA during the gel-filtration.

The ACh-like substance in peak I_1 is of quite interest. Its actions on the ABRM of <u>Mytilus</u> and the heart of <u>Meretrix</u> are similar to those of ACh. The ACh blockers antagonize the actions on these muscles. I have observed that authentic ACh has an elution volume being almost the same as that of peak I_1 on the gel filtration through Sephadex G-15. Although these findings suggest that the bioactive substance in peak I_1 is ACh, the following facts do not support it: peak I_1 shows an inhibitory action on the body-wall muscle, while ACh shows an excitatory action; treatment of the ABRM with eserine does not result in any potentiation of contractile response of the muscle to peak I_1; treatment of peak I_1 fraction with AChE does not affect its contractile action on the ABRM. Further, the substance seems not to be choline. This is because of that choline does not show any inhibitory action on twitch contraction of the body-wall muscle. At high concentrations, choline as well as ACh shows contractile action on the muscle. In contrast, peak I_1 inhibits twitch contraction of the muscle in a dose-dependent manner, and hence, at high concentrations, it shows marked inhibitory action. From these results, the substance in peak I_1 is suspected not to be ACh but to be a new cholinergic substance or a mixture of ACh and other bioactive substance(s). In Chapter 4, I show that the substance in peak I_1 is a mixture of a bioactive peptide and ACh.

One of the three catecholamine-like substances in the flow-through is quite unstable. This substance markedly potentiates twitch contraction of the body-wall muscle. These facts suggest that it is DA.

The other two substances (substances in peak I_2 and I_3) show NA-like pharmacological properties. They inhibit twitch contraction of the body-wall muscle and potentiate phasic contraction of the ABRM. These actions are antagonized by the α-blocker phentolamine. They relax catch tension in the ABRM. These findings suggest that the substances may be NA and AD. However, unlike the actions of NA and AD, those of peak I_2 on the body-wall muscle and the ABRM are not recovered after washing out the antagonist phentolamine. Therefore, it cannot be ruled out the possibility that the peak I_2 substance is neither NA nor AD. It might be another phenylethanolamine-type catecholamine.

2-4-2 Bioactive Substances in The Retained Materials

From the results in the present experiments, it is probable that the retained material contains at least three bioactive substances. One of them has contractile activity on the body-wall muscle and the other two substances have contraction-inhibiting activity and contraction-potentiating activity on the muscle, respectively. All of them might be novel peptidic substances. As shown in Chapter 5, I isolated five novel neuropeptides from the retained material in the aqueous acetic acid extract of the ventral nerve cords of the animal.
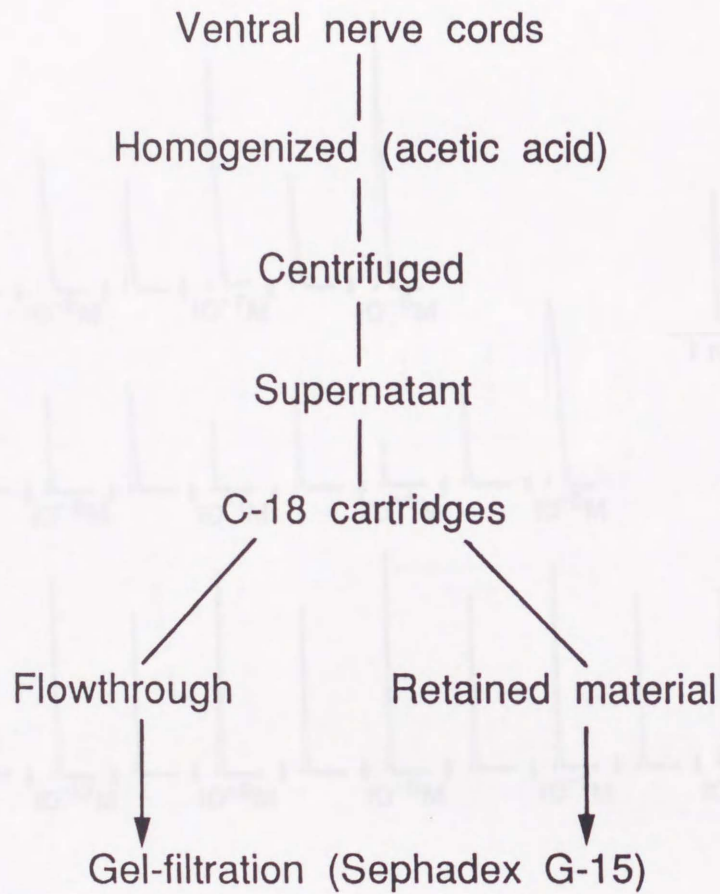
Ventral nerve cords

|

Homogenized (acetic acid)

|

Centrifuged

|

Supernatant

|

C-18 cartridges

Flowthrough   Retained material

Gel-filtration (Sephadex G-15)

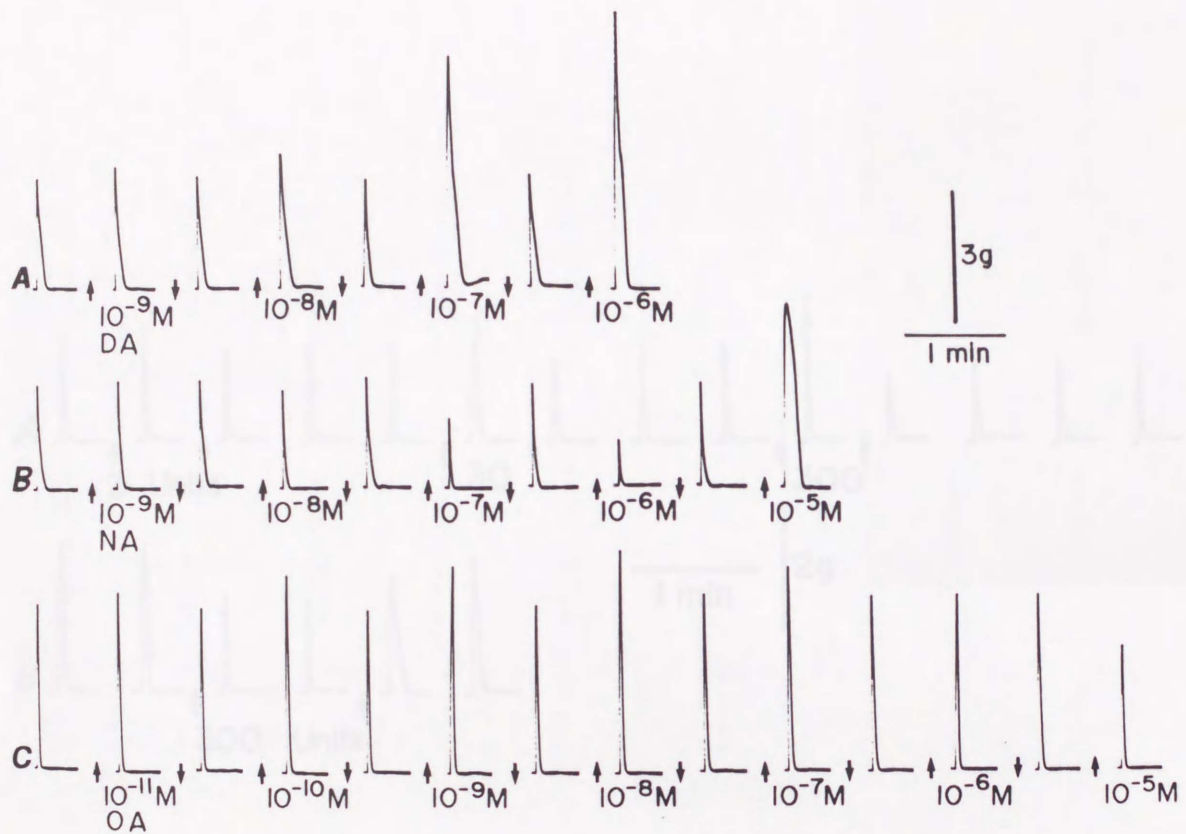Fig. 2.1.   The procedures of fractionation of the nerve-cord ex-
tract.

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Fig. 2.2. Effects of some biogenic amines on twitch contraction of the body-wall muscle of Urechis. A: effect of dopamine (DA). B: effect of noradrenaline (NA). C: effect of octopamine (OA). Twitch contraction was evoked by applying an electrical pulse (15 V, 3msec) of stimulation to the muscle at 10 min intervals. Each dose of biogenic amines was introduced 8 min prior to evoking the contraction in it and washed out soon after recording the contraction.
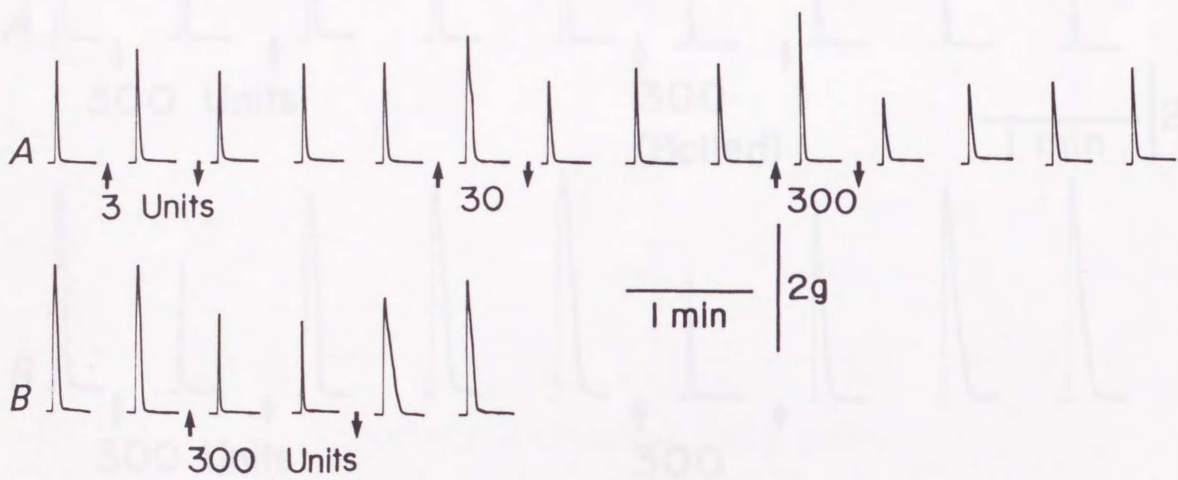
Fig. 2.3.   Activities of the flowthrough on twitch contraction
of the body-wall muscle of Urechis.   A: potentiating activity of
the normal flowthrough.   B: inhibitory activity of the flow-
through kept for 2 hr at room temperature (20°C) at pH 7.8.   The
other procedures are the same as in Fig. 1.1.

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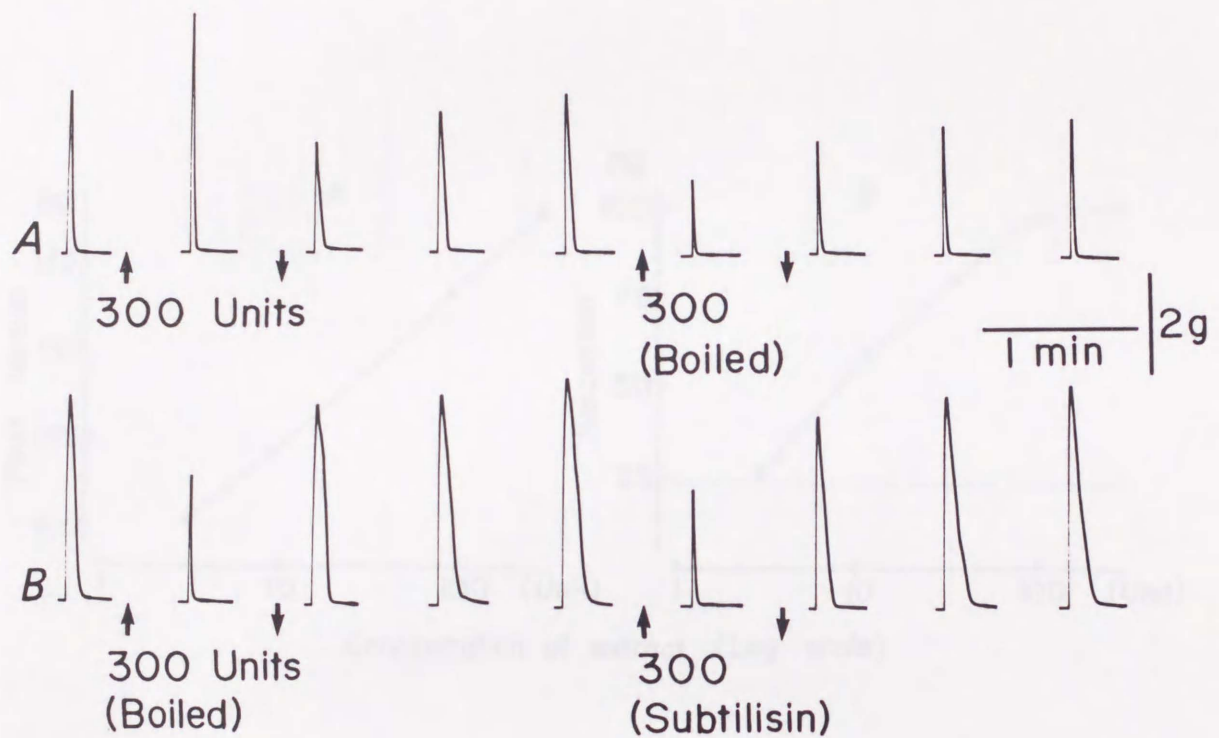
Fig. 2.4. Effects of boiling and subtilisin-treatment of the flowthrough on its activities on twitch contraction of the body-wall muscle of Urechis. A: effect of boiling. After confirmed the potentiating activity of the normal flowthrough (the second contraction), the activity of the flowthrough boiled for 10 min at pH 7.8 was examined (the sixth contraction). B: effect of subtilisin treatment. After confirmed the inhibitory activity of the flowthrough boiled for 30 min at pH 7.8 (the second contraction), the activity of the subtilisin-treated flowthrough was examined (the sixth contraction). The other procedures are the same in Fig. 2.1.

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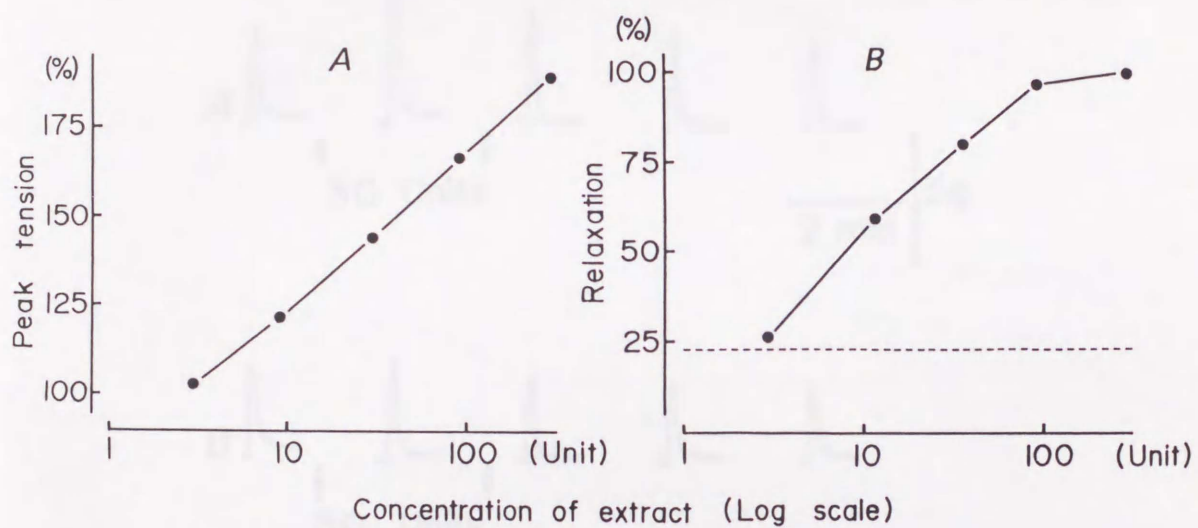
Fig. 2.5. Activities of the flowthrough on the ABRM of <u>Mytilus</u>. A: potentiating activity on the peak tension of phasic contraction of the ABRM. The contraction was evoked by applying repetitive electrical pulses (15 V, 3 msec, 10 Hz, for 5 sec) of stimulation at 10 min intervals. Each peak tension was shown as a percentage of the control peak tension. B: relaxing activity on catch tension of the ABRM. Catch tension was induced by applying 10^{-4} M ACh for 2 min at 20 min intervals. Five minutes after washing out ACh, the flowthrough was applied for another 5 min. Relaxation was expressed as a percentage: relaxed tension during 5 min application of a dose X 100 / tension just before applying the dose. Broken line shows the percentage of relaxation in the control catch tension.
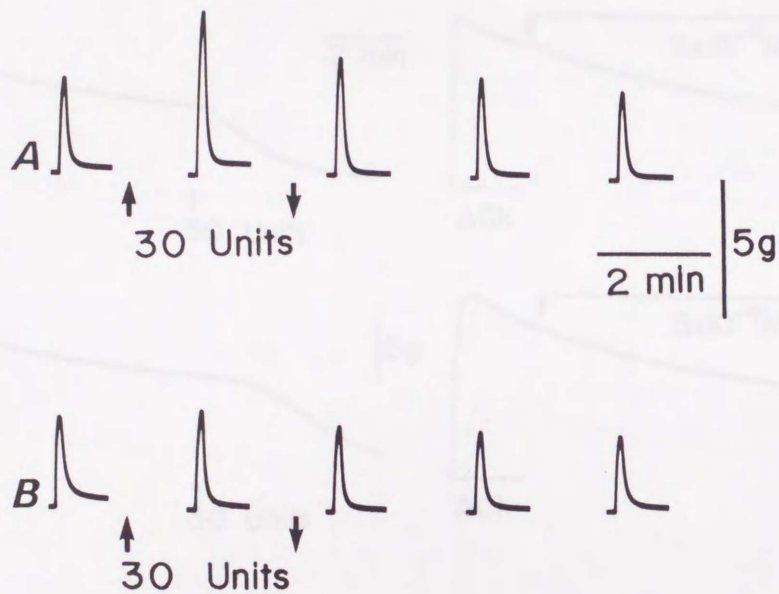
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Fig. 2.6. Phentolamine block of potentiating effect of the flowthrough on phasic contraction of the ABRM of _Mytilus_. A: potentiating effect of the flowthrough in the normal ASW. B: block of the potentiating effect by 10^{-5} M phentolamine. A and B were obtained from one and the same preparation. Between A and B, phentolamine was introduced. The other procedures are the same as in Fig. 2.4A.
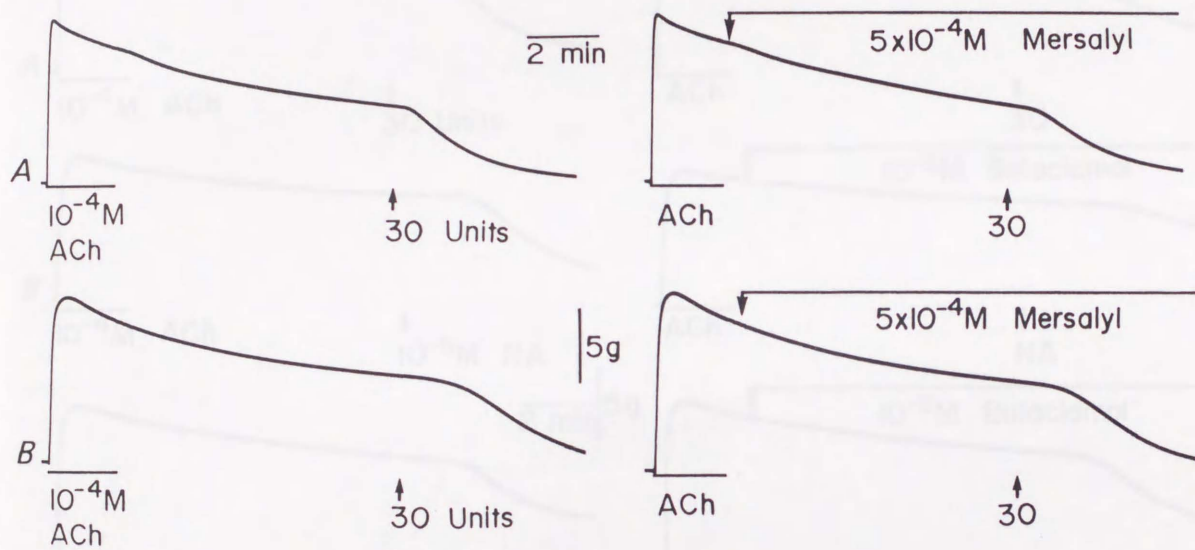
Fig. 2.7.   Effect of mersalyl on relaxation of catch tension of
the ABRM of Mytilus in response to the flowthrough. Mersalyl was
introduced soon after washing out ACh (downward arrows). The
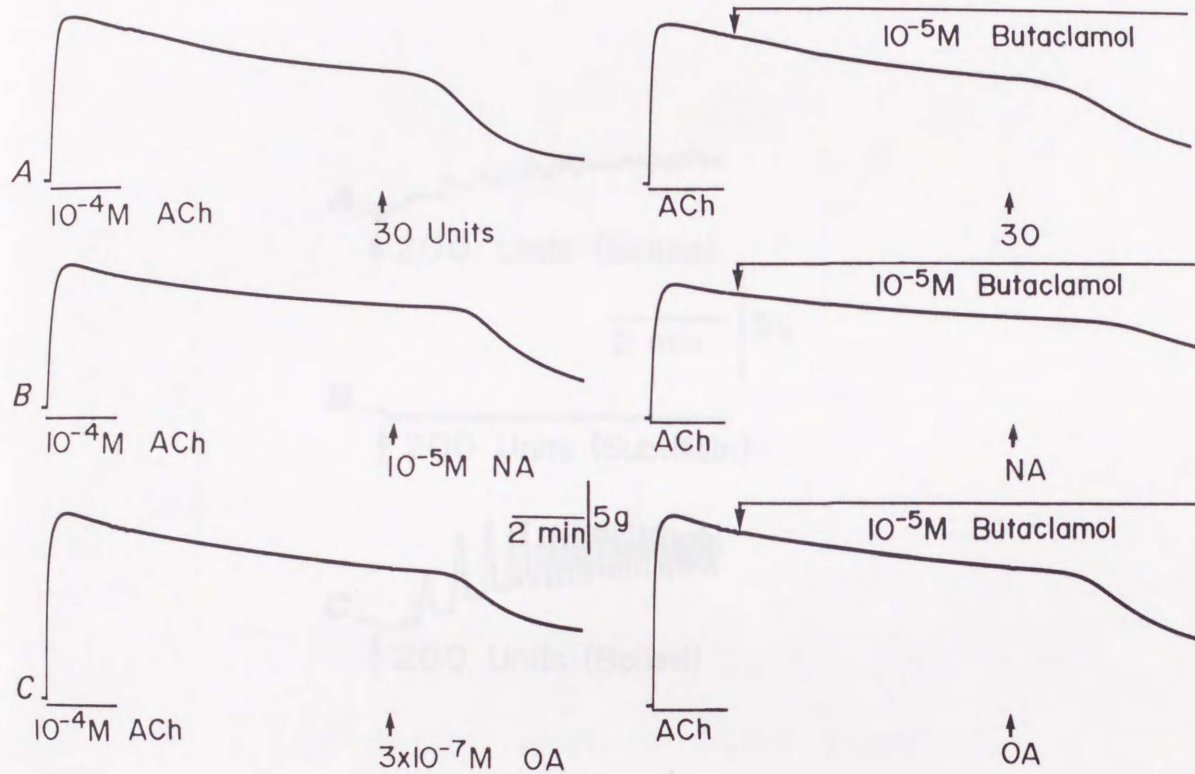other procedures are the same as in Fig. 2.4B.

Fig. 2.8. Effect of butaclamol on relaxation of catch tension of the ABRM of Mytilus. A: effect on relaxation in response to the flowthrough. B: effect on relaxation in response to nora-drenaline (NA). C: effect on relaxation in response to octopa-mine (OA). Butaclamol was introduced soon after washing out ACh (downward arrows). The other procedures are the same as in Fig. 2.4B.
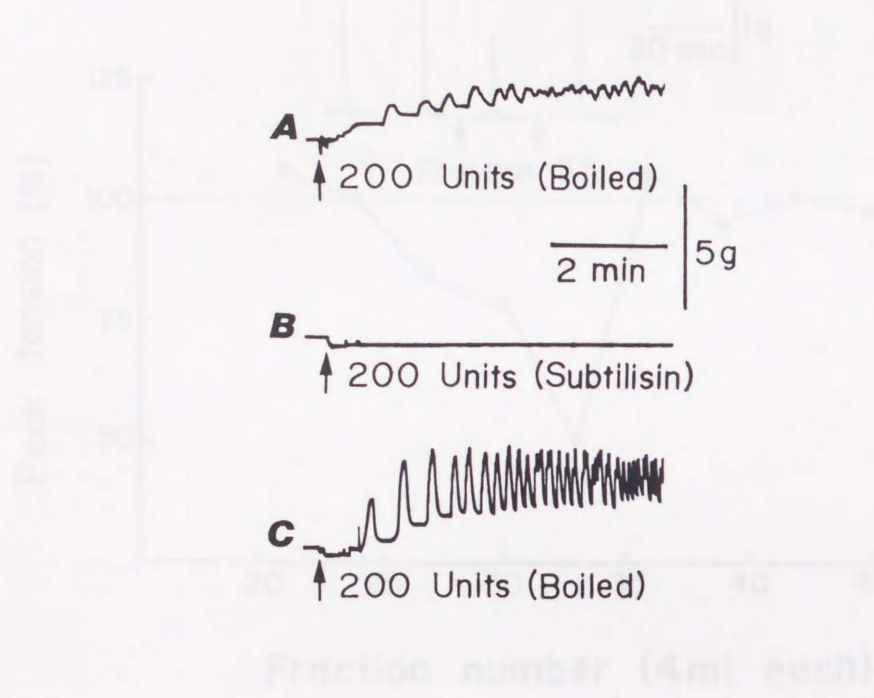
Fig. 2.9. Contractile activity of the retained material on the body-wall muscle of <u>Urechis</u>, and elimination of the activity by treatment of the material with subtilisin. A and C: activities of the control material. B: activity of the subtilisin-treated material.
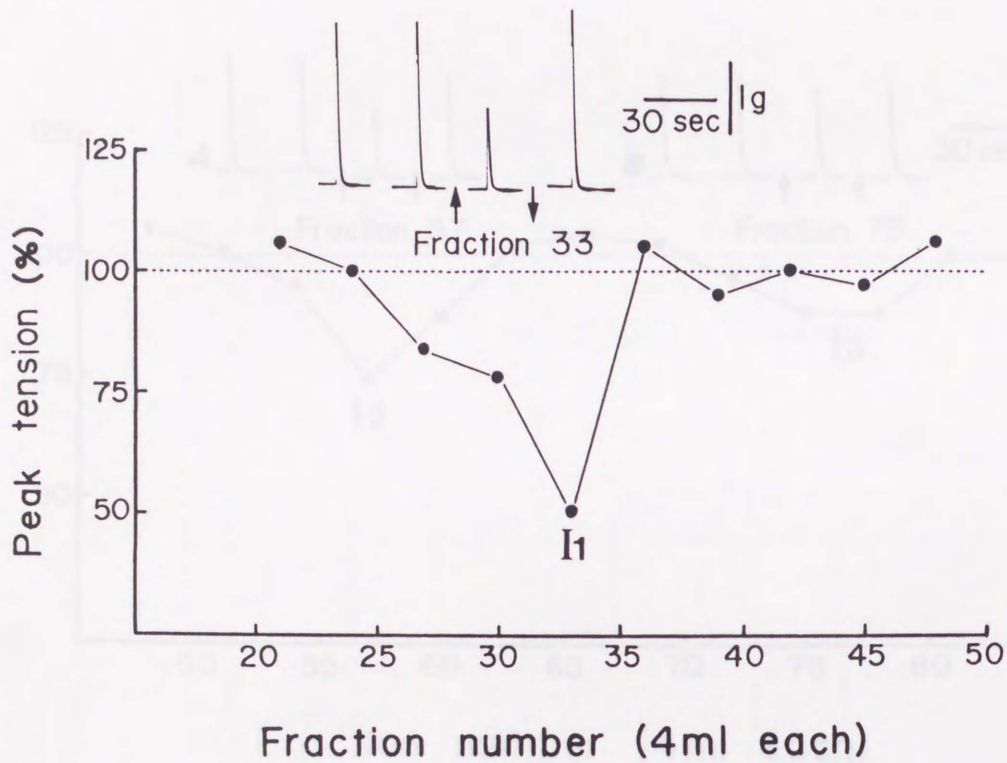
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Fig. 2.10.   Inhibitory activities of peak I_1 fractions (1,500 unit each) on twitch contraction of the body-wall muscle of _Urechis_. Each peak tension was shown as a percentage of the control peak tension. The other procedures are the same as in Fig. 2.1. The insert shows inhibitory activity of fraction 33.
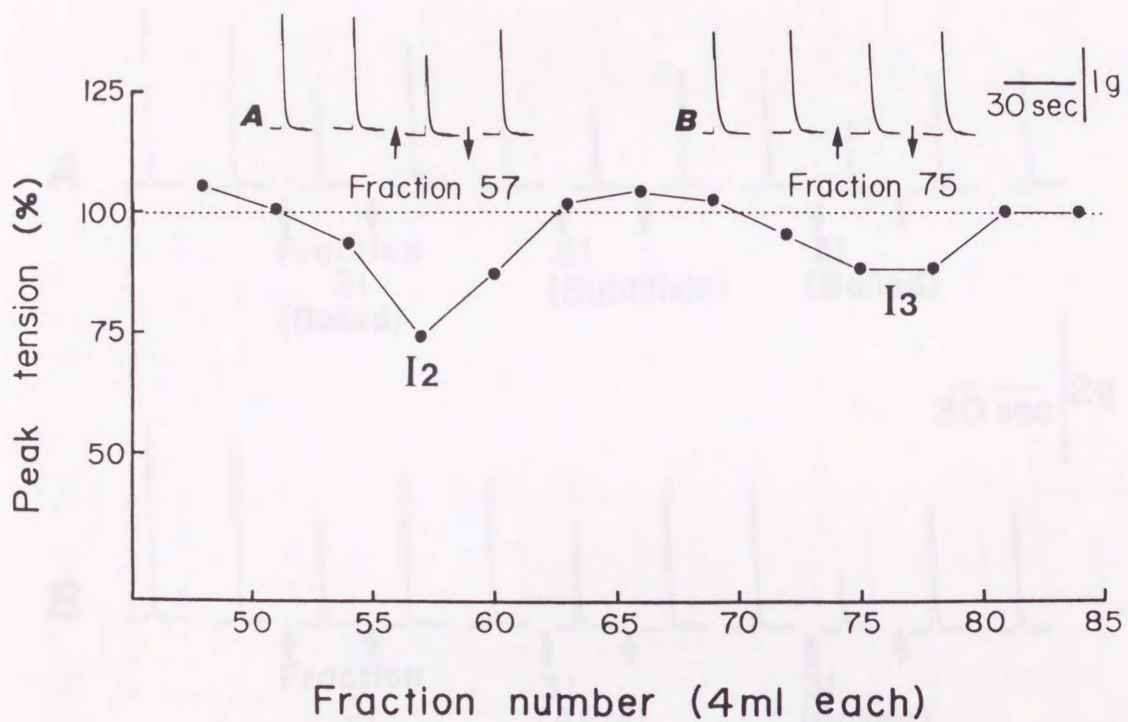
Fig. 2.11. Inhibitory activities of fractions (1,500 units each) of peak I_2 and I_3 on twitch contraction of the body-wall muscle of Urechis. The procedures are the same as in Fig. 2.9. Inserts A and B show inhibitory activities of fraction 57 and 75, respectively.
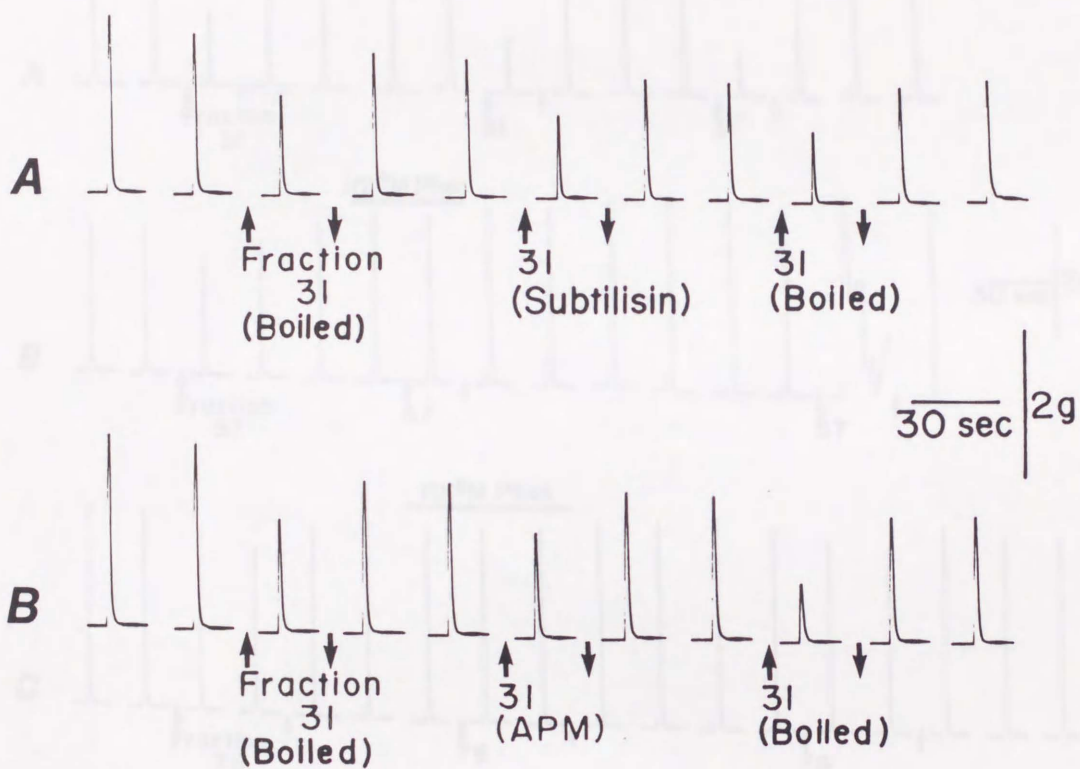
Fig. 2.12.   Inhibitory activity of a peptidase-treated peak I_1 fraction (fraction 31, 3,000 units) on twitch contraction of the body-wall muscle of Urechis. A: The activity of the fraction treated with subtilisin. B: The activity of the fraction treated with aminopeptidase M (APM). The procedures are the same as in Fig. 3B.
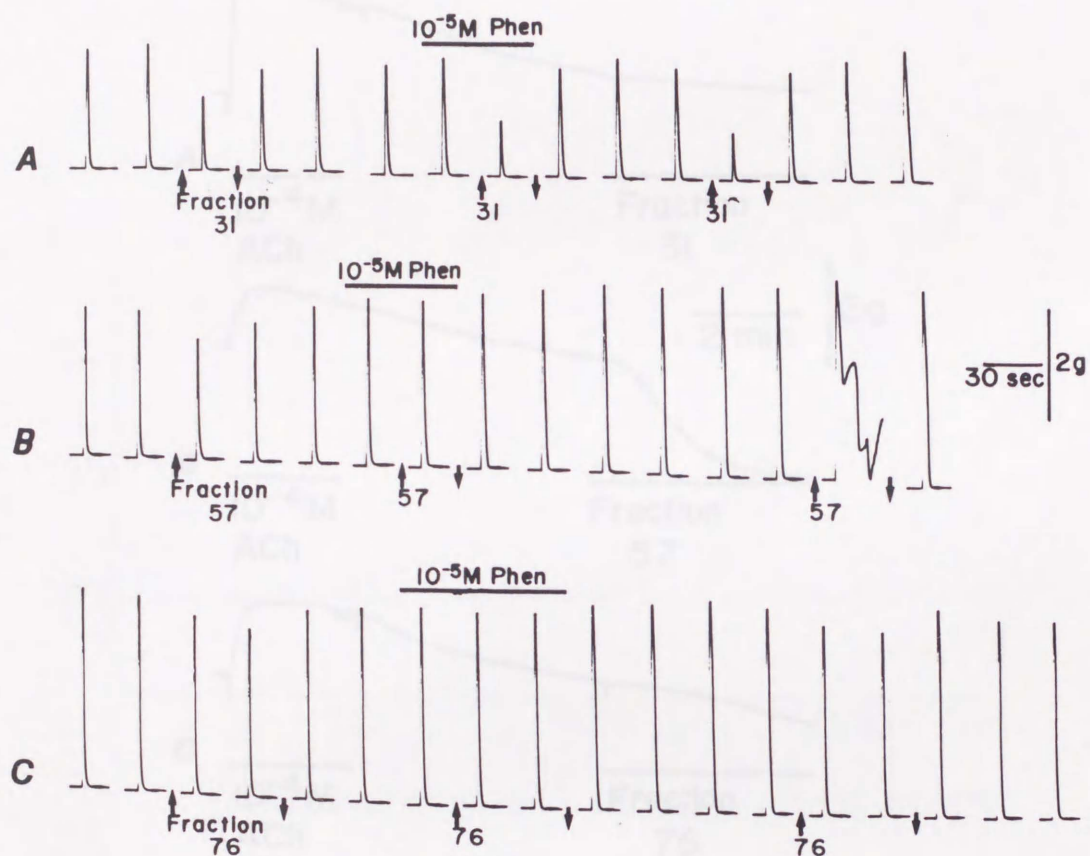
33

Fig. 2.13. Effect of phentolamine on inhibitory activities of fractions (3,000 units each) of peak I_1, I_2 and I_3 on twitch contraction of the body-wall muscle of Urechis. A: effect on the activity of a peak I_1 fraction (fraction 31). B: effect on the activity of a peak I_2 fraction (fraction 57). C: effect on the activity of a peak I_3 fraction (fraction 76). Phentolamine (Phen) was applied during the intervals indicated by the horizontal lines. The other procedures are the same as in Fig. 2.1.
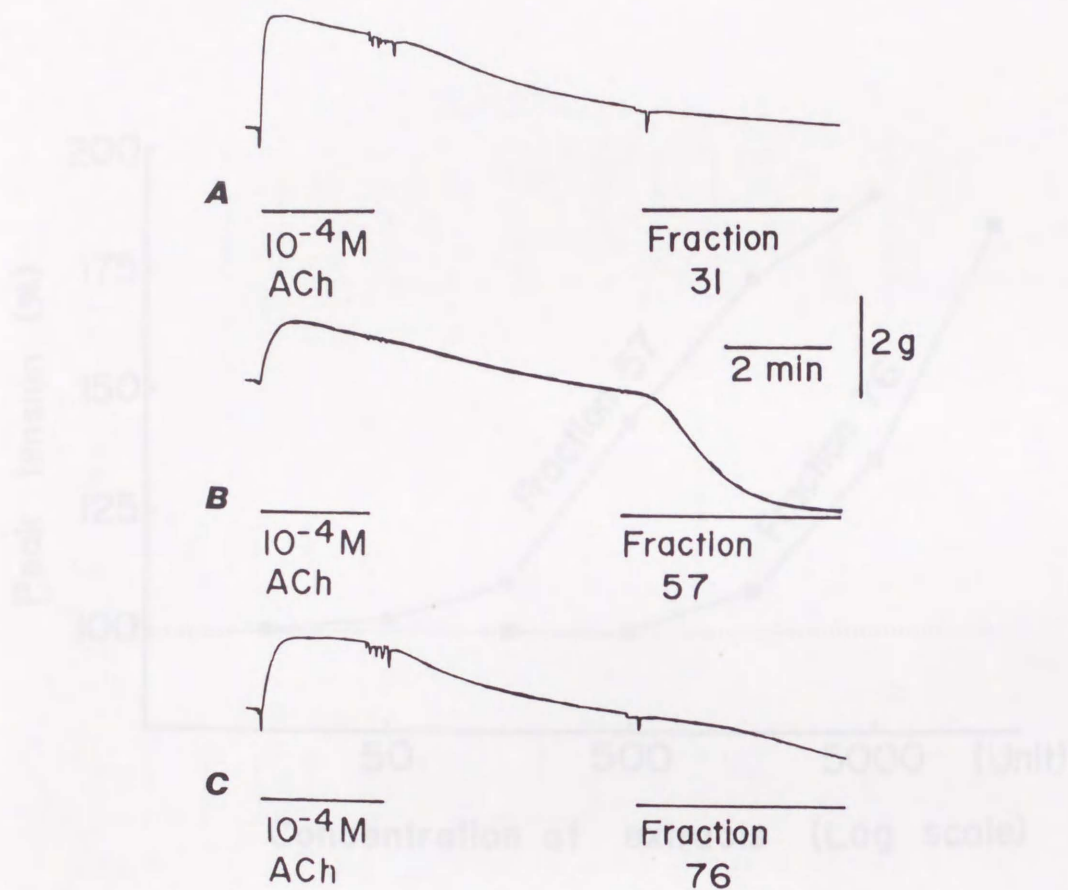
Fig. 2.14.   Activities of fractions (3,000 units each) of peak
I_1, I_2 and I_3 on catch tension in the ABRM of <u>Mytilus</u>.   A: activ-
ity of a peak I_1 fraction (fraction 31).   B: activity of a peak
I_2 fraction (fraction 57).   C: activity of a peak I_3 fraction
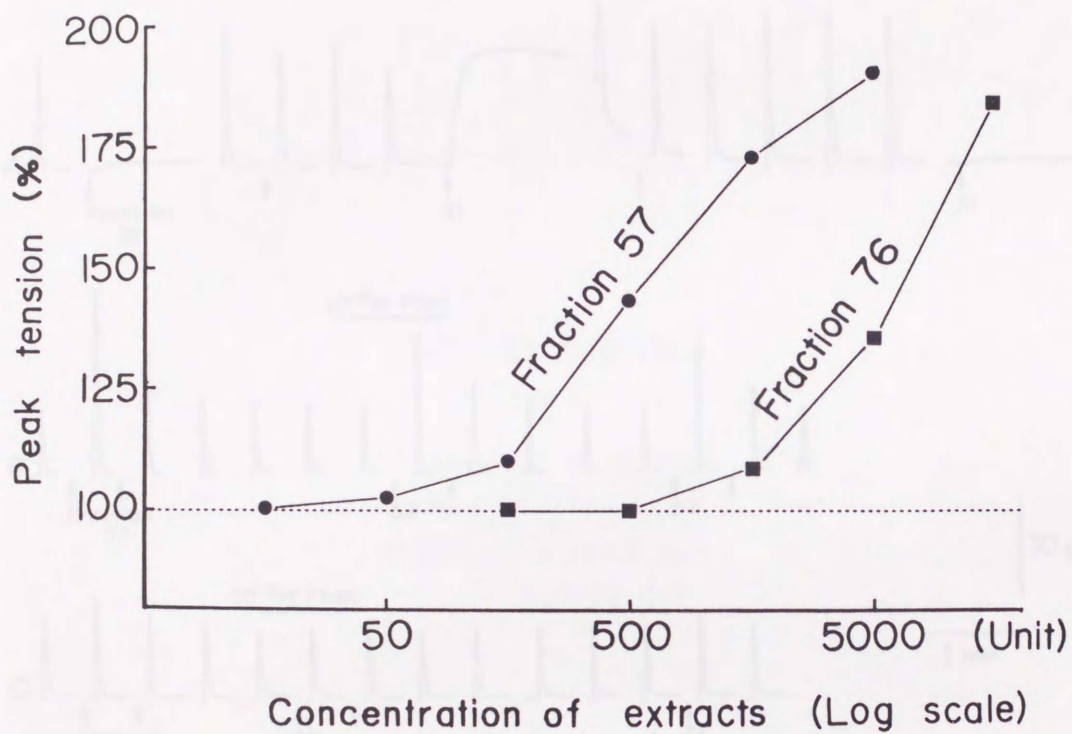(fraction 76).   The procedures are the same as in Fig. 2.4B.

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Fig. 2.15.   Potentiating activities of fractions (fraction 57 and 76) of peak I_2 and I_3 on phasic contraction of the ABRM of *Mytilus*. The procedures are the same as in Fig. 4B.

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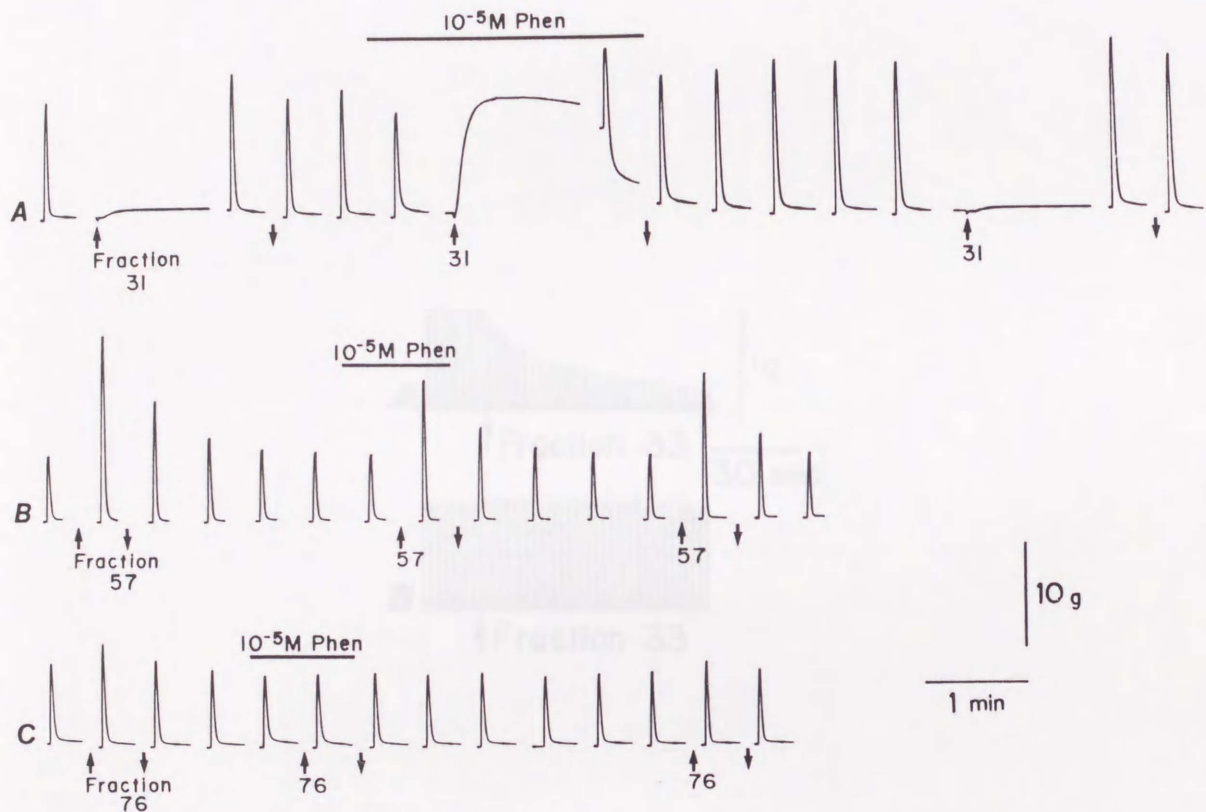
<u>Fig. 2.16.</u>   Effect of phentolamine on potentiating activities of fractions (3,000 units each) of peak I_1, I_2 and I_3 on phasic contraction of the ABRM of <u>Mytilus</u>. A: effect on the activity of a peak I_1 fraction (fraction 31). B: effect on the activity of a peak I_2 fraction (fraction 57). C: effect on the activity of a peak I_3 fraction (fraction 76). Phentolamine (Phen) was applied during the intervals indicated by the horizontal lines. The other procedures are the same as in Fig. 4A. Note that peak I_1 substance evokes contraction of the ABRM by itself, and the contraction is markedly potentiated by phentolamine.

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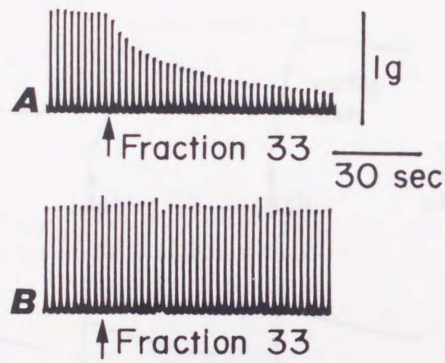<u>Fig. 2.17.</u>   Inhibitory activity of a peak I_1 fraction (fraction 33, 3,000 units) on spontaneous heart beat of <u>Meretrix</u> (A), and block of the inhibitory activity by 10^{-5} M benzoquinonium (B). Between A and B, the heart was washed for 10 min with normal ASW. Benzoquinonium was introduced 5 min prior to recording B.
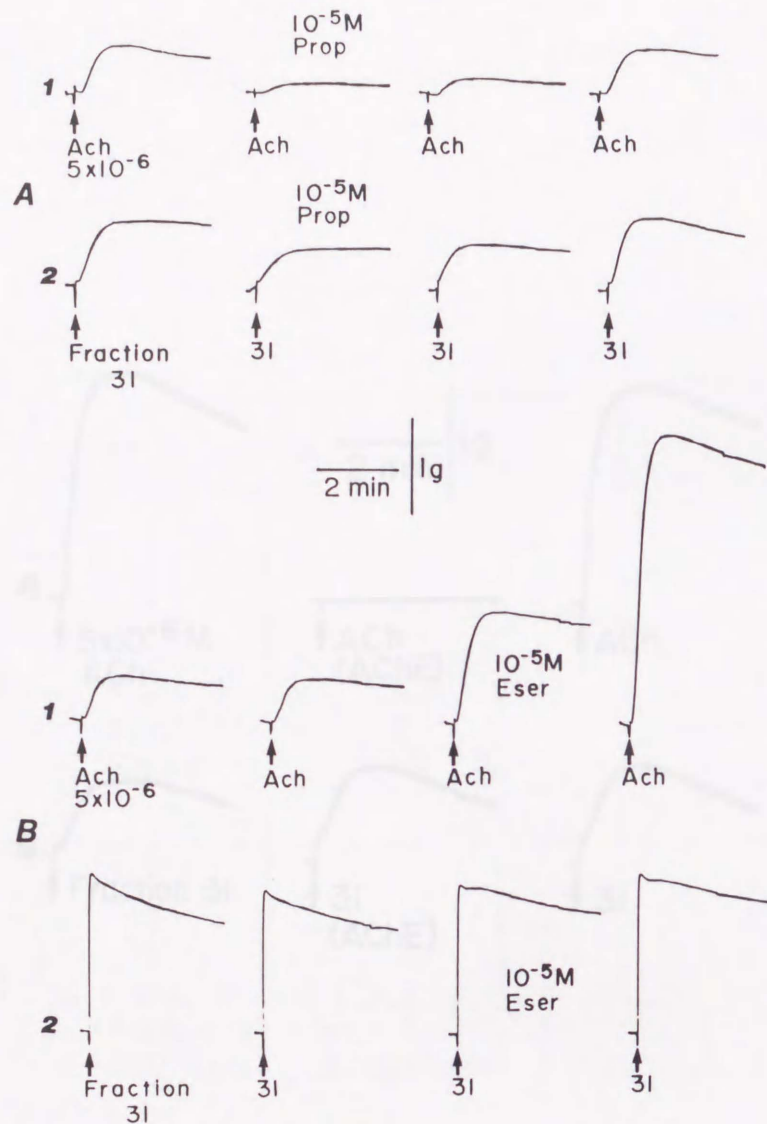
Fig. 2.18 Effects of propantheline and eserine on contractions of the ABRM of Mytilus in response to ACh and a peak I_1 fraction (Fraction 31, 3,000 units). A: effect of 10^{-5} M propantheline (Prop) on contractions by ACh (A1) and fraction 31 (A2). B: effect of eserine (Eser) on contractions by ACh (B1) and fraction 31 (B2). In both A and B, ACh or fraction 31 was applied to the muscle for 3-4 min at 15 min intervals. In A, propantheline was applied 10 min prior to the second contraction in each column and washed out soon after the contraction. In B, eserine was applied 10 min prior to the third contraction and washed out soon after the contraction.
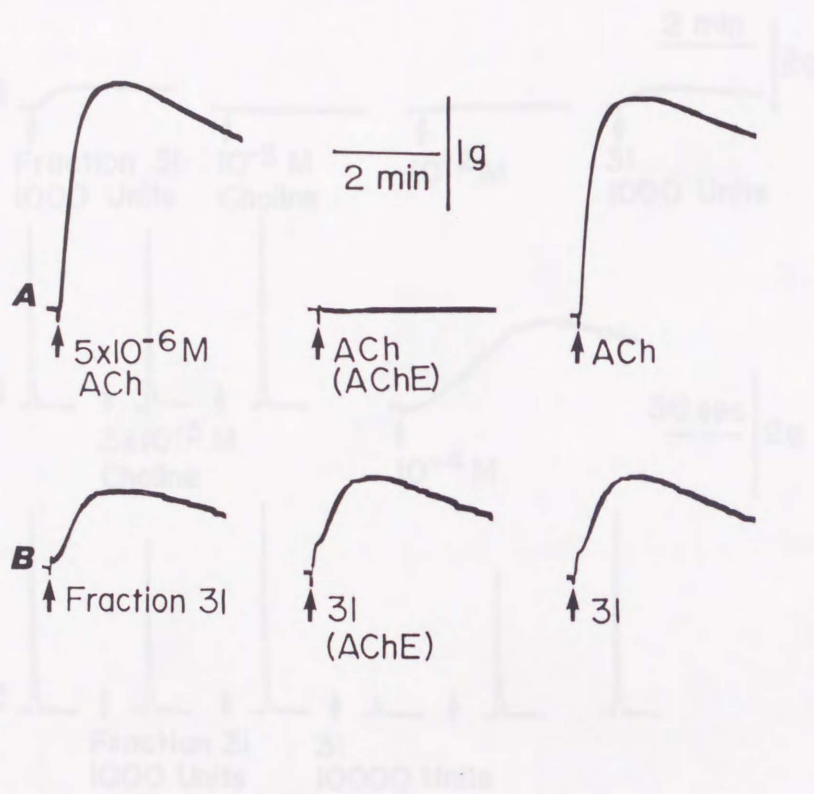
Fig. 2.19. Effects of AChE-treatment of ACh and a peak I_1 fraction (fraction 31, 3,000 units) on their contractile actions on the ABRM of _Mytilus_. A: effect of the treatment of ACh. B :effect of the treatment of fraction 31. In each column, ACh or fraction 31 was applied for 3.5 min at 15 min intervals, and the AChE-treated sample was applied at the second arrow and washed out soon after the second record.

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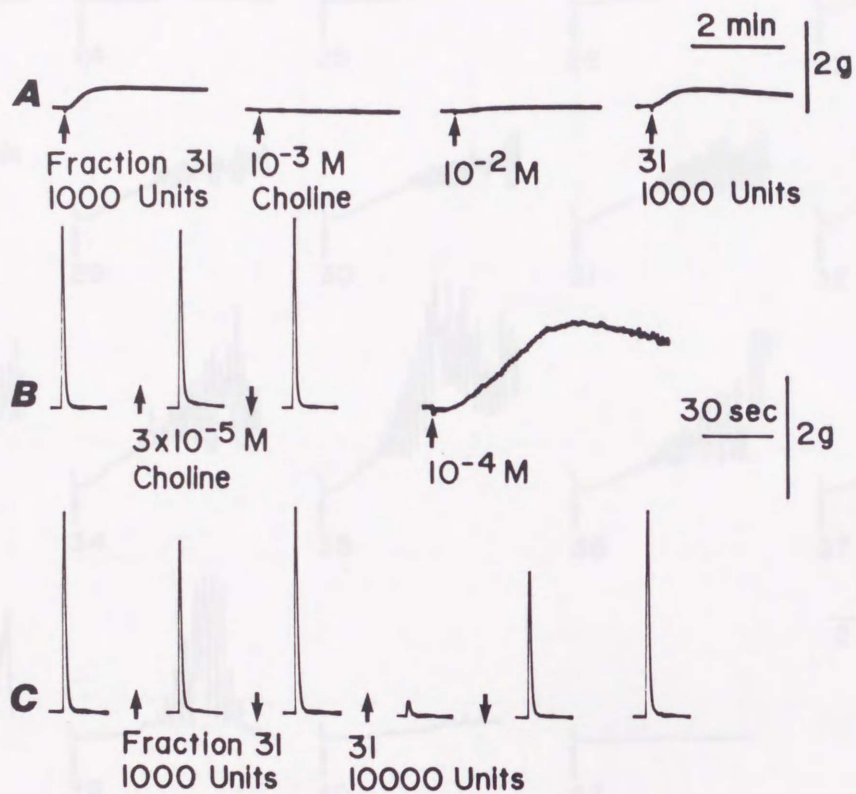
Fig. 2.20. Effects of choline and peak I_1 fraction (fraction 31) on the ABRM of Mytilus and the body-wall muscle of Urechis. A: effect of choline and fraction 31 on the ABRM. B: effect of choline on the body-wall muscle. Note that at 10^{-4} M choline evokes contraction by itself. C: effect of fraction 31 on twitch contraction of the body-wall muscle. Note the marked inhibitory effect of fraction 31 at 10,000 units.
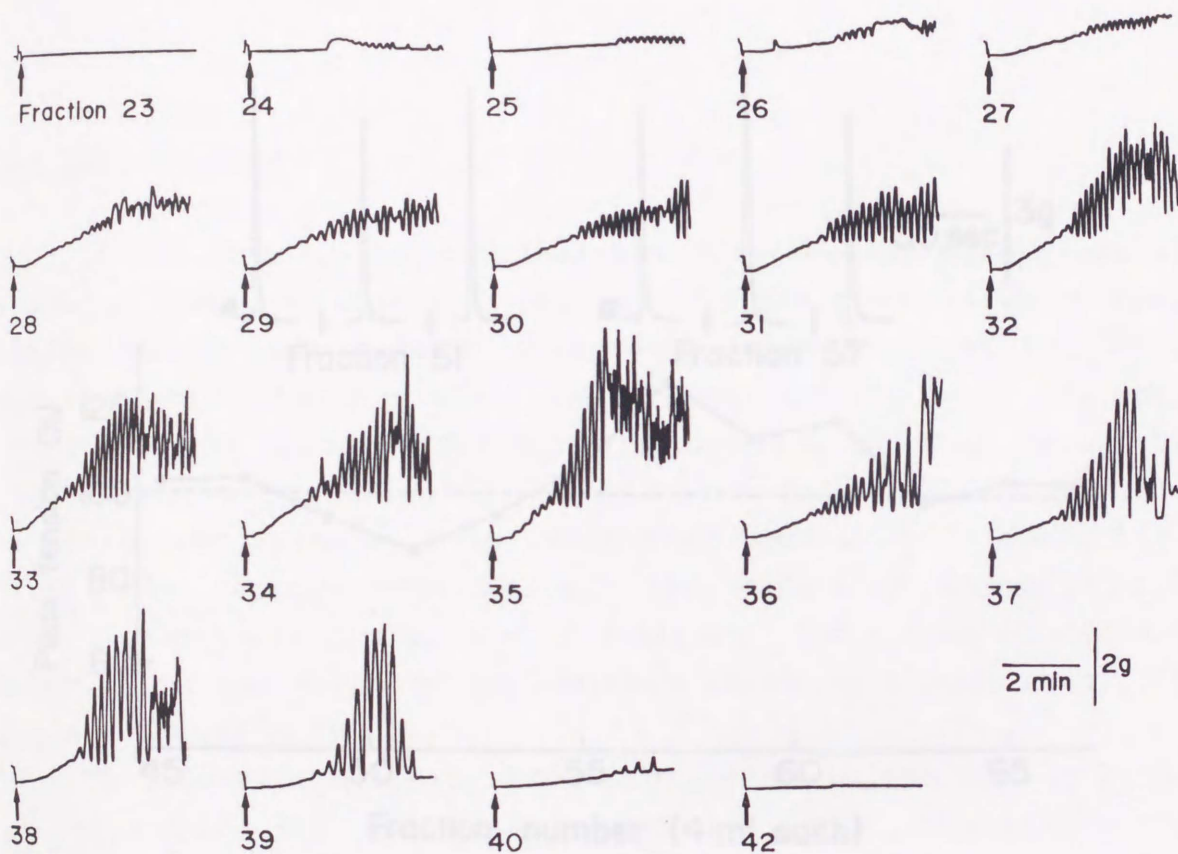
Fig. 2.21. Contractile activities of gel-filtrated fractions 23 - 42 (3,000 unit each) of the retained material on the body-wall muscle of _Urechis_. Fractions were applied for 3 min at 10 min intervals and washed out soon after the records.
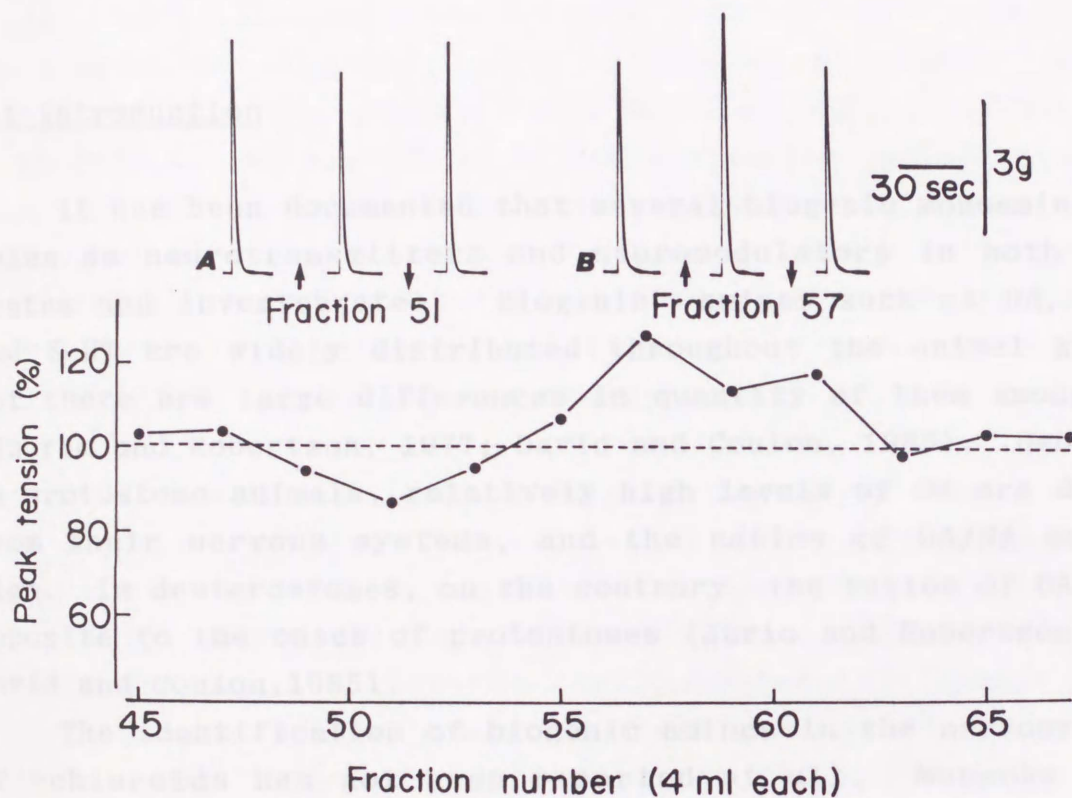
Fig. 2.22. Modulatory activities of the gel-filtrated fractions 45-67 (3,000 units each) of the retained material on twitch contraction of the body-wall muscle of Urechis. The procedures are the same as in Fig. 2.9. Inserts A and B show inhibitory activity of fraction 51 and potentiating effect of fraction 57, respectively.

CHAPTER 3

CATECHOLAMINES AND RELATED BIOACTIVE AMINES

3-1 Introduction

It has been documented that several biogenic monoamines play
roles as neurotransmitters and neuromodulators in both verte-
brates and invertebrates. Biogenic amines such as DA, NA, OA
and 5-HT are widely distributed throughout the animal kingdom,
but there are large differences in quantity of them among phyla
(Juorio and Robertson, 1977; David and Coulon, 1985). Generally,
in protostome animals, relatively high levels of OA are detected
from their nervous systems, and the ratios of OA/NA are also
high. In deuterostomes, on the contrary, the ratios of OA/NA are
opposite to the cases of protostomes (Jurio and Robertson, 1977;
David and Coulon,1985).

The identification of biogenic amines in the nervous system
of echiuroids has not been reported at all. Muneoka et al.
(1981) and Muneoka and Kamura (1982a) have suggested that ACh may
be an excitatory transmitter while NA may be an inhibitory trans-
mitter at the neuromuscular junctions in the body wall muscle of
Urechis unicinctus. Further, I suggested that there exist at
least four bioactive amine-like substances, one ACh-like and
three catecholamine-like substances, in the ventral nerve cord of
the animal (Chapter 2). In the present experiments, I attempted
simultaneous determination of biogenic amines in the ventral
nerve cord of Urechis by using ion-pair reversed-phase HPLC
coupled with a high sensitive electrochemical detector (ECD).

A number of methods have been reported for determination of

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catecholamines in tissues and body fluids of animals: radioenzy-matic methods (Cuello et al., 1973; Weise and Kopin, 1976; Vla-chakis et al., 1981; Peskind et al., 1986), gas-liquid chromatog-raphy with electron-capture detection (Wong et al., 1973; Imai et al., 1973), gas chromatography with mass spectrometry (Zambotti et al., 1975; Ehrhardt and Schwartz, 1978), HPLC with fluores-cence detection (Schwedt, 1977; Nimura et al., 1980) and HPLC with ECD (Moyer and Jiang, 1978; Hallman et al., 1978). Among these methods, except HPLC-ECD, radioenzymatic method is the most sensitive one to detect catecholamines in small samples. In this technique, however, procedures for making sample preparation are very complex (Davies and Molyneux, 1982). HPLC-ECD technque is a simple method to detect a small amount of catecholamines and their metabolites in tissues (Wagner et al., 1979; Krstulovic, 1982).

Determination of tryptophan and its metabolites including 5-HT in brain and other tissues has also been performed by using various methods such as thin-layer chromatography (Edvinsson et al., 1972), amino acid analysis (Wilkinson, 1976), gas-liquid chromatography with electron-capture detection (Degen et al., 1972), gas chromatography with mass spectrometry (Garrick et al., 1983), radio immunoassay (Wilkinson et al., 1977; Kennaway et al., 1977), HPLC with UV absorbance detection (Fornstedt, 1978; Riley et al., 1979), HPLC with fluorescence spectrometry (Grushka and Kikta, 1977; Anderson and Purdy, 1979) and HPLC with ECD (Penzio and Jonsson, 1979; Koch and Kissinger, 1979; Mefford and Barchas, 1980). The use of ion-pair reversed-phase HPLC coupled with ECD allows the simultaneous determination of small amounts of catecholamines, indolamines and their metabolites with a single injection (Warsh et al., 1982; Mefford et al., 1982; Taylor et al., 1983). Furthermore, OA can also be determined simultaneously by this technique (Ehrenström and Berglind, 1988; Bakary et al., 1988; Nagao and Tanimura, 1989).

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3-2 Materials and Methods

3-2-1 Preparation of tissue sample

The ventral nerve cords (260 mg) isolated from six specimens of Urechis were homogenized with glass homogenizer in 1 ml of 1% acetic acid at 0°C. The homogenate was centrifuged at 8,000g for 20 min at 4°C. The supernatant was filtrated through a 0.45 μm filter and diluted to 60 ml with the solution which was also used for the mobile phase of HPLC. An aliquot of the extract (0.1 ml) containing 1/100 nerve-cord equivalents was applied to HPLC analysis.

3-2-2 Preparation of Standard Solution

Standard solution (0.1 ml) containing 10 pmoles NA, 10 pmoles AD, 10 pmoles DA, 10 pmoles 3,4-dihydoroxyphenylacetic acid (DOPAC), 40 pmoles OA, 2 pmoles 5-hydroxyindole-3-acetic acid (5-HIAA) and 10 pmoles 5-HT was prepared by diluting the stock solution of a mixture of these substances just before HPLC analysis. DOPAC and 5-HIAA are major metabolites of DA and 5-HT, respectively. The stock solution was filtrated through a 0.45 μm filter and stored at -30°C.

3-2-3 HPLC System and Chromatographic Procedures

The HPLC system used in the present study consisted of a dual-piston pump (EP-10, Eicom), a degasser (DG-100, Eicom) and an amperometric ECD (ECD-100, Eicom) equipped with a graphite carbon electrode (WE-3G, Eicom) and a C-18 reversed-phase column (Eicompak MA-5ODS, 4.6 X 150 mm, Eicom) kept at 25 °C in a column incubater (CTC-100, Eicom). The detector potentials in the experiments by other investigators for immediate oxidization of catecholamines, 5-HT and OA have been reported to be 0.6 V (Krstulovic, 1982), 0.72 V (Kim et al., 1983) and 0.95 V (Nagao

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and Tanimura, 1989) versus the Ag/AgCl reference electrode, respectively. Therefore, in this experiments, the detector potential was set at 0.95 V for simultaneous determination of biogenic monoamines.

The mobile phase was 20% methanol in 0.1 M potassium phosphate buffer (pH 3.5 with H_3PO_4) containing 100 μM Na_2EDTA and 400 mg/l sodium octanesulfonate (SOS) as an ion-pair reagent (Nagao and Tanimura, 1989). Methanol and the mobile-phase buffer were filtrated through 0.22 μm filter. Their mixture was degassed with a water-aspirator. The flow rate was 1.0 ml/min.

3-3 Results

Figure 3.1 shows the chromatograms of NA, AD, DA and OA. These amines showed different retention times under the same chromatographic condition. After the retention times of DOPAC, 5-HT and 5-HIAA, in addition to the above amines, were measured, the standard solution (a mixture of the seven substances) was applied to the HPLC (Fig. 3.2). All of the seven substances showed characteristic retention times. The retention times (min) of the amines and the metabolites were as follows; NA: 3.7, AD: 4.1, OA: 4.6, DOPAC: 5.1, DA: 6.3, 5-HIAA: 7.1 and 5-HT: 12.5.

The chromatogram of the tissue sample (1/100 nerve-cord equivalent) was shown in Fig. 3.3. The identification of each peak was performed by comparing the retention times of the peaks with those in the case of the standard solution. Five biogenic amines, DA, NA, AD, OA and 5-HT were detected, though detected amounts of AD and OA were very small. Two metabolites, DOPAC and 5-HIAA were not detected. Estimated amounts of DA, NA, AD, OA and 5-HT in a nerve cord were 1,760 pmoles, 528 pmoles, 8.70 pmoles, 35.3 pmoles and 324 pmoles, respectively. Estimated amount (ng) of these amines in 1g of the nerve cords are shown in table 3.1.

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<u>3-4 Discussion</u>

As shown in Table 3.1, relatively large amounts of DA, NA and 5-HT are contained in the ventral nerve cord of <u>Urechis</u>. It seems that DA is the most abundant catecholamine in the nerve cord. In contrast, the amounts of AD and OA are very small. In Chapter 2, it was suggested that the inner circular body-wall muscle has at least five classes of receptors for biogenic amines; one is contractile receptors for ACh, another is inhibitory receptors for NA, and the other three are contraction-potentiating receptors for DA, OA and 5-HT, respectively. From the results of the experiments, it is considered that two catecholamines, DA and NA, and 5-HT have functions as neurotransmitters in the neuromuscular system of <u>Urechis</u>. However, the roles of AD and OA in the nervous system, if any, are considered to be minor.

Determination of biogenic amines and examinations of their pharmacological actions have been carried out in many animals. The amounts of the biogenic amines, DA, NA, OA and 5-HT, in some arthropods, an annelid, an echiuroid, some molluscs, an echinoderm and a mammal are summarized in Table 3.2 (see David and Coulon, 1985). There is evidence for DA as neurotransmitter in most of the animal phyla (Leak and Walker, 1980). In protostomes, especially in arthropods, annelids and molluscs, OA is utilized as a neurotransmitter and a neuromodulator rather than NA which is the major phenylethanolamine-type chemical messenger in deuterostomes (Walker and Kerkut, 1978; Leak and Walker, 1980; Gardner and Walker, 1982; David and Coulon, 1985). Further, in protostomes, relatively high levels of OA are detected from their nervous systems, and the ratios of OA/NA is also high. In deuterostomes, however, the ratios of OA/NA is opposite to those in protostomes (Table. 3.2).

Occurrence of abundant NA in the nerve cord of <u>Urechis</u> which

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is a member of protostomes is very interesting from a phylogenic point of view. Further, the actions of OA and NA on the inner circular body-wall muscle of <u>Urechis</u> are opposite, in spite of that the actions of NA are qualitatively similar to those of OA in the tissues of other protostomes examined.

I suggested in Chapter 2 that a novel phenylethanolamine-type catecholamine may be contained in the ventral nerve cord of <u>Urechis</u>. The two large peaks, which could not be identified were observed in the present ECD experiments (Fig. 3). Both of the two substances might be novel phenylethanolamine-type biogenic amines, though it is also possible that they are metabolites or precursors of catecholamines or 5-HT.

<u>Table 3.1</u> Estimated amounts of biogenic amines in the ventral nerve cord of <u>Urechis</u>.

Amines	ng/1 nerve cord	ng/g of fresh tissue
Dopamine	279.0	6430
Noradrenaline	89.3	2060
Adrenaline	1.6	37
Octopamine	5.5	125
Serotonin	57.1	1310

Table 3.2   Quantitative distribution of biogenic amines, and octopa-
mine/noradrenaline ratios in nervous tissues of invertebrates and
vertebrates. (data were reproduced from David and Coulon, 1985, except
those of Urechis and Mytilus)

Phyla	Class	Genus	DA	NA	OA (ng/g tissue)	5-HT	OA/NA
Arthropoda	Crustacea	Pacifastacus	200	120	540	-	4.5
	Insecta	Scistocerca	870	110	2430	-	22
		Periplaneta	1196	166	1760	-	11
Annelida	Oligocaeta	Lumbricus	1800	800	3600	-	4.5
Echiura		Urechis	6430	2060	125	1320	0.06
Mollusca	Pelecypoda	Mytilus	523	270	1253	87	4.6
	Gastropoda	Helix	9490	141	142	4130	1.0
	Cephalopoda	Sepia	6940	3200	220	2520	0.07
		Octopus	13300	4780	1200	5590	0.25
Echinodermata	Asteroidea	Pycnopodia	3954	2133	260	<30	0.12
Vertebrata	Mammalia	Rattus					
	16-days	embryo	16	16.4	26*	-	1.9
		adult	613	432	3.9	-	0.01

Note, data of Mytilus was obtained from extract of the ABRM.

*, in ng/mg protein

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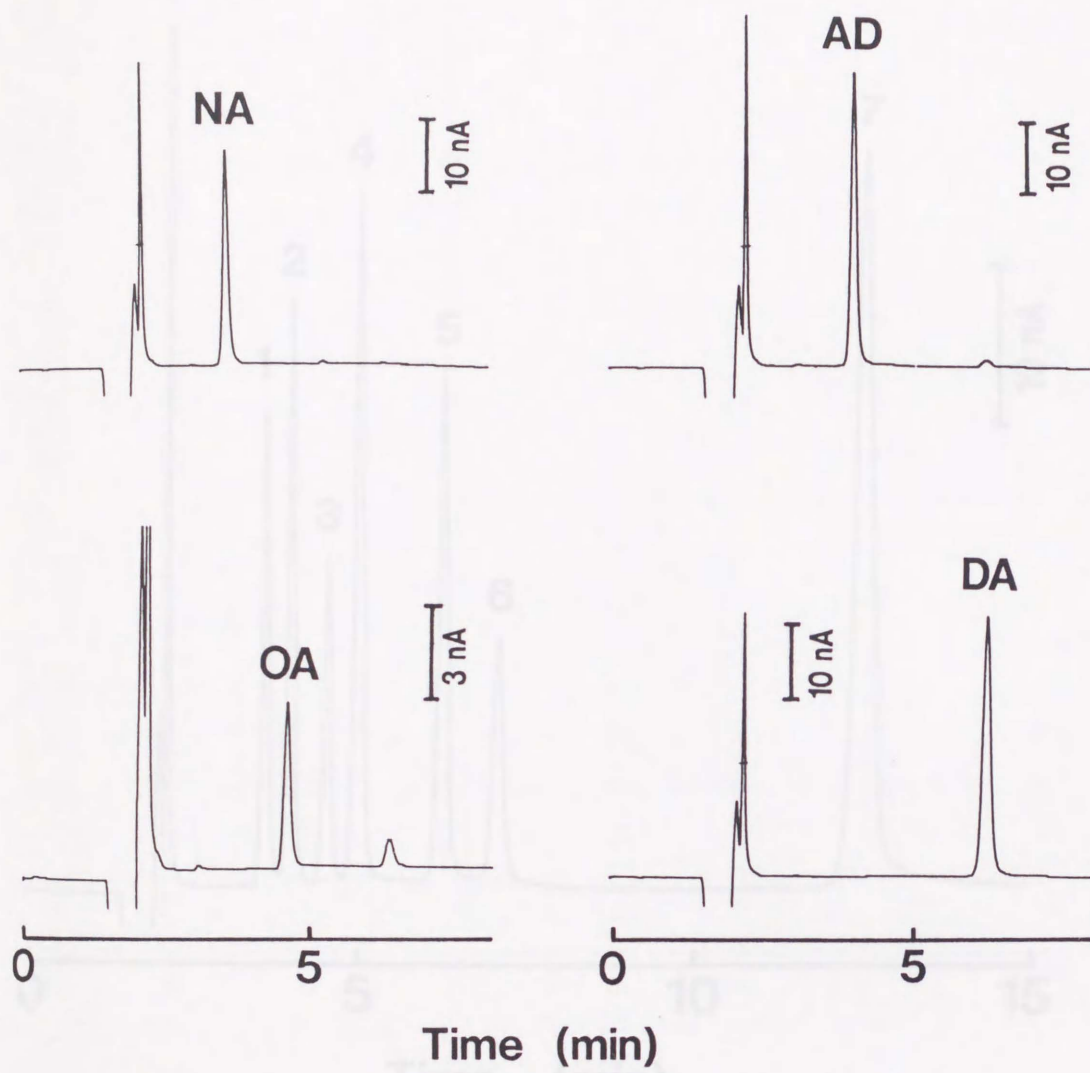
Fig. 3.1. Chromatograms of noradrenaline (NA), adrenaline (AD), octopamine (OA) and dopamine (DA). The amount of each amine is 10 pmoles. Chromatographic conditions are given in text.
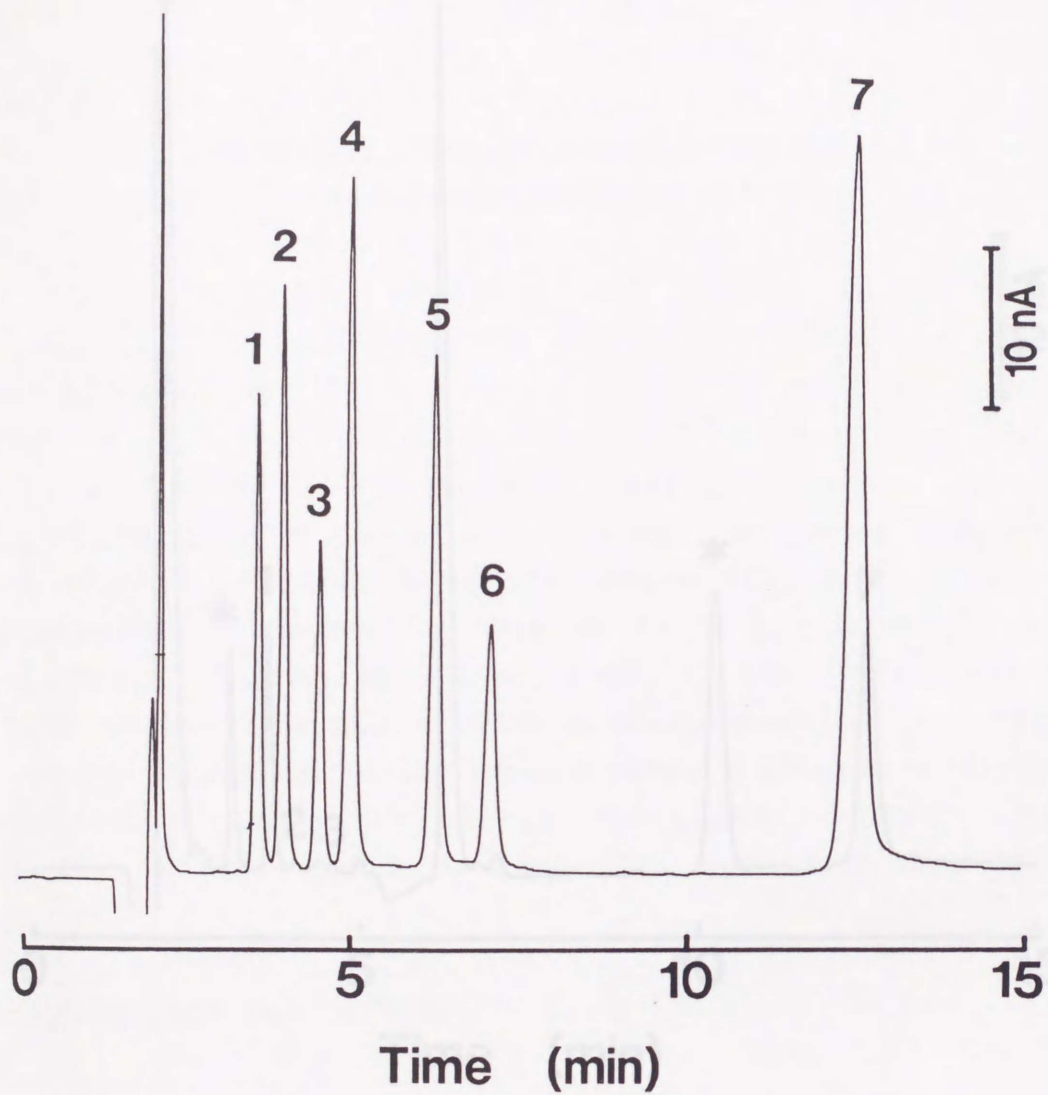
Fig. 3.2. A typical chromatogram of the standard solution (a mixture of the biogenic amines and the metabolites). Chromatographic conditions are given in text. Peak identification: 1, noradrenaline (10 pmoles); 2, adrenaline (10 pmoles); 3, octopamine (40 pmoles); 4, DOPAC (10 pmoles); 5, dopamine (10 pmoles); 6, 5-HIAA (2 pmoles); 7, serotonin (10 pmoles)

53
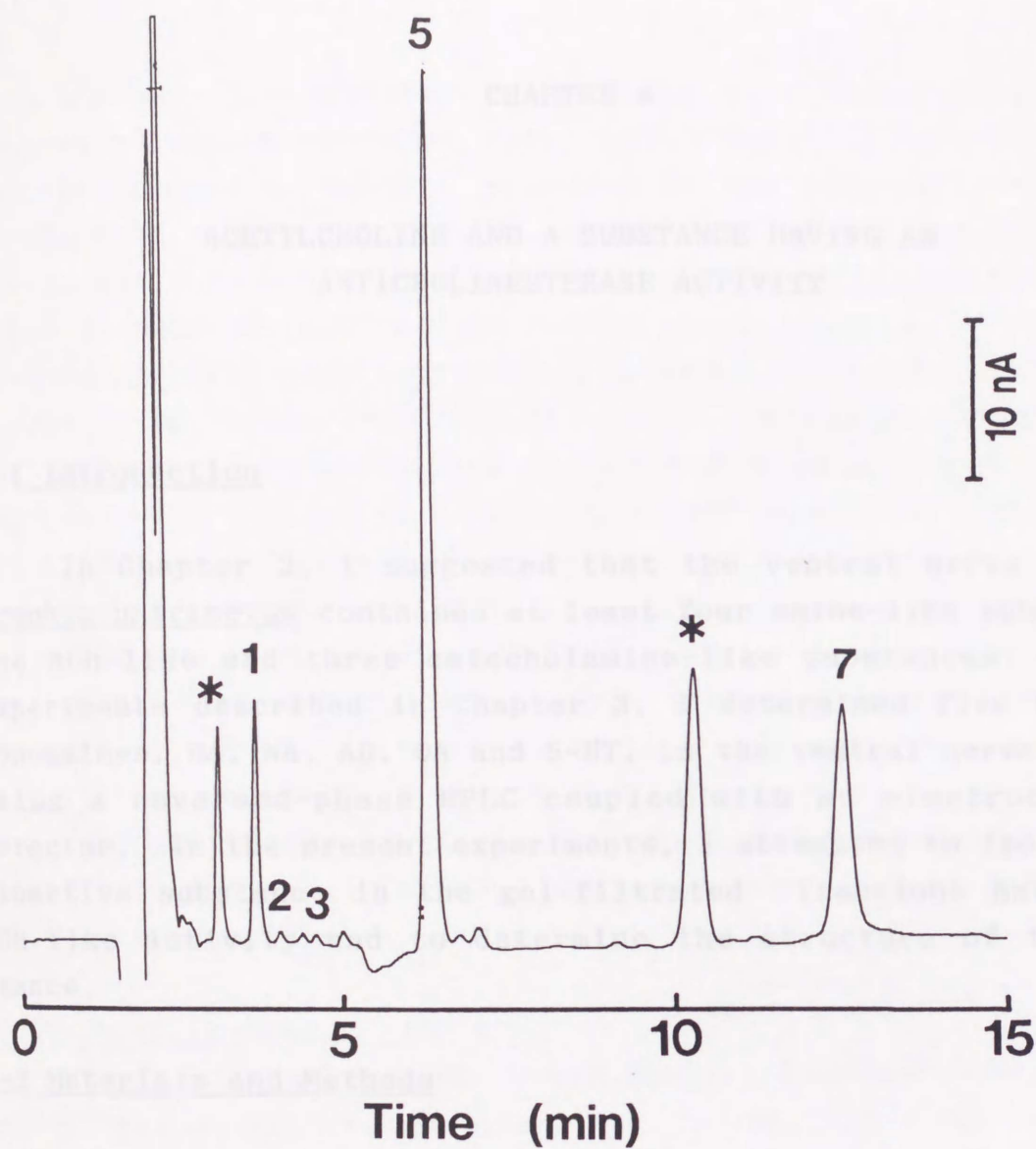
<u>Fig. 3.3.</u>   Chromatogram of biogenic amines in the ventral nerve cords (1/100 animal equivalent) of <u>Urechis</u>.   Chromatographic conditions are given in text. Peak identification: 1, noradrena- line; 2, adrenaline; 3, octopamine; 5, dopamine; 7, serotonin; *, unidentified peaks.

CHAPTER 4

ACETYLCHOLINE AND A SUBSTANCE HAVING AN
ANTICHOLINESTERASE ACTIVITY

4-1 Introduction

In Chapter 2, I suggested that the ventral nerve cord of Urechis unicinctus contained at least four amine-like substances, one ACh-like and three catecholamine-like substances. In the experiments described in Chapter 3, I determined five biogenic monoamines, DA, NA, AD, OA and 5-HT, in the ventral nerve cord by using a reversed-phase HPLC coupled with an electrochemical detector. In the present experiments, I attempted to isolate the bioactive substance in the gel-filtrated fractions having the ACh-like activity and to determine the structure of the substance.

4-2 Materials and Methods

4-2-1 Extraction and Separation of Bioactive Substances

Aqueous acetic acid extract of the ventral nerve cords (85g) of Urechis (1,800 animals) was forced through C-18 cartridges (Waters Sep-pak). The flowthrough was concentrated and gel-filtrated by using a Sephadex G-15 column (2.5 X 40 cm). The biological activity of each fraction (4 ml) was assayed on twitch contraction of the inner circular body-wall muscle of the animal. I obtained three peaks (peak I_1, I_2 and I_3) of twitch-inhibiting activity (see Chapter 2). Peak I_1 showed an ACh-like activity on some molluscan muscles.

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The active fractions of peak I_1 (fractions 25-39) were evaporated and re-extracted with 100% ethanol. The re-extracted material showed an inhibitory action on the body-wall muscle and an ACh-like contractile action on the ABRM of Mytilus edulis and the radula retractor muscle of Fusinus ferrugineus (Fig. 4.1). These actions were identical to those of peak I_1. The re-extracted material was concentrated and applied to a C-18 reversed-phase column (Asahipak ODP-50, 6.0 X 250 mm, Asahi Chemical Industry). The column was eluted with a 120-min linear gradient of 0-60% acetonitrile in 10 mM phosphate buffer at pH 6.9 (Fig 4.2). I obtained two active peaks (peak 1 and 2). Peak 1 showed an ACh-like contractile activity on the ABRM of Mytilus. The substance which showed ACh-like activity in peak 1 was termed native ACh-like substance. Peak 2 showed a potent inhibitory activity on twitch contraction of the body-wall muscle of Urechis. The native ACh-like substance in peak 1 was shown to be ACh. The inhibitory substance in peak 2 was shown to be a novel tripeptide, H-Ala-Leu-Thr-OH. The peptide was termed ALT according to its structure.

4-2-2 Determination of ACh

The fractions of peak 1 were concentrated and applied to a gel-filtration HPLC column (Asahipak GS-320, 7.6 X 500 mm, Asahi Chemical Industry) and eluted with water containing 0.01% TFA at pH 2.2. The native ACh-like substance was eluted between 32 min and 35 min after injection (flow rate was 0.5 ml/min). As shown in Fig. 4.3, the retention time of the native ACh-like substance was identical to that of authentic ACh on a cation-exchange column (TSKgel SP-5PW, 7.5 X 75 mm, Tosoh). In this experiment, the elution was performed with a 40-min linear gradient of 0-0.8 M NaCl in 0.01% TFA. The activities of the fraction, which was eluted at the identical position with the peak of ACh, and authentic ACh were compared by using the inner circular body-wall

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muscle of _Urechis_, the ABRM of _Mytilus_ and the radula retractor muscle of _Fusinus_.

4-2-3 Purification of ALT

The inhibitory peptide ALT in peak 2 was purified through five more HPLC-separation steps. At the second step, the fractions of peak 2 were applied to a gel-filtration column (Asahipak GS-320) and eluted with water containing 0.01% TFA (Fig. 4.5A). The active fraction was then applied to another C-18 reversed-phase column (TSKgel ODS80T$_M$) and eluted with a 60-min linear gradient of 0-60% acetonitrile in 0.1% TFA (Fig. 4.5B). At the fourth step, I used a C-8 reversed-phase column (Finepak C$_8$5). The column was eluted with a 60-min linear gradient of 0-20% acetonitrile in 0.1% TFA (Fig. 4.6A). The active fraction was applied to the C-18 reversed-phase column used at the third step and eluted isocratically with 10.5% acetonitrile in 0.1% TFA (Fig. 4.6B). The final purification was performed by applying the active material again to the same C-18 reversed-phase column and eluting with 9.5% acetonitrile (Fig. 4.7).

4-2-4 Bioassay and Pharmacology

After each purification steps of ALT, biological activities of the fractions were examined on twitch contraction of the isolated small bundle (1.5 mm in width and 20 mm length) of the inner circular body-wall muscle of _Urechis_. The twitch contraction was elicited at 10 min intervals by stimulating the muscle bundle with an electrical pulse (15 V, 3 msec). Each bioassay material was applied to the bundle 8 min prior to the electrical stimulation. The ABRM of _Mytilus_ and the radula retractor muscle of _Fusinus_ were used to assay of the ACh-like substance. In the case of the ABRM assay, each bioassay material was applied to the muscle at 15 min intervals. The method of isolating and tension recording from the muscles were essentially the same as those

described in Chapter 2.

The activities of ALT were examined not only on twitch contraction of the muscle but also on contractions of the muscle in response to ACh, UEP$_A$ and UEP$_C$. UEPs (Urechis excitatory peptides) are the bioactive peptides which were purified from the retained material of the nerve cords of Urechis. They show a potent contractile activity on the muscle (see the next Chapter).

4-2-5 Structure Determination of ALT

The purified substance was subjected to amino acid sequence analysis by automated Edman degradation with a gas-phase sequencer (Applied Biosystems 477A) coupled with a PTH-amino acid analyzer (Applied Biosystems 120A), quantitative amino acid analysis (Hitachi L-8500) and fast atom bombardment mass spectrometric (FAB-MS) analysis (JEOL JMS HX-100). Thus, probable structure of the peptide was determined. In order to confirm the structure, the peptide was synthesized at the Peptide Institute (Osaka Japan). The behavior of the synthesized peptide on a C-18 reversed-phase and a C-8 reversed-phase HPLC was compared with that of the native peptide. Furthermore, the relationships between concentration and action of the synthetic and native peptides were also compared by using the inner circular body-wall muscle of Urechis.

4-2-6 Assay of Anti-AChE Activity of ALT

ACh produces a contraction in the ABRM of Mytilus at 10^{-6} M or higher (Twarog, 1954). The effect of AChE is able to observe by comparing the peak tension of contraction elicited by ACh with that elicited by AChE-treated ACh. Therefore, I used the ABRM to examine the effect of ALT on the activity of AChE. AChE used in the experiments was from vobine erythrocytes, and it was purchased from Wako Pure Chemical Industries. An appropriate amount of the stock solution of AChE was added to ACh or a mixture of

ACh and ALT. The final concentrations of AChE, ACh and ALT were 10^{-5} g/ml, 10^{-3} M and 10^{-3} M, respectively. The solution pH was adjusted to 7.8 by Tris-HCl. Incubations were carried out at 36°C for 120 min. The incubated samples were diluted 100 folds by ASW and applied to the ABRM.

4-2-7 Structure-activity Relationship of ALT

In order to examine the structure-activity relationship of ALT, three analogue peptides were synthesized. Their structures were as follows: H-Gly-Leu-Thr-OH (GLT), H-Leu-Thr-OH (LT) and H-Ala-Leu-OH (AL). The concentration-action relationships of the three analogue peptides on twitch contraction of the body-wall muscle of Urechis were compared with that of ALT. The effects of these analogues on the activity of AChE were also examined by the same procedures as those in the case of ALT.

4-2-8 Salines

The physiological saline for Urechis and Mytilus was ASW (see Chapter 2). The saline for Fusinus was low-Mg^{2+} (20 mM Mg^{2+}) ASW. This was made by replacing a part of $MgCl_2$ in the normal ASW with osmotically equivalent NaCl. In some experiments on the body-wall muscle of Urechis, high-Mg^{2+} (100 mM Mg^{2+}) ASW was used. This saline was made by replacing a part of NaCl in the normal ASW with osmotically equivalent $MgCl_2$.

4-3 Results

4-3-1 ACh-like activity

The ACh-like activity in peak I_1 was separated from the twitch-inhibiting activity at the first HPLC purification step (Fig. 4.2). The ACh-like substance and authentic ACh showed identical behavior on the cation-exchange HPLC (Fig. 4.3) and also showed identical effects on the inner circular body-wall

muscle of <u>Urechis</u>, the ABRM of <u>Mytilus</u> and the radula retractor muscle of <u>Fusinus</u> (Fig. 4.4). Thus, I concluded that the ACh-like substance in the peak I_1 is ACh.

4-3-2 The inhibitory peptide, ALT

The other purified substance in peak I_1 showed a potent inhibitory action on the inner circular body-wall muscle of <u>Urechis</u> (Fig. 4.7). The quantitative amino acid analysis of the purified substance showed the following amino acid composition normalized on Leu=1.0: Thr 0.9, Ala 0.7, Leu 1.0. The determined sequence and the detected amount (pmoles) of each amino acid in the sequence analysis were as follows: Ala(1537)-Leu(1709)-Thr(749). A molecular ion peak in the FAB-MS spectrum of the substance was at 304.1 m/z (M+H)$^+$. Based on these results, the structure of the substance was proposed to be H-Ala-Leu-Thr-OH.

The synthetic and native peptides showed identical behavior on both the C-18 reversed-phase and the C-8 reversed-phase HPLC (Fig. 4.8). The concentration-action relationships for the synthetic and native peptides in inhibiting twitch contraction of the body-wall muscle were also identical (Fig. 4.9). Thus, the structure of the peptide was confirmed to be H-Ala-Leu-Thr-OH (ALT).

ALT showed an inhibitory effect on contraction elicited by ACh in the body-wall muscle of <u>Urechis</u> (Fig. 4.10A). Further, contractions of the muscle in response to UEP$_A$ and UEP$_C$ were also inhibited by ALT (Fig. 4.10B,C). It is known that neuromuscular transmission is blocked by high-Mg^{2+} saline (see, Yanagawa <u>et al</u>., 1988; Minakata <u>et al</u>., 1992). In high-Mg^{2+} ASW (100 mM Mg2+), twitch contraction of the body-wall muscle in response to an electrical pulse having relatively long duration and strong intensity (5 msec, 25 V) was also inhibited by ALT (Fig. 4.11).

As shown in Fig. 4.12, ALT was suggested to inhibit the

activity of bovine AChE.

4-3-3 Structure-activity Relationship of ALT

The structure-activity relationship of ALT was examined by comparing the activity of ALT with three analogue peptides, GLT, AL and LT. GLT showed a twitch-inhibiting activity on the body-wall muscle of Urechis (Fig. 4.13). The concentration-action relations of four peptides including ALT are shown in Fig. 4.14. The activity of GLT was of dose-dependence, though the peptide was less potent than ALT. The other analogues, AL and LT, did not show any inhibitory activity even at high concentrations such as 10^{-5} M.

The effects of ALT and its three analogues on the activity of bovine AChE were examined in the same muscle. As shown in Fig. 4.15, the peptides were suggested to inhibit AChE activity. The potency order for the AChE-inhibiting activity was ALT > LT > GLT > AL.

4-4 Discussion

In the experiments in Chapter 2, I obtained three peaks (peak I_1, I_2 and I_3) of bioactivity from the flowthrough of the aqueous acetic acid extract of the ventral nerve cords of Urechis by using a gel-filtration column. The peaks showed an inhibitory action on twitch contraction of the inner circular body-wall muscle of Urechis The fractions of peak I_1 also showed an ACh-like activity on the ABRM of Mytilus and the heart of Meretrix lusoria. Peak I_1, however, did not show ACh-like activity on the body-wall muscle of Urechis. Furthermore, the ACh-like activity of peak I_1 was not eliminated by incubating it with AChE. Therefore, I speculated that the bioactive substance in peak I_1 was not ACh itself but a new cholinergic substance or a mixture of ACh and other substance(s).

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The results obtained in the present experiments indicate that peak I_1 contains both ACh and an inhibitory peptide. Muneoka et al. (1981) have suggested that ACh may be an excitatory transmitter at the neuromuscular junctions in the body-wall muscle of Urechis. The results of the present experiments support this notion.

In contrast to that a few result has been reported on the physiological and pharmacological studies of echiuroids, a number of reports are concerned with annelids which is a phylum closely related to the Echiura (for reviews; Tashiro and Kuriyama, 1978; Leake and Waker, 1980; Gardner and Waker, 1982). It has been suggested that ACh acts as an excitatory transmitter at the neuromuscular junctions in the somatic muscles of annelids. The body-wall strips of several species of the Annelida have been shown to be capable of responding to physiological concentration of ACh with a contraction. ACh contraction is poteniated by anti-AChE agents in the body-wall muscle of leech (Bhattacharya and Feldberg, 1958; Flacke and Yeoh, 1968a, 1968b) and earthworm (Botsford, 1941), and is blocked by cholinergic blocking agents (Andersson and Fange, 1967). ACh has excitatory action on certain neurons of the central nervous system of leech (Kerkut and Waker, 1967). Further, ACh, choline acetyltransferase and AChE are shown to be present in the nervous system of annelids (Sargent, 1977; Stenersen, 1979). These facts lead us to suppose that the principal excitatory neurotransmitter in the Echiura is ACh. From the present study, this seems to be the case.

The present work is of the first determination of ACh in the nervous system of the echiuroid, Urechis.

An inhibitory peptide was purified from Peak I_1 and its structure was determined to be H-Ala-Leu-Thr-OH. The amino acid sequence of the peptide is not homologous to any other peptides previously reported. ALT has a potent inhibitory action on twitch contraction of the body-wall muscle of Urechis, but does

not show any inhibitory action on molluscan muscles, such as the ABRM of Mytilus, the radula retractor muscle of Fusinus and the heart of Meretrix lusoria. ALT also showed an inhibitory action on contractions of the body-wall muscle of Urechis in response to ACh and UEPs.

Since twitch contraction of the body-wall muscle in response to strong electrical stimulation in high Mg^{2+} ASW is also inhibited by ALT, it is probable that the site of action of ALT is not presynaptic but postsynaptic. The facts that ALT inhibits contractions by ACh and UEPs also support this notion. It has been suggested that NA is an inhibitory neurotransmitter in the body-wall muscles of Urechis (Muneoka and Kamura, 1982a). I have shown that a large amount of NA is present in the ventral nerve cord of the animal (Chapter 2). ALT may be an inhibitory co-transmitter in the muscle.

ALT showed an inhibitory effect on bovine-AChE activity, though the hydrolysis of ACh by AChE was not completely inhibited by the peptide. Three analogue peptides, GLT, AL and LT, were examined on twitch contraction of the body-wall muscle and on AChE activity. Only GLT showed an inhibitory activity on twitch contraction though it was less potent than ALT. However, all the analogues inhibited AChE activity. From these results, it is considered that the active site of ALT for inhibiting contraction of the body-wall muscle of Urechis is different from that for inhibiting AChE activity.

It is very interesting that ALT and the analogue peptides inhibit AChE activity. ALT shows the most potent anti-AChE activity. A more potent AChE inhibitor might be able to make by changing the structure of ALT.
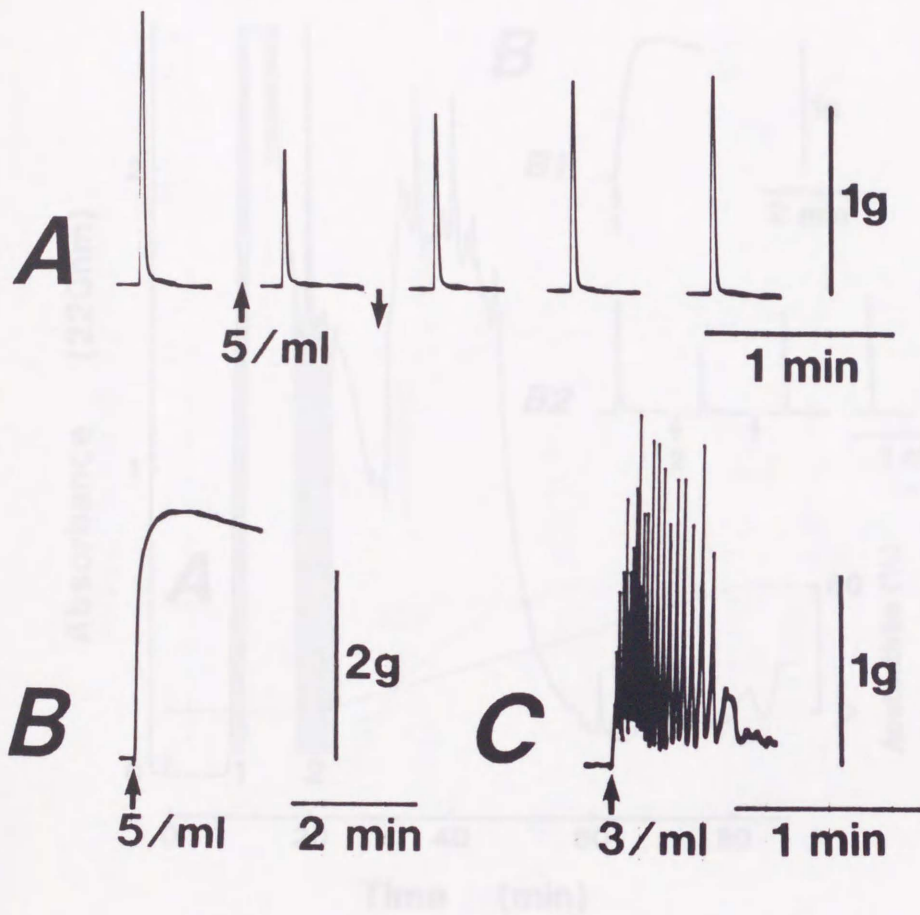
Fig. 4.1. Activities of the re-extracted peak I_1. A: inhibitory activity on twitch contraction of the body-wall muscle of Urechis. Twitch contraction was evoked by applying an electrical pulse (15 V, 3 msec) of stimulation to the muscle at 10 min intervals. The sample was introduced 8 min prior to evoking the contraction and washed out soon after recording the contraction. B: contractile activity on the ABRM of Mytilus. C: contractile activity on the radula retractor muscle of Fusinus.
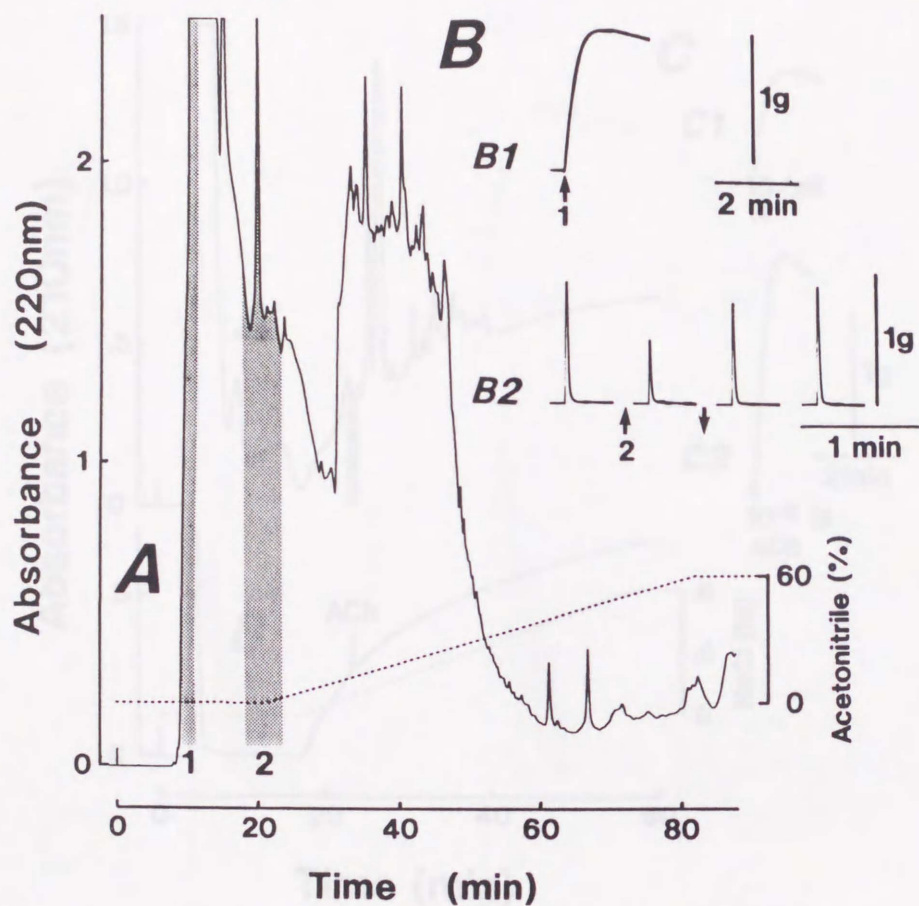
<u>Fig. 4.2.</u>   The first step of HPLC purification of the re-extracted peak I$_1$.   A: chromatogram.   HPLC conditions are given in text.   Two active peaks were eluted (stippled areas).   The former was termed peak 1, and latter was termed peak 2.   B:activities of peak 1 and 2.   B1: contractile activity of peak 1 on the ABRM of <u>Mytilus</u>.   B2: inhibitory activity of peak 2 on twitch contraction of the body-wall muscle of <u>Urechis</u>.   The procedures are the same as in Fig. 4.1.
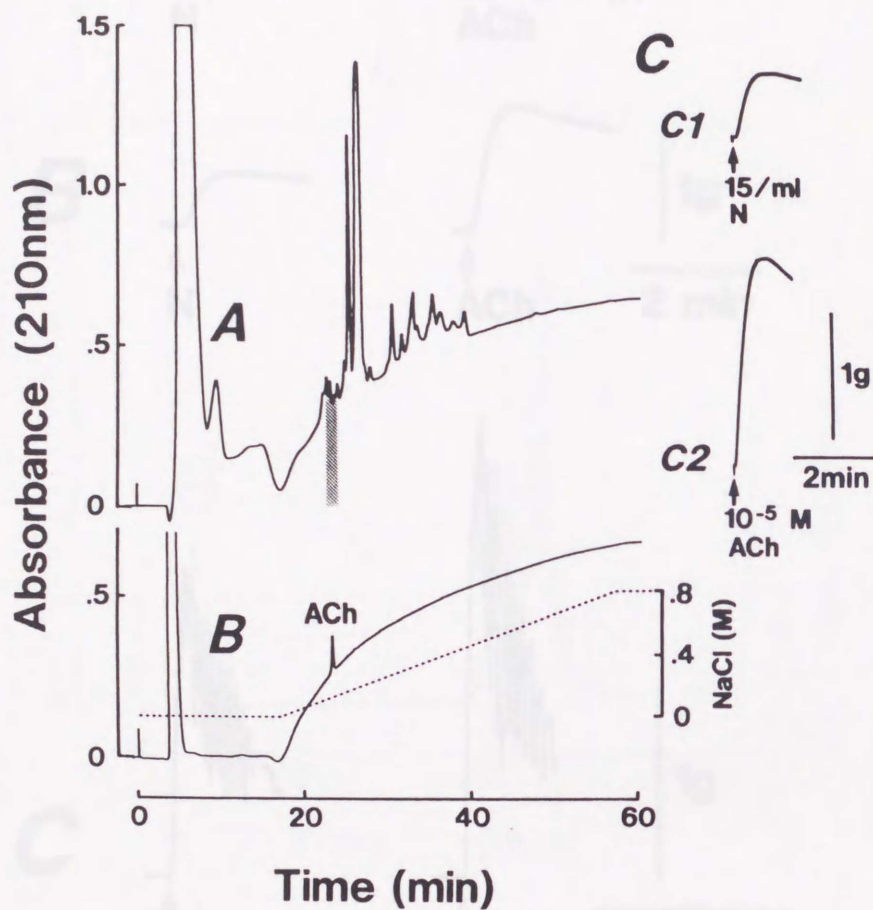
Fig. 4.3.   Comparison between properties of native ACh-like
substance (N) of peak 1 and those of ACh.   A: chromatogram of the
fraction of peak 1 on a cation-exchange HPLC.   HPLC conditions
are given in text.   The ACh-like substance was eluted in stippled
area.   B: chromatogram of 300 nmoles of ACh.   C1: contractile
activity of the fraction of the stippled area in A on the ABRM of
Mytilus.   Dose is 15 nerve cods/ml equivalent.   C2: contractile
activity of 10^{-5} M ACh obtained in B on the ABRM of Mytilus.
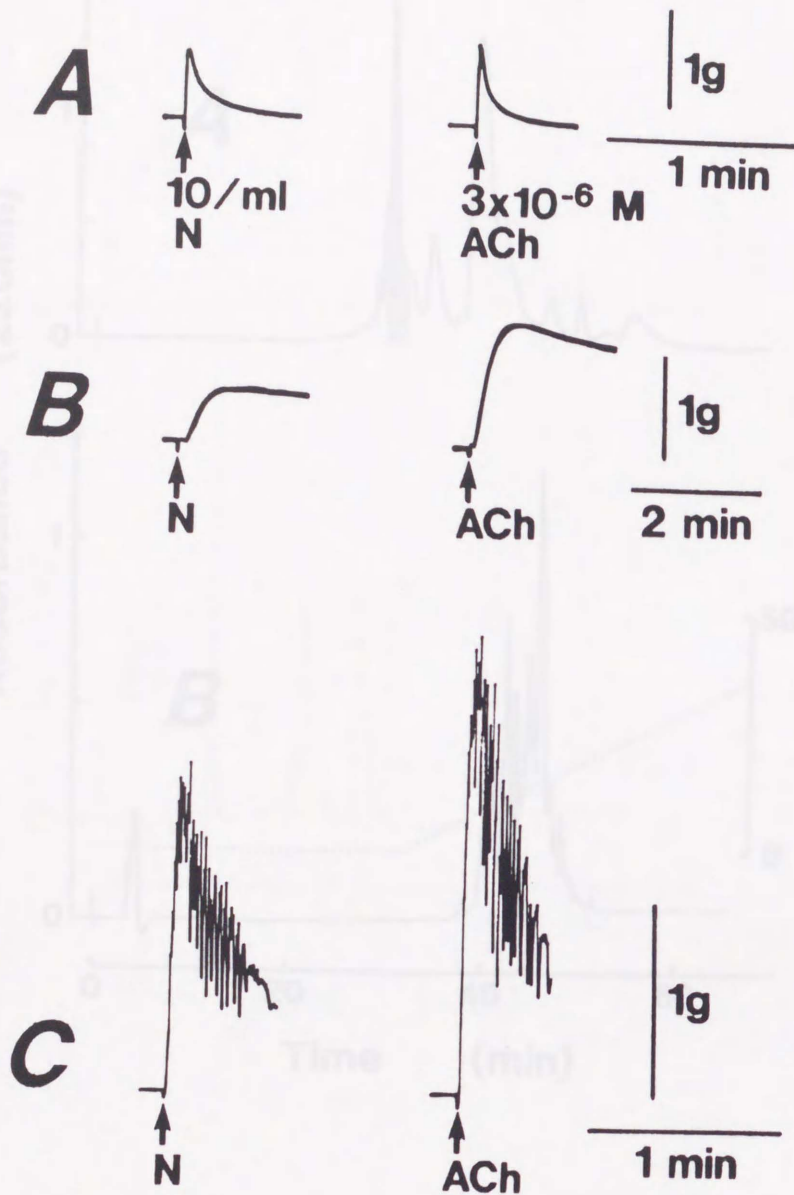
<u>Fig. 4.4.</u>   Activities of the native ACh-like substance (N) and
ACh on the body-wall muscle of <u>Urechis</u> (A), the ABRM of <u>Mytilus</u>
(B) and the radula retractor muscle of <u>Fusinus</u> (C). Dose of the
native ACh-like substance was 10 nerve cords/ml equivalent.
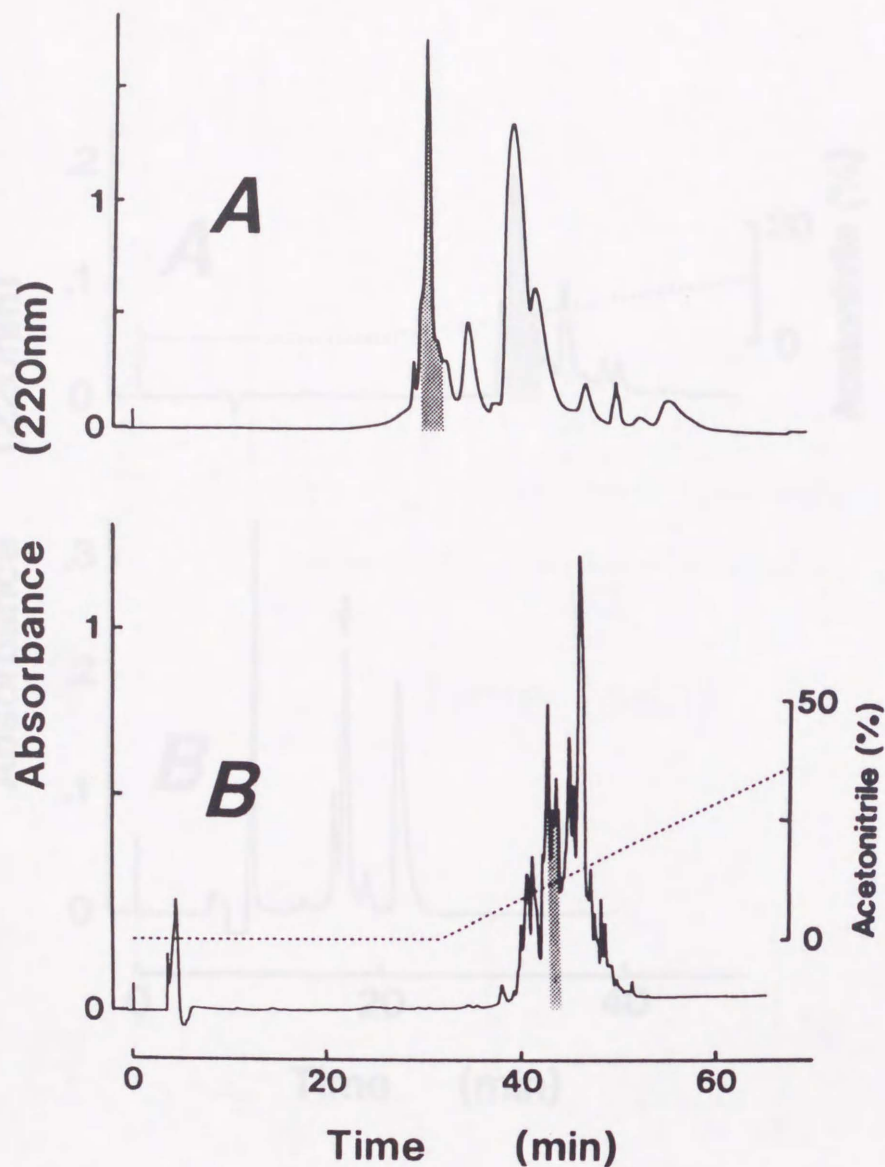
Fig. 4.5. Chromatograms of the second step (A) and the third step (B) of HPLC purification of ALT. A: gel-filtration column. B: C-18 reversed-phase column. At each step, the inhibitory substance on twitch contraction of the body-wall muscle of Ure-chis was eluted at the stippled area. HPLC conditions are given in text.

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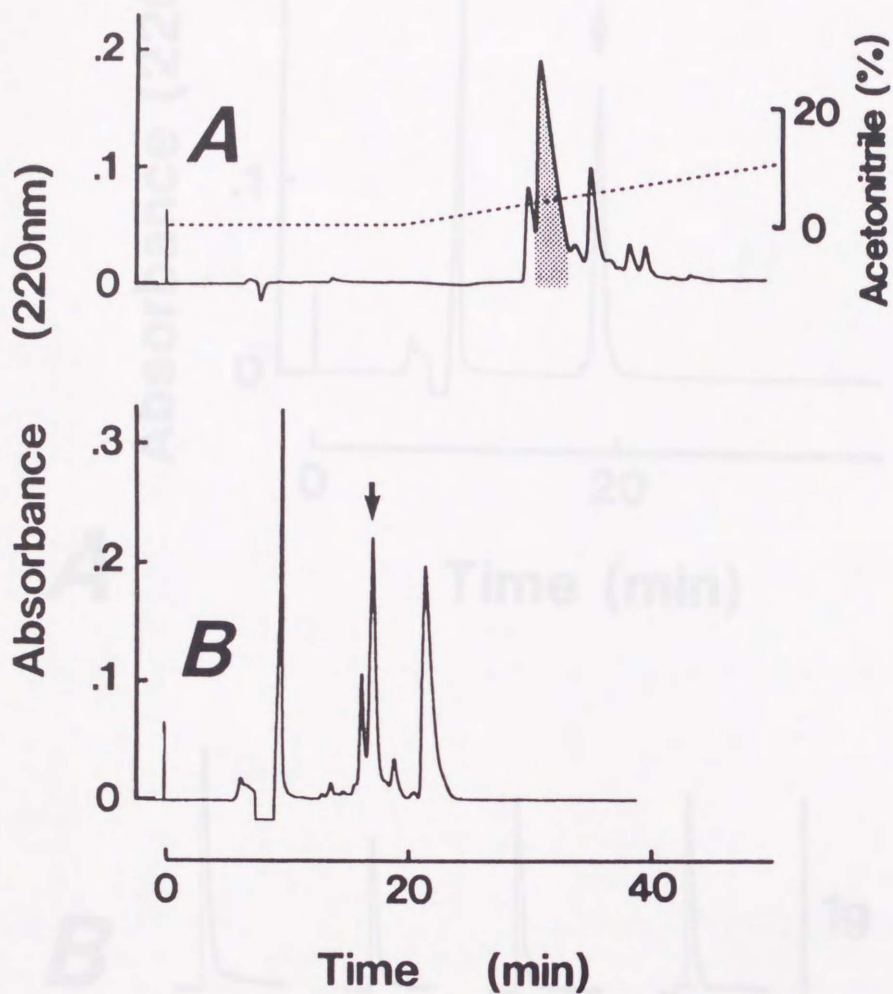
Fig. 4.6.   Chromatograms of the fourth step (A) and the fifth step (B) of HPLC purification of ALT.   A: C-8 reversed-phase column.   The inhibitory substance was eluted at the stippled area.   B: C-18 reversed-phase column.   The absorbance peak indicated by arrow corresponded to activity peak.   HPLC conditions are given in text.

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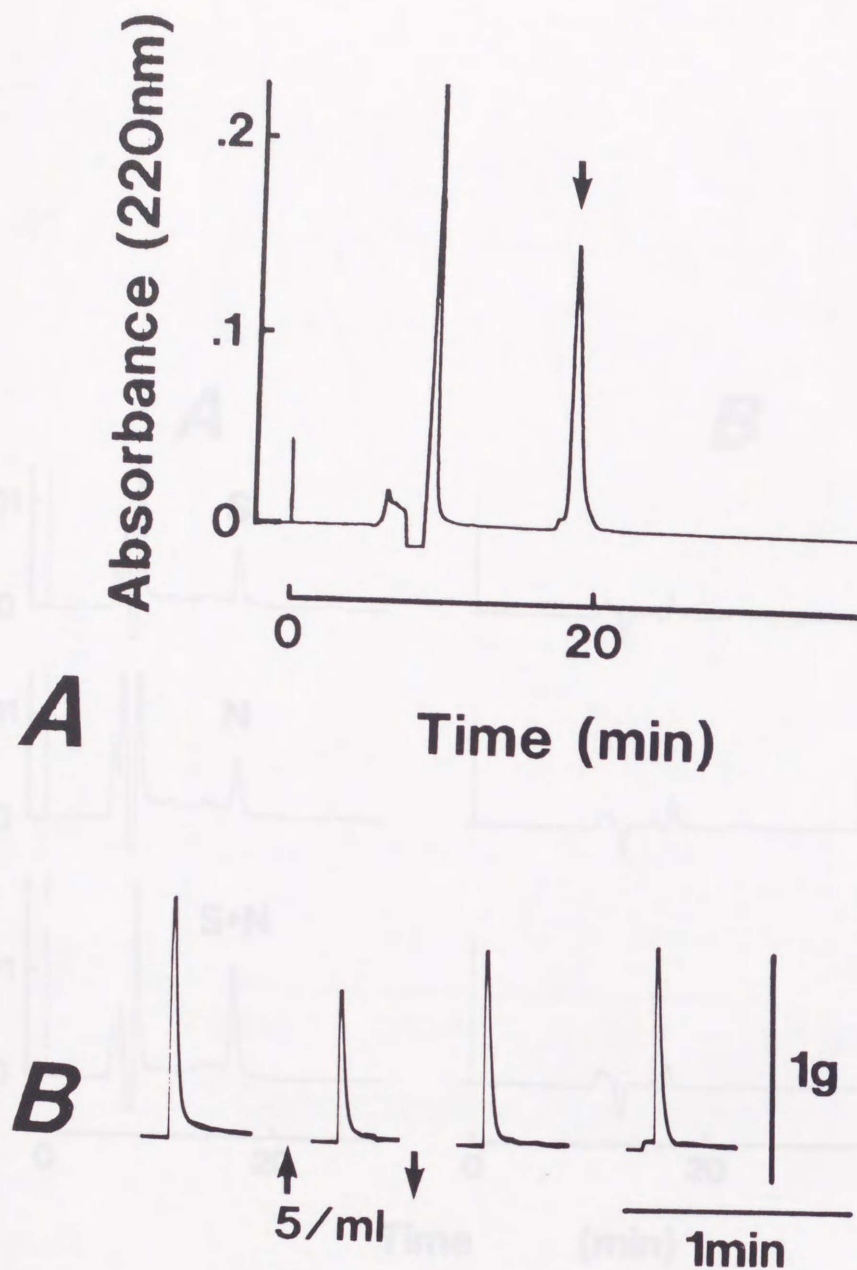
Fig. 4.7. The sixth (final) step of purification of ALT. A: chromatogram. The single absorbance peak indicated by arrow corresponded to the activity peak. HPLC conditions are given in text. B: inhibitory activity of the purified ALT on twitch contraction of the body-wall muscle of Urechis. Dose was 5 nerve cords/ml equivalent. The other procedures are the same as in Fig. 1.
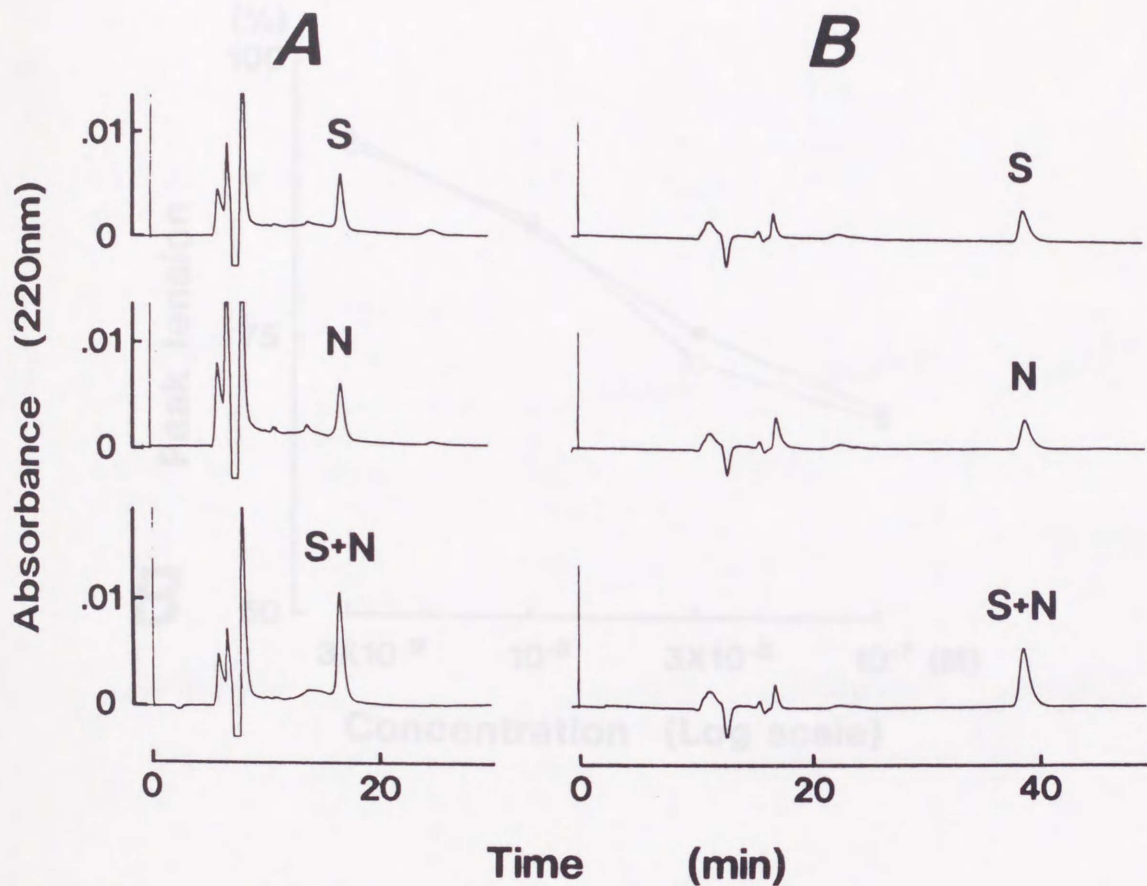
Fig. 4.8. Comparison between behavior of the synthetic (S) and that of native (N) ALT on HPLC. A: C-18 reversed-phase HPLC. The column was eluted isocratically with 9% acetonitrile in 0.1% TFA (pH 2.2). B: C-8 reversed-phase HPLC. The column was eluted with 3% acetonitrile in 0.1% TFA. S+N, a mixture of the synthetic and native ALT.
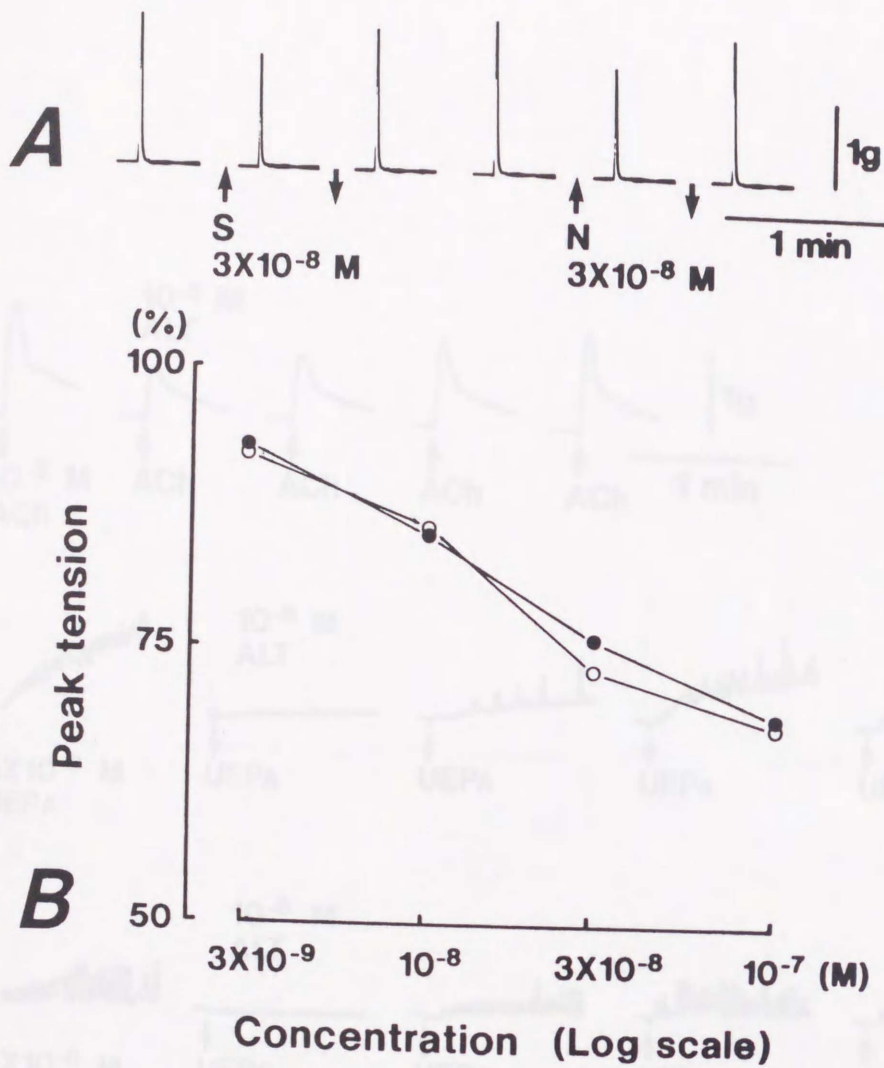
Fig. 4.9. Comparison between inhibitory effect of the synthetic and that of the native ALT on twitch contraction of the body-wall muscle of Urechis. A: activity of 3 X 10^{-8} M of synthetic (S) and native (N) ALT on twitch contraction of the muscle. B: concentration-action relationships of the synthetic (closed circle) and native (open circle) ALT. The procedures for recording the twitch contraction are the same as in Fig. 1. The concentrations of the native peptide were estimated from the results of the amino acid analysis.
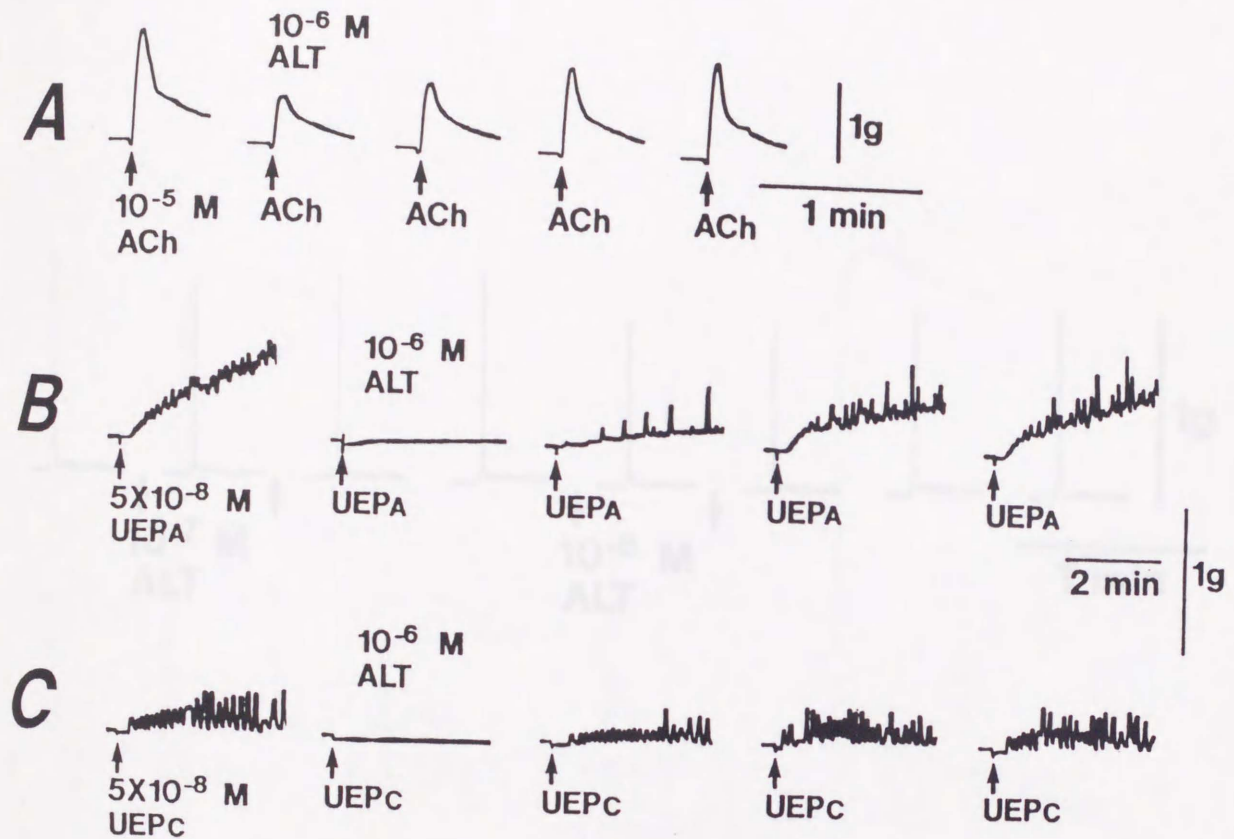
Fig. 4.10. Effect of ALT on contractions induced by ACh (A), UEP$_A$ (B) and UEP$_C$ (C) in the body-wall muscle of <u>Urechis</u>. ACh (10^{-5} M) was applied to the muscle for 30 sec at 15 min intervals. UEP$_A$ (5 X 10^{-8} M) and UEP$_C$ (5 X 10^{-8} M) were applied to the muscle for 3 min at 15 min intervals. 10^{-6} M of ALT was applied 10 min prior to the second contraction in each column and washed out soon after the contraction.
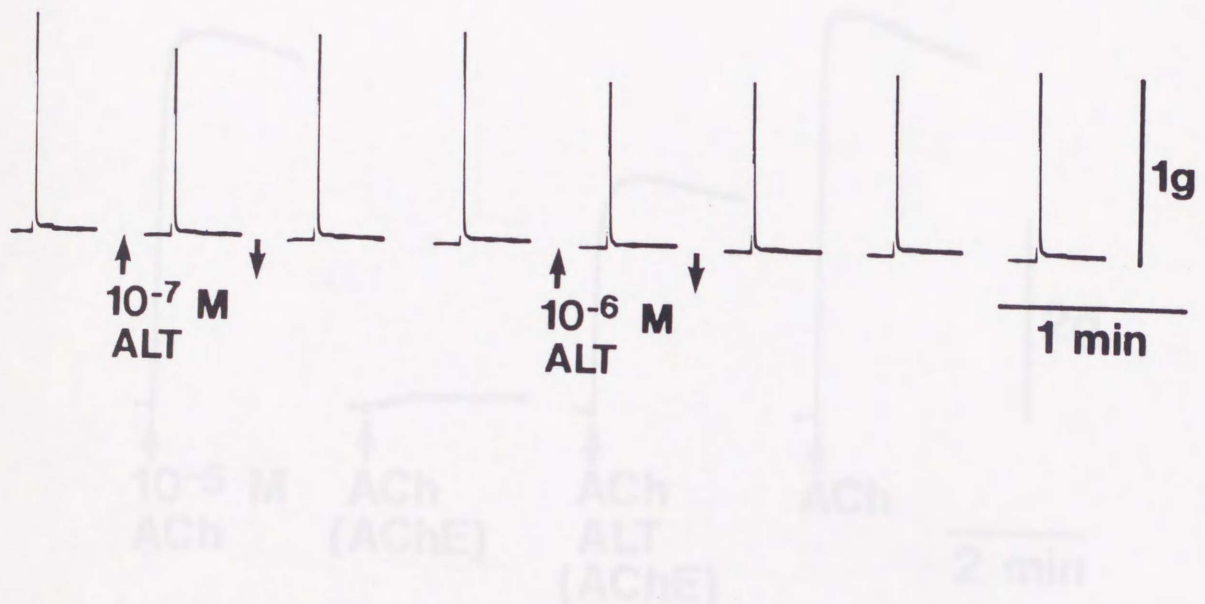
Fig. 4.11. Activity of ALT on twitch contraction of the body-wall muscle of Urechis in high-Mg^{2+} ASW. Other procedures are the same as in Fig. 1.
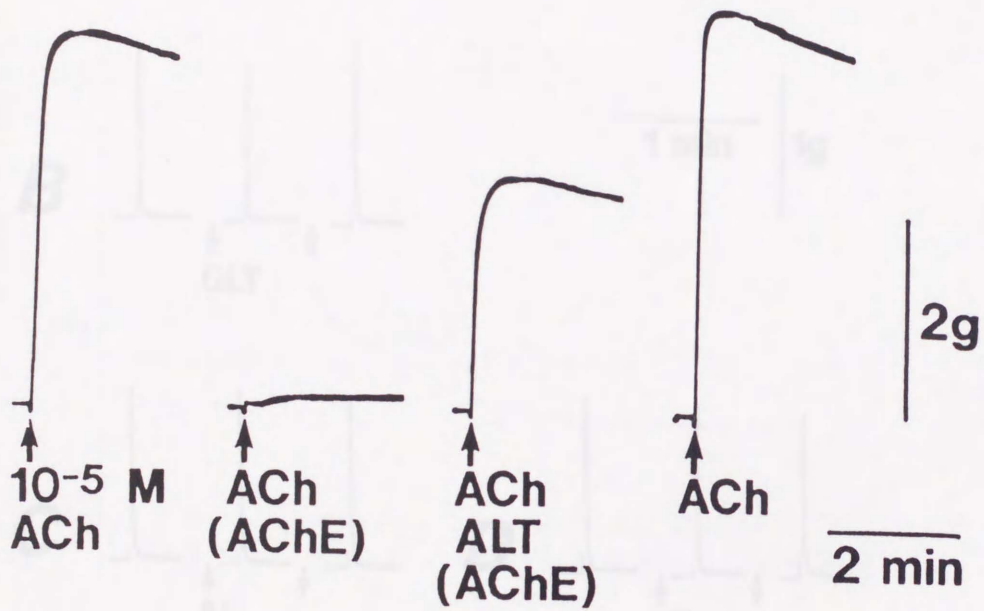
Fig. 4.12.   Contractile effects of AChE-treated ACh and AChE-treated mixture of ACh and ALT on the ABRM of _Mytilus_.
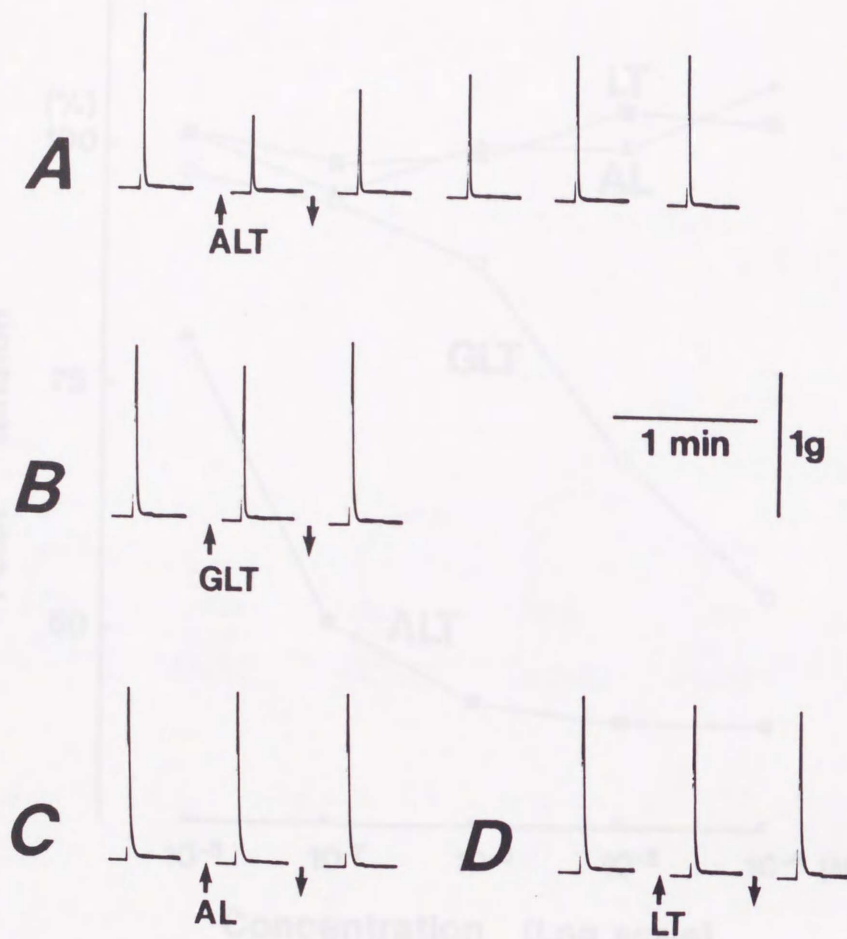
Fig. 4.13. Effects of 10^{-7} M of ALT and its analogues (GLT, AL and LT) on twitch contraction of the inner circular body-wall muscle of Urechis. The procedures for recording the twitch contraction are the same as in Fig. 1.

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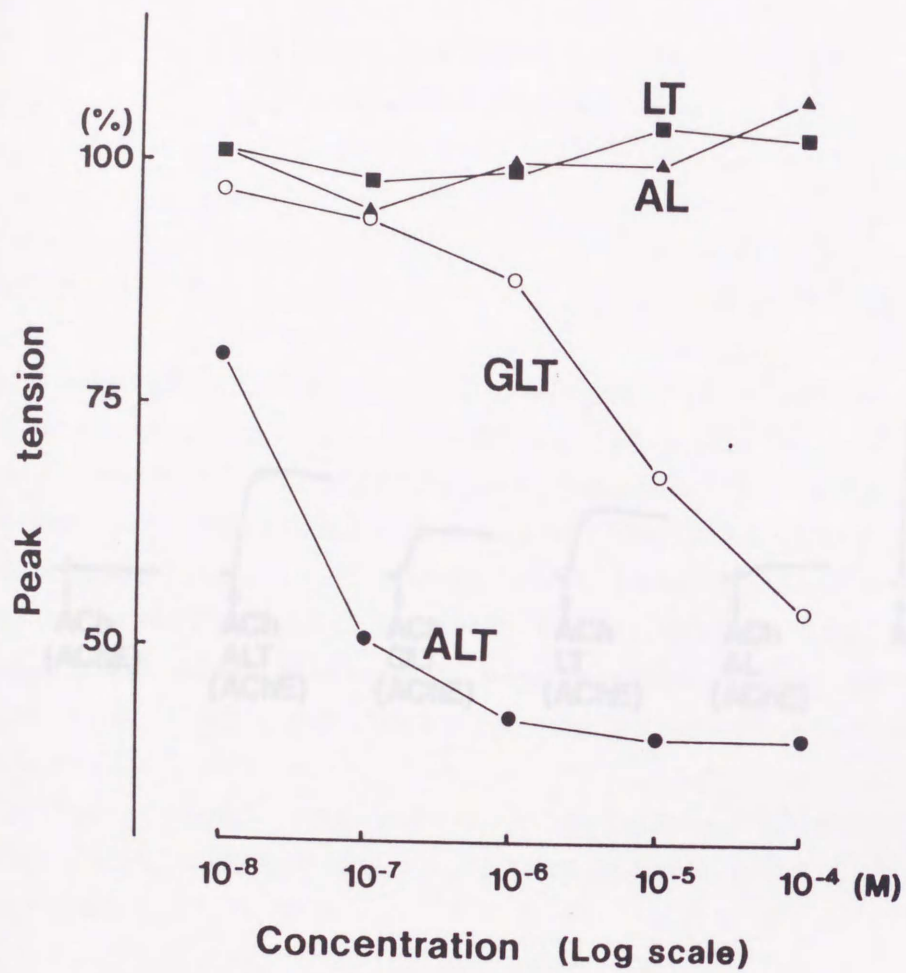
Fig. 4.14. Concentration-action relationships of ALT (closed circle), GLT (open circle), AL (closed triangle) and LT (closed square) on twitch contraction of the body-wall muscle of Urechis. The procedures for recording the twitch contractions are the same as in Fig. 1.

BIOACTIVE PEPTIDES EXTRACTED WITH
AQUEOUS ACETIC ACID

4.1 Introduction

In Chapter 3, I showed the biological activities of aqueous acetic acid extracts of the central nerve cords of *Mytilus* on the isolated inner circular bodywall muscle of the same, and suggested that the material retained by the C-18 cartridges contains peptidic substances. That is, the retained material showed a strong acetylcholine-like and non-acetylcholine activities on the muscle, and its bioactivities were drastically destroyed by treating the material with the peptidase inhibitor.

In the present experiments, I attempted to purify the bioactive peptidic substances in the retained material by using an HPLC system.

4.2 Materials and Methods

| ACh | ACh | ACh | ACh | ACh | ACh | ACh |
| 10⁻⁵ M | (AChE) | ALT (AChE) | GLT (AChE) | LT (AChE) | AL (AChE) | |

1g
2 min

Fig. 4.15.   Contractile effects of AChE-treated ACh and AChE-treated mixtures of ACh and ALT-related peptides (ALT, GLT, AL and LT) on the ABRM of *Mytilus*. The procedures are the same as in Fig. 12.

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CHAPTER 5

BIOACTIVE PEPTIDES EXTRACTED WITH
AQUEOUS ACETIC ACID

5-1 Introduction

In Chapter 2, I showed the biological activities of aqueous acetic acid extracts of the ventral nerve cords of <u>Urechis</u> on the isolated inner circular body-wall muscle of the worm, and suggested that the material retained by the C-18 cartridges contains peptidic substances. That is, the retained material showed a potent contractile activity and twitch-modulating activities on the inner circular body-wall muscle, and these activities were completely destroyed by treating the material with the peptidase subtilisin.

In the present experiments, I attempted to purify the bioactive peptidic substances in the retained material by using an HPLC system.

5-2 Materials and Methods

5-2-1 Extraction and Separation of the Bioactive Peptides

Aqueous acetic acid extracts from the ventral nerve cords of <u>Urechis</u> (1,500 animals) were applied to C-18 cartridges (Sep-Pak, Waters). The retained material eluted with 50% methanol was then applied to a gel-filtration column (Sephadex G-15), and fractions of 4ml each were collected. The biological activities of the fractions were examined on the isolated inner circular body-wall muscle of <u>Urechis</u>. I obtained a contractile peak (fraction 24-

40) and two twitch-contraction-modulatung peaks, one is of inhibition and the other is of potentiation, as described in Chapter 2. Although the maximal activities of the inhibitory and potentiating peaks were found at fractions 51 and 57, respectively, the two peaks seemed to be eluted partially overlapping with each other. Therefore, we divided all the active fractions into two groups, group A (fraction 24-40) and group B (fraction 49-62). The former was the group having contractile activity and the latter was the group having modulating activities. The active fractions of each group were pooled and then subjected to an HPLC system (TRI ROTAR-VI, Jasco) to purify the bioactive substances in it. The columns used for the purification were two kinds of reversed-phase column (C-8 and C-18 column), a cation-exchange column and an anion-exchange column. The methods of bioassay in these purification experiments were the same as those used in Chapter 2. As shown in Fig 5.1, thirteen and twelve bioactive peaks were separated from group A and B, respectively. The structures of six peptides were determined from these groups. They were two contractile peptides (Urechis excitatory peptides, UEP, A and C) and four twitch-contraction-inhibiting peptides (FSAFRADLamide, REFWK, FRVF and FRF).

5-2-2 Purification of UEP_A, UEP_C and FSAFRADLamide

UEP_A---The contractile peptides UEP_A was purified from group A through four HPLC-separation steps. At the first step, a C-18 reversed-phase column (Finepak SIL $C_{18}S$, 4.6 X 150 mm, Jasco) was used. The column was eluted with a 60-min linear gradient of 0-60% acetonitrile in 0.1% TFA at pH 2.2 (Fig. 5.2). I obtained three peaks (peak A, B and C) of contractile activity (Fig. 5.3). UEP_A was isolated from the fractions of peak A, and UEP_C and FSAFRADLamide were isolated from peak B. Their purification procedures are summarized in Fig. 5.4. The fractions of peak A were subjected to the next separation step of HPLC with a

cation-exchange column (TSKgel SP-5PW, 7.5 X 75 mm, Tosoh). The column was eluted with a 60-min linear gradient of 0.1-0.4 M NaCl in 10 mM phosphate buffer (pH 6.8). A contractile peak was obtained at around 0.26 M NaCl. At the third step, I used the other C-18 reversed phase column (TSKgel ODS80T$_M$, 4.6 X 150 mm, Tosoh). The column was eluted with a 50-min linear gradient of 15-25% acetonitrile in 0.1% TFA. I obtained an absorbance peak which corresponded to the peak of contractile activity (Fig. 5.5A). Final purification was performed by applying the active material again to the reversed-phase column used at the first step and eluting isocratically with 19.5% acetonitrile in 0.1% TFA (Fig. 5.5B).

UEP$_C$---As mentioned above, three peaks of contractile activity were observed at the first step of purification of UEP$_A$, and the peptide was obtained from peak A. The contractile peptide UEP$_C$ was obtained from peak B. In the next step of purification of UEP$_C$, peak B was applied to an anion-exchange column (TSKgel DEAE-5PW, 7.5 X 75 mm, Tosoh) and eluted with a 60-min linear gradient of 0-0.3 M NaCl in 10 mM Tris buffer (pH 9.5). Contractile activity was observed in two groups of fractions, flow-through fractions and fractions eluted at around 0.1 M NaCl. The fractions of the former peak was found to have an inhibitory peptide, FSAFRADLamide in addition to a contractile peptide which was termed UEP$_B$. However, UEP$_B$ was failed to purify. The active material in the fractions of the latter peak eluted at around 0.1 M NaCl was then applied to a reversed-phase column (TSKgel ODS80T$_M$) and eluted with an 80-min linear gradient of 5-25% acetonitrile in 5 mM phosphate buffer (pH 6.8). An active peak which corresponded to an absorbance peak was obtained (Fig. 5.6A). The active peak was subjected to the final HPLC separation. The column (Finepak SILC$_{18}$S) was eluted with an isocratic system consisting of 22% acetonitrile and 0.1% TFA (Fig. 5.6B).

FSAFRADLamide---As described above, the inhibitory peptide

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FSAFRADLamide was isolated from the flowthrough fractions at the second step of purification of UEP$_C$. At the third step of purification of FSAFRADLamide, the flowthrough fractions was applied to a C-18 reversed-phase column (TSKgel ODS80T$_M$) and eluted with a 50-min linear gradient of 0-50% acetonitrile in 0.1% TFA. The contractile peak was then applied to a cation-exchange column (TSKgel SP-5PW) and eluted with a 60-min linear gradient of 0.1-0.4 M NaCl in 10 mM phosphate buffer (pH 6.7). Biological activities were observed in two groups of fractions, twitch-contraction-inhibiting activity in the flowthrough fractions and contractile activity (UEP$_B$) in the fractions eluted at around 0.2 M NaCl. The inhibitory material in the former group of fractions was then applied to a C-8 reversed-phase column (Finepak C$_8$5, 4.6 X 250 mm, Jasco) and eluted with a 60-min linear gradient of 10-40% acetonitrile in 0.1% TFA. After two more purification steps by using the C-18 reversed-phase column used at the third step, the active peak was subjected to final HPLC separation (Fig. 5.4). The column (Finepak C$_8$5) was eluted isocratically with 25.5% acetonitrile in 0.1% TFA (Fig. 5.7).

5-2-3 Purification of REFWK, FRVF and FRF

REFWK---The twitch-contraction inhibiting peptides, REFWK, FRVF and FRF, were purified from group B of the gel-filtration fractions through four HPLC purification steps. The fractions of group B were pooled, concentrated and loaded onto a C-18 reversed phase column (Finepak SIL C$_{18}$S). The column was eluted with a 60-min linear gradient of 0-60% acetonitrile in 0.1% TFA (Fig. 5.8C). I obtained four bioactive peaks (peaks D, E, F and G). Peak D and F were shown to have an inhibitory effect on twitch contraction of the inner circular body-wall muscle of Urechis, and peak E and G were shown to have a potentiating effect on the contraction (Fig. 5.8A,B). REFWK was isolated from the fractions of peak D, and FRVF and FRF were isolated from peak F (Fig. 5.9).

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At the second purification step of REFWK purification, a cation-exchange column (TSKgel SP-5PW) was used, and the column was eluted with a 70-min linear gradient of 0-0.7 M NaCl in 10 mM phosphate buffer (pH 6.8). The active peak was then applied to another C-18 reversed-phase column (TSKgel ODS80T$_M$) and eluted isocratically with 18% acetonitrile in 0.1% TFA. The final purification was performed by applying the active material to the other C-18 reversed-phase column (Asahipak ODP-50, 6.0 X 250 mm, Asahi Chemical Industry) and eluting isocratically with 21% acetonitrile in 0.1% TFA (Fig. 5.10).

FRVF and FRF---Peak F was applied to a cation-exchange column (TSKgel SP-5PW) and eluted with a 70-min linear gradient of 0-0.7 M NaCl in 10 mM phosphate buffer (pH 6.8). I obtained two bioactive peaks, one eluted at around 0.03 M NaCl and the other at around 0.06 M NaCl. FRVF was isolated from the former peak and FRF from the latter peak.

The former peak was then applied to a C-18 reversed-phase column (TSKgel ODS80T$_M$). The column was eluted isocratically with 22% acetonitrile in 0.1% TFA. The final purification of FRVF was performed by applying the active material to the other reversed-phase column (Asahipak ODP-50) and eluting isocratically with 22.5% acetonitrile in 0.1% TFA (Fig. 5.11).

The latter peak obtained by the cation-exchange HPLC was applied to a reversed-phase column (TSKgel ODS80T$_M$) and eluted with 22% acetonitrile in 0.1% TFA. The final purification of FRF was performed by applying the active material to the same reversed-phase column and eluting with 21% acetonitrile in 0.1% TFA (Fig. 5.12).

5-2-4 Bioassay

After each HPLC purification step, biological activities of the fractions were examined on the isolated small bundle (1.5 mm in width and 20 mm in length) of the inner circular body-wall

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muscle of <u>Urechis</u>. The methods of isolating and tension record-
ing from the muscle bundle were essentially the same as those
described in Chapter 2. Twitch inhibiting activities were exam-
ined on twitch contraction of the muscle. Twitch contraction was
elicited at 10 min intervals by stimulating the muscle bundle
with an electrical pulse (15 v, 3 msec). Each bioassay material
was applied to the bundle 8 min prior to the electrical stimula-
tion.

5-2-5 Enzyme Treatment

After adjusted to pH 7.8 by adding Tris-HCl (10 mM), ali-
quots of the purified substances were incubated with subtilisin
(10^{-5} g/ml) for 30 min at 30°C. The incubated materials were
then boiled for another 30 min. The control material was only
boiled for 30 min after adjusted to pH 7.8. The enzymes were
purchased from Boehringer Mannheim Biochemica.

5-2-6 Structure Determination.

The purified peptidic substances were subjected to amino
acid sequence analysis by the automated Edman degradation method
with a gas-phase sequencer (Applied Biosystems 470A) coupled with
a PTH-amino acid analyzer (Applied Biosystems 120A), quantitative
amino acid analysis (Hitachi L-8500) and fast atom bombardment
mass spectrometric (FAB-MS) measurement (JEOL JMS HX-100). Thus,
the probable structures of the peptides were determined. One of
the two excitatory peptides, UEP$_C$, was synthesized by a solid-
phase peptide synthesizer (Applied Biosystems 430A) followed by
HF cleavage and HPLC purification, All of the four inhibitory
peptides were also synthesized at the Peptide Institute (Osaka,
Japan). The behavior of the synthetic peptides, except FSAFRAD-
Lamide, on a reversed-phase and an ion-exchange HPLC was com-
pared with that of the native peptides. Further, the relation-
ships between dose and effect for the synthetic and native pep-

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tides were also compared by using an inner circular body-wall muscle of Urechis.

5-2-7 Pharmacological Experiments

The synthetic peptides of FRVF and FRF were tested not only on twitch contraction of the inner circular body-wall muscle but on proline contraction of the longitudinal and lateral body-wall strips of Urechis. The body-wall strips were dissected by cutting the body-wall lengthwise (longitudinal strip) or crosswise (lateral strip). The longitudinal strip was obtained from the ventral part of the body and the lateral strip from the middle part. The dissected strips were 2.0-2.5 mm in width and 20-25 mm in length. The methods for recording of tension changes from them were the same as those of the inner circular body-wall muscle (see Chapter 2). UEP_A and UEP_C were tested on phasic contraction of the ABRM of the bivalve mollusc Mytilus edulis, twitch contractions of the radula retractor muscle of the prosobranch mollusc Fusinus ferrugineus, spontaneous contractions of the heart of the bivalve mollusc Meretrix lusoria, twitch contraction of the longitudinal body-wall muscle of the polichaeta annelid Marphysa sanguinea and spotaneous contractions of the hind gut of the insect Gryllus bimaculatus. FSAFRADLamide, REFWK, FRVF and FRF were also tested on the ABRM of Mytilus, the radula retractor muscle of Rapana thomasiana and spontaneous contractions of the small intestine of the African clawed toad Xenopus laevis. The methods employed for the ABRM and the radula retractor muscles were the same as those of Muneoka and Twarog (1977) and Muneoka and Kobayashi (1980), respectively. The preparation of the hind gut of Gryllus and the small intestine of Xenopus were made by dissecting them out at about 20 mm length and tying with cotton threads at both ends. The preparation was suspended in a 10-ml aerated organ bath filled with a physiological saline. The peptides were applied to the preparation by

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injecting their stock solutions into the aerated bath.

The pharmacological experiments, as well as the bioassay experiments, were carried out at room temperature (20-25°C).

5-2-8 Salines

The physiological saline for the muscles of Urechis, Mytilus, Meretrix, Rapana and Marphysa, was ASW (see Chapter 2). The saline for the muscles of Fusinus was low-Mg^{2+} (20 mM Mg^{2+}) ASW (see Chapter 4). The composition of the physiological saline for the hind gut of Gryllus was as follows: 103 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$, 17 mM $MgCl_2$, 10 mM sucrose and 10 mM Hepes-NaOH (pH 6.5). The composition of the saline for the intestine of Xenopus was as follows: 110 mM NaCl, 1.8 mM KCl, 1.2 mM $CaCl_2$, 10 mM glucose and 10 mM Hepes-NaOH (pH 7.4).

5-3 Results

5-3-1 The Excitatory Peptides, UEP$_A$ and UEP$_C$

The contractile activities of the purified substances on the inner circular body-wall muscle of Urechis were not affected by boiling, but were completely eliminated by treating them with the peptidase subtilisin, indicating that they were peptidic substances. Therefore, I termed them Urechis excitatory peptide A and C (UEP$_A$ and UEP$_C$).

Quantitative amino acid analysis of UEP$_A$ showed the following amino acid composition normalizing on Lys = 2.0: $Asp_{2.1}$ $Ser_{1.9}$ $Gly_{2.2}$ $Ala_{4.0}$ $Leu_{0.8}$ $Tyr_{0.9}$ $Lys_{2.0}$. The determined sequence and detected amount (pmoles) of each amino acid were as follows:Ala(578)-Lys(145)-ND-Ser(126)-Gly(250)-Lys(129)-Trp(96)-Ala(343)-Asn(177)-Ser(91)-Tyr(148)-Leu(164)-ND-Ala(181)-Gly(104)-Ala(164)-Asn(58), in which ND means "not detected". Asn is detected as Asp in the amino acid analysis. Trp is usually not detected in amino acid analysis, because it is easily oxidized

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during the analysis. Cys is not detected in either amino acid or amino acid sequence analysis; therefore there is no inconsistency between the result of the amino acid analysis and that of the amino acid sequence analysis. The amino acid residues which were not detected in the amino acid sequence analysis were assumed to be Cys. Supporting this assumption, 1.6 of $CysO_3H$ (normalized on Lys = 2) were detected when the amino acid analysis was performed on the active substance treated with performic acid.

A molecular ion peak in the FAB-MS spectrum of the active substance was at 1734.7 m/z $(M+H)^+$, suggesting that the substance is cyclized with a S-S bond between the two cysteine residues and that its C-terminus is free, not amidated. Thus, I propose that the structure of UEP_A is as follows.

H-Ala-Lys-Cys-Ser-Gly-Lys-Trp-Ala-Asn-Ser-Tyr-Leu-Cys-
    Ala-Gly-Ala-Asn-OH
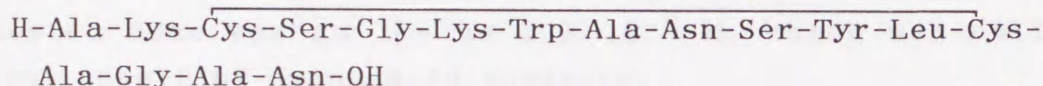
Quantitative amino acid analysis of normal and oxidized (performic-acid-treated) UEP_C showed the following amino acid compositions, respectively (normalized on Leu = 1.0): $Asp_{2.2}$ $Thr_{1.7}$ $Ile_{0.9}$ $Leu_{1.0}$ $Phe_{1.3}$ and $Asp_{2.2}$ $Thr_{1.7}$ $Ile_{1.0}$ $Leu_{1.0}$ $Phe_{1.0}$ $CysO_3H_{2.0}$. The determined sequence and detected amount (pmoles) of each amino acid in the amino acid sequence analysis were as follows: Thr(35)-Phe(98)-ND-Thr(21)-Ile(52)-Asp(18)-Leu(24)-Asn(54). From these results, the sequence of UEP_C is suggested to be Thr-Phe-Cys-Thr-Ile-Asp-Leu-Asn-Cys. That is, the peptide has a Cys at each of the third and last (ninth) positions.

A molecular ion peak in the FAB-MS spectrum of UEP_C was at 1027.4 m/z $(M+H)^+$, suggesting that it is cyclized with a S-S bond between the two cysteine residues and that the C-terminus is free. Thus, I propose that the structure of UEP_C is as follows.

H-Thr-Phe-Cys-Thr-Ile-Asp-Leu-Asn-Cys-OH
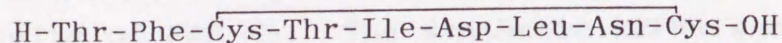
The synthetic UEP_C showed a potent contractile activity on the inner circular body-wall muscle of Urechis. The dose-re-

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sponse relation of the peptide was found to be identical with that of the native UEP$_C$ (Fig. 5.13). As shown in Fig. 5.14, a mixture of the synthetic peptide and the native peptide showed a single absorbance peak when applied to the C-18 reversed-phase column and the cation-exchange column (Fig. 5.14).

UEP$_C$ showed contractile action on the inner circular body-wall muscle at 5 X 10^{-9} M or higher concentrations. However, the peptide, even at 10^{-5} M, did not show any contractile or contraction modulating activity on the ABRM of Mytilus, the radula retractor muscle of Fusinus, the heart of Meretrix, the longitudinal muscle of Marphysa and the hind gut of Gryllus. Using the native UEP$_A$, I also examined its effect on the above muscles of the non-echiuroid animals but I could not find any effect, though the tested dose was as low as 5 X 10^{-8} M, which was estimated from the result of amino acid analysis.

5-3-2 The Inhibitory Peptides, FSAFRADLamide, REFWK, FRVF and FRF

The results of the structure analyses of all of the inhibitory peptides were shown in Table 5.1. The results indicate that their probable structures are as follows.

H-Phe-Ser-Ala-Phe-Arg-Ala-Asp-Leu-NH2 (FSAFRADLamide)

H-Arg-Glu-Phe-Trp-Lys-OH (REFWK)

H-Phe-Arg-Val-Phe-OH (FRVF)

H-Phe-Arg-Phe-OH (FRF)

In the amino acid sequence analysis of FSAFRADLamide, Leu residue was not detected. I concluded, however, that this peptide have Leu-NH$_2$ at the C-terminal part from the results of the amino acid analysis and FAB-MS measurement.

The penultimate amino acid residue of REFWK, which was not detected in amino acid sequence analysis, was presumed to be Trp from the results of FAB-MS. The synthetic REFWK showed a potent inhibitory activity on twitch contraction of the body-wall muscle. The dose-response relation of the peptide was found to be

identical with that of the native REFWK (Fig. 5.15). As shown in Fig. 5.16, the synthetic and native peptides showed identical behavior on both the reversed-phase and cation-exchange HPLC.

The structures of FRVF and FRF were also confirmed by comparing chemical and pharmacological properties of the synthetic peptides with those of the native peptides. In either cases of FRVF (Fig. 5.17) and FRF (Fig. 5.18), the synthetic and native peptides showed identical behavior on both the reversed-phase and cation-exchange HPLC. Dose-response relations for the synthetic and native peptides in inhibiting twitch contraction of the body-wall muscle were also nearly identical (Fig. 5.19). I termed them FR peptides from the similarity of the N-terminal parts of their structures.

In either cases of FRVF and FRF, inhibitory actions on the inner circular body-wall muscle were not potent. Even at 10-7 M, the degree of inhibition of the contraction was 25% or less. However, the threshold of the action was as low as 10^{-9} M, and the effect increased with increase in concentration of the peptides in both of the cases.

Muneoka et al. (1981) have shown that the body-wall strips of Urechis is capable of responding to various amino acids, such as proline, sarcosine, beta-alanine and taurine, with contraction and that proline is the most potent contractile amino acid. Further, Muneoka et al (1981). have suggested that the contractions in response to the amino acids are brought about by their actions on the epidermal chemoreceptors, which are supposed to be connected with the body-wall muscle fibres by a subepidermal nerve network.

In the present study, I also examined the effects of the FR peptides on proline-induced contractions of the longitudinal and lateral body-wall strips. The contraction of the longitudinal strip is considered to be due to the response of the longitudinal body-wall muscle, and that of the lateral strip is considered to

be due to the responses of the inner and outer circular body-wall muscles.

As shown in Fig. 5.20, the proline contraction of the lateral body-wall strip, as well as twitch contraction of the isolated inner circular body-wall muscle, was inhibited by FRVF and FRF. On the contrary, the contraction of the longitudinal body-wall strips was potentiated by the peptides. These result suggest that the peptides inhibit contractions of the circular body-wall muscles while potentiate contraction of the longitudinal muscle by acting directly on muscle fibres or by acting on nerve elements in the body-wall. However, the possibility that one of the sites of the actions of the peptides is chemoreceptors for proline cannot be ruled out.

Effects of the four inhibitory peptides were also examined on phasic contraction of the ABRM of Mytilus, twitch contractions of the radula retractor muscle of Rapana and spontaneous contractions of the small intestine of Xenopus. FSAFRADLamide, REFWK and FRF, even at 10^{-5} M, did not show any effect on the contractions of the above muscles. Only FRVF showed a potentiating effect on spontaneous contractions of the small intestine of Xenopus. The threshold concentration was found to be approximately 10^{-7} M (Fig. 5.21).

5-4 Discussion

In the present study, I isolated six novel neuropeptides from aqueous acetic acid extracts of the ventral nerve cords of Urechis unicinctus by using the inner circular body-wall muscle of Urechis as the bioassay system. Two out of the six peptides, UEP$_A$ and UEP$_C$, were excitatory peptides which showed a contractile action on the body-wall muscle. The remaining four peptides, FSAFRADLamide, REFWK, FRVF and FRF, showed an inhibitory

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action on twitch contraction of the body-wall muscle.

The structures of both of UEP_A and UEP_C are not homologous to any other vertebrate and invertebrate peptides. The peptides do not appear to be members of any other previously identified peptide family. However, UEP_C seems to bear some resemblance to the crustacean cardioactive peptide (CCAP), as shown below.

$$UEP_C \quad Thr-Phe-\overline{Cys-Thr-Ile-Asp-Leu-Asn-Cys}-OH$$
$$CCAP \quad Pro-Phe-\overline{Cys-Asn-Ala-Phe-Tyr-Gly-Cys}-NH_2$$

Both of the peptides are nonapeptides and have a S-S bond between Cys^3 and Cys^9. Further, the second amino acid residue is Phe in both of them. The most different point between them is the C-terminus structures. The C-terminus of CCAP is amidated but that of UEP_C is free.

CCAP was first isolated from the pericardial organs of a shore crab, Carsinus maenas (Stangier et al., 1987). The peptide showed an excitatory action on the crab heart. Later, CCAP was shown to be present in other parts of the nervous system of Carcinus by using a radioimmunoassay method (Stangier et al., 1988) and an immunocytochemical method (Dirksen and Keller, 1988). Furthermore, this peptide was also found in the nervous system of an insect, Locusta migratoria (Stangier et al., 1989). Therefore, CCAP seems to act as a neurotransmitter and/or a neuromodurator in Carsinus nervous system. The peptide may be widely distributed in arthropods. The Echiura is closely related to the Annelida, and the Annelida is a phylum phylogenically related to the Arthropoda. Annelid worms might have a peptide related to both UEP_C and CCAP. Although UEP_C does not show any effect on the body-wall muscle of the annelid Marphysa and on the hind gut of the insect Gryllus, it is interesting to examine more precisely the effects of UEP_C, CCAP and their analogues, especially C-terminus-free CCAP and C-terminus-amidated UEP_C, on the muscles of echiuroids, annelids and arthropods.

It has been suggested that ACh may be an excitatory neuro-

transmitter in the body-wall muscle of Urechis (Muneoka et al., 1981). Indeed, ACh itself was found in the ventral nerve cord of Urechis (see Chapter 3). In the present study, it was shown that UEP_A and UEP_C have a potent contractile action on the inner circular body-wall muscle. These peptides might also be excitatory transmitters in the muscle. ACh might be the principal excitatory transmitter and the peptides might be excitatory co-transmitters.

Inhibiting activity of the synthetic FSAFRADLamide on twitch contraction of the body-wall muscle of Urechis was less potent than that of the native peptide. However, from the results of the structure analyses, the sequence of FSAFRADLamide seems to be correct. Therefore, it is suspected that the native FSAFRADLamide might contain D-amino acid residue(s).

Several bioactive peptides isolated from animal tissues have been shown to contain a D-amino acid residue. For example, the opioid peptides, dermorphins and deltorphins, isolated from frog skin possess either a D-ala[2] or a D-Met[2] (Montecucchi et al., 1981; Richter et al., 1987; Erspamer et al., 1989; Mor et al., 1989, 1991). The other examples are neuropeptides, achatin-I (Kamatani et al., 1989) and fulicin (Ohta et al., 1991) isolated from the ganglia of the pulmonate mollusc Achatina fulica, and Mytilus-FFRFamide (Fujisawa et al., 1992) isolated from the ABRMs of the bivalve mollusc Mytilus edulis. Peptides containing D-amino acid residue(s) might be present widely in animal kingdom. It is expected that FSAFRADLamide contains D-amino acid residue(S).

Although the inhibitory actions of the FR peptides, FRVF and FRF, on twitch contraction of the inner circular body-wall muscle of Urechis were not potent, they showed inhibiting activity on proline contractions of the lateral body-wall strip of Urechis and potentiating activity on that of the longitudinal body-wall strips. Opposite actions of neuromediator candidates on antago-

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nistic muscles are also known in some other muscles. In the prosobranch mollusc <u>Rapana</u>, serotonin and octopamine inhibit contraction of the radula retractor while potentiate that of the radula protractor (Kobayashi and Muneoka, 1980; Muneoka and Kobayashi, 1980). In <u>Fusinus</u>, the neuropeptide APGWamide potentiates contraction of the radula retractor and inhibits that of the radula protractor (Kuroki <u>et al</u>., 1990). The FR peptides may be physiological neuromodulators controlling the movement of the body wall.

Recently, I isolated a novel neuropeptides, H-Phe-Arg-Thr-Phe-OH (FRTF), from the ganglia of pulmonate mollusc <u>Helix poma</u><u>tia</u> by using the ABRM of <u>Mytilus</u> as a bioassay system (Ikeda, unpublished). FRVF and FRTF are clearly related with each other. Only the penultimate amino acid residues of them are different. I propose that FRTF is a member of the FR peptide family. Furthermore, FRTF as well as FRVF showed a potentiating effect on spontaneous contractions of the small intestine of <u>Xenopus</u>. Although there is no available evidence for that FR peptide(s) or related substance(s) is involved physiologically in the regulation of contractions of the <u>Xenopus</u> intestine, I expect that such peptide(s) is present in <u>Xenopus</u>.

In the present experiments, it was also suggested that at least 19 bioactive peptides other than the above 6 peptides present in the ventral nerve cord of <u>Urechis</u>. Some of the peptides were found to have biological activities not only on the inner circular body-wall muscle of <u>Urechis</u> but also on the ABRM of <u>Mytilus</u> and the heart of bivalve mollusc <u>Meretrix lusoria</u>. However, the amounts of these peptides were too small to purify and to apply to the structure analysis experiments. Therefore, as reported in the next Chapter, I tried further purification and structure determination of bioactive peptides in the ventral nerve cord of <u>Urechis</u> by changing the methods of extraction and purification.

Table 5.1.   Results of structure analyses of the inhibitory peptidic substances purified from the aqueous acetic acid extract.

_____

FSAFRADLa   AAS (pmoles):   Phe-Ser-Ala-Phe-Arg-Ala-Asp
                            121 43 126 63 67 73 92
            AAA (Phe=2.0): Asp, Ser, Ala, Leu, Phe, Arg
                            1.4 1.4 2.1 1.4 2.0 1.4
            FAB-MS: 925.7 m/z (M+H)$^+$
            Structure: H-Phe-Ser-Ala-Phe-Arg-Ala-Asp-Leu-NH$_2$

REFWK   AAS (pmoles):   Arg-Glu-Phe-ND-Lys
                             8 33 35   2
            AAA (Phe=1.0): Glu, Phe, Lys, Arg
                            1.3 1.0 1.1 1.0
            FAB-MS: 765.3 m/z (M+H)$^+$
            Structure: H-Arg-Glu-Phe-Trp-Lys-OH

FRVF   AAS (pmoles):   Phe-Arg-Val-Phe
                            355 112 148 17
            AAA (Phe=2.0): Val, Phe, Arg
                            1.0 2.0 1.1
            FAB-MS: 568.3 m/z (M+H)$^+$
            Structure: H-Phe-Arg-Val-Phe-OH

FRF   AAS (pmoles):   Phe-Arg-Phe
                            73 34   2
            AAA (Phe=2.0): Phe, Arg
                            2.0 1.0
            FAB-MS: 469.2 m/z (M+H)$^+$
            Structure: H-Phe-Arg-Phe-OH

_____

AAS, amino acid sequence analysis; AAA, amino acid analysis; FAB-MS, fast atom bombardment mass spectrometric measurement.
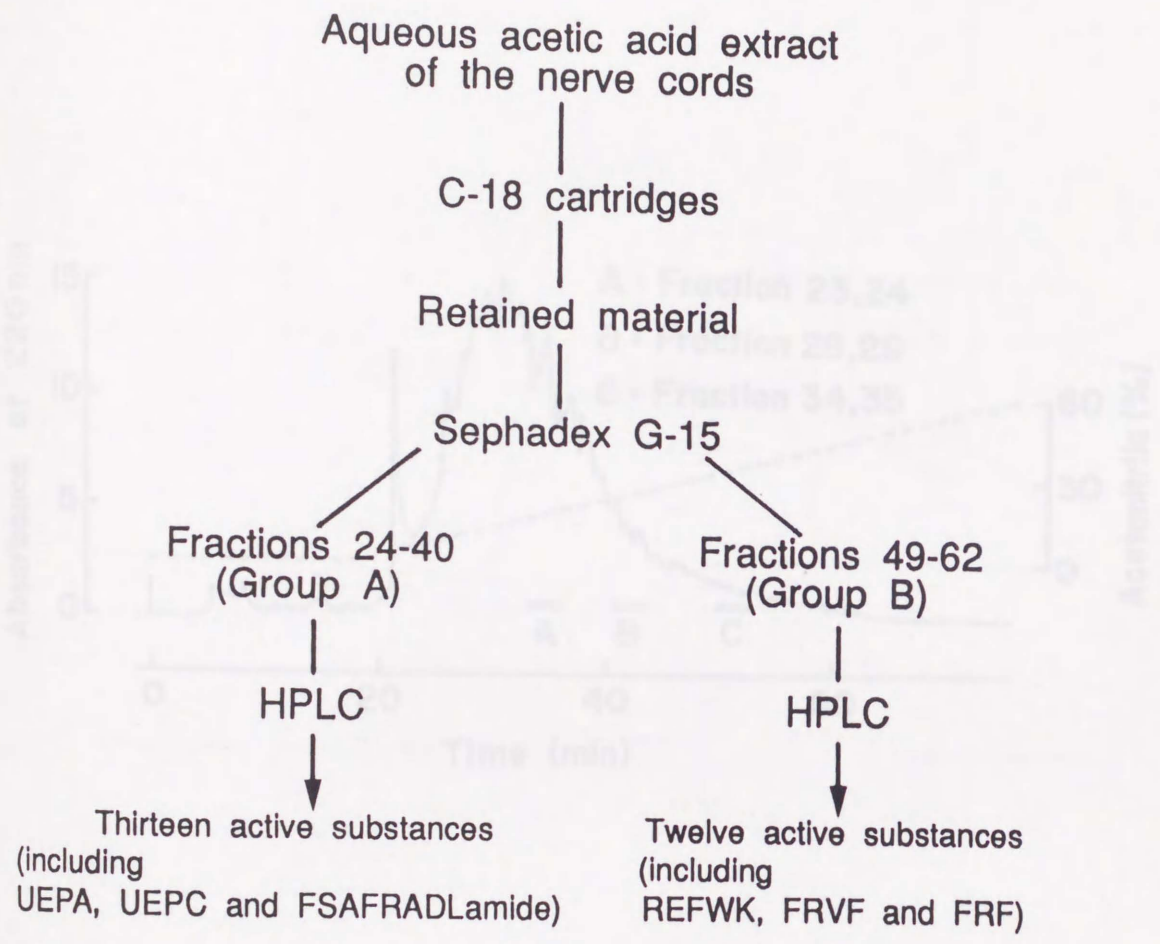
```
                 Aqueous acetic acid extract
                      of the nerve cords
                              |
                              |
                        C-18 cartridges
                              |
                              |
                        Retained material
                              |
                              |
                       Sephadex  G-15
                          /          \
                        /              \
             Fractions 24-40        Fractions 49-62
               (Group A)              (Group B)
                    |                      |
                    |                      |
                  HPLC                   HPLC
                    |                      |
                    v                      v
         Thirteen active substances   Twelve active substances
        (including                    (including
        UEPA, UEPC and FSAFRADLamide)  REFWK, FRVF and FRF)
```

<u>Fig. 5.1.</u>   The isolation procedures for bioactive peptides
in the aqueous acetic acid extract of the ventral nerve cords of
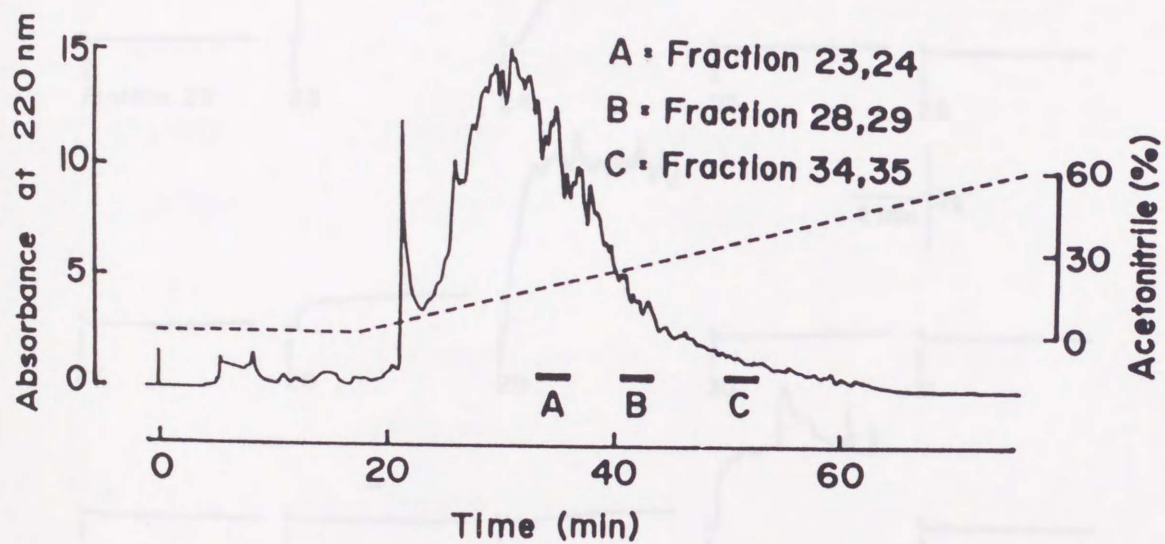<u>Urechis</u>.

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Fig. 5.2. Chromatogram of the first HPLC separation step of group A. The fractions of the three peaks (A, B and C) showed a contractile activity on the body-wall muscle of <u>Urechis</u>. HPLC conditions are given in text.
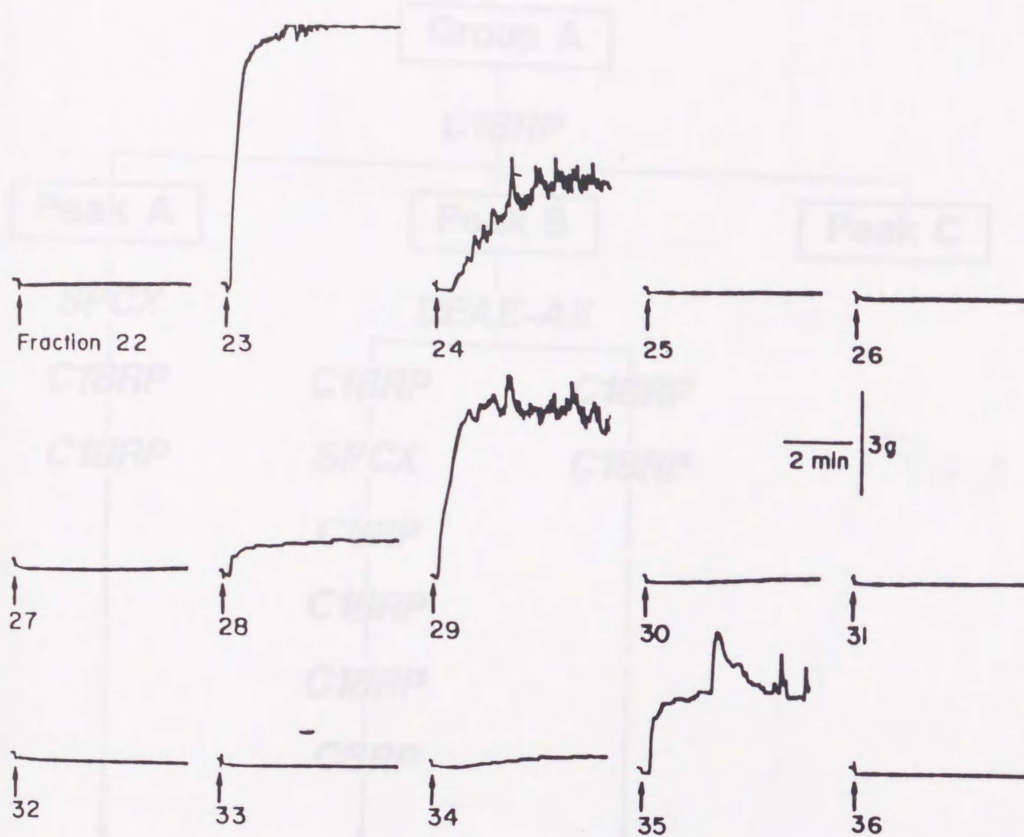
Fig. 5.3.   Contractile action of the fractions of the three peaks(A, fraction 23,24; B, fraction 28,29; C, fraction 34,35) of the first HPLC separation step on the body-wall muscle of Urechis.   Each bioactive material was applied to the muscle for 5 min at 10 min interval and washed out soon after the contraction.   Dose is 6 nerve cords/ml equivalent.
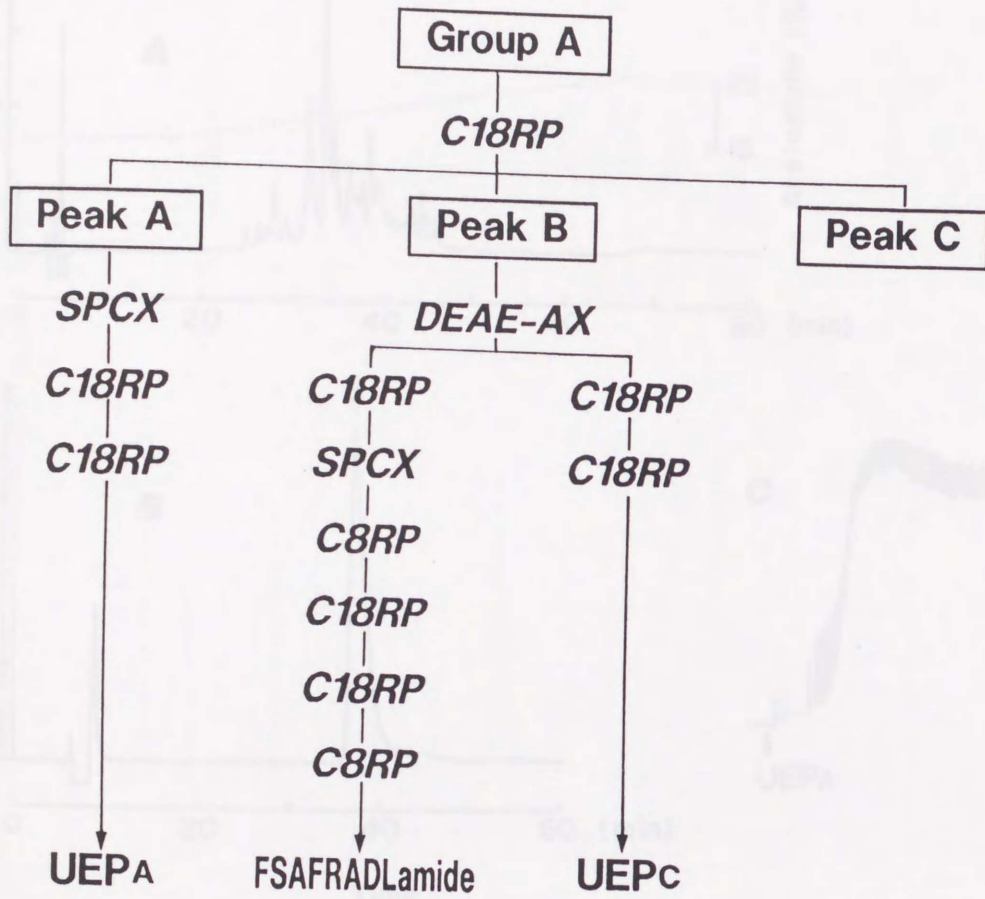
```
                        ┌──────────┐
                        │ Group A  │
                        └──────────┘
                           C18RP
          ┌──────────────────┼──────────────────┐
    ┌──────────┐        ┌──────────┐        ┌──────────┐
    │  Peak A  │        │  Peak B  │        │  Peak C  │
    └──────────┘        └──────────┘        └──────────┘
       SPCX                DEAE-AX
      C18RP          ┌────────────┐
      C18RP        C18RP         C18RP
                   SPCX          C18RP
                   C8RP
                   C18RP
                   C18RP
                   C8RP
      UEPA      FSAFRADLamide     UEPC
```

Fig. 5.4.   HPLC purification procedures for UEP_A, UEP_C and FSA-FRADLamide in group A.   C18RP, C-18 reversed-phase column; SPCX, cation-exchange column; DEAE-AX, anion-exchange column; C8RP, C-8 reversed-phase column.
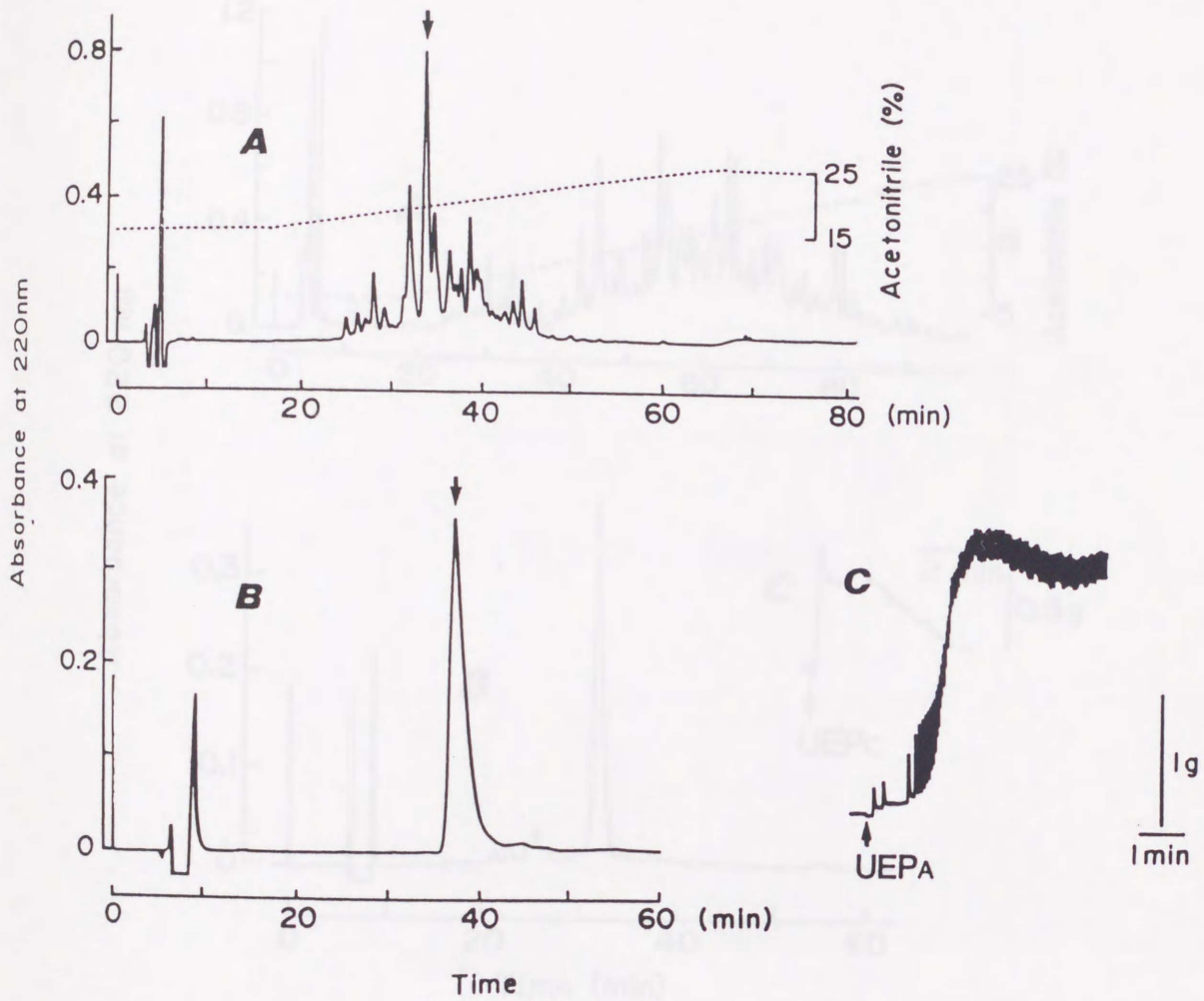
Fig. 5.5. Chromatograms of the third step (A) and the fourth (last) step of HPLC purification of UEP$_A$ and the contractile activity of the purified UEP$_A$ on the body-wall muscle of Urechis (C). The absorbance peaks indicated by arrows correspond to activity peaks. HPLC conditions are given in text.
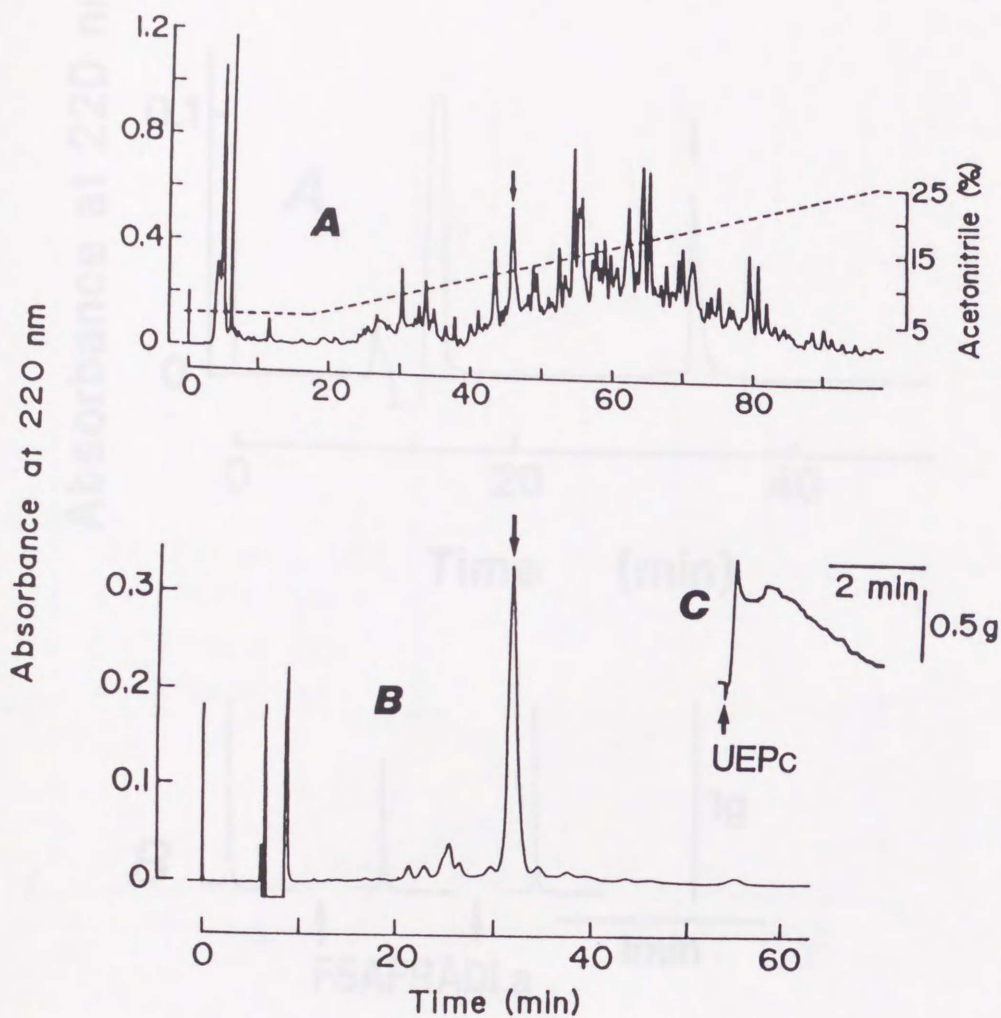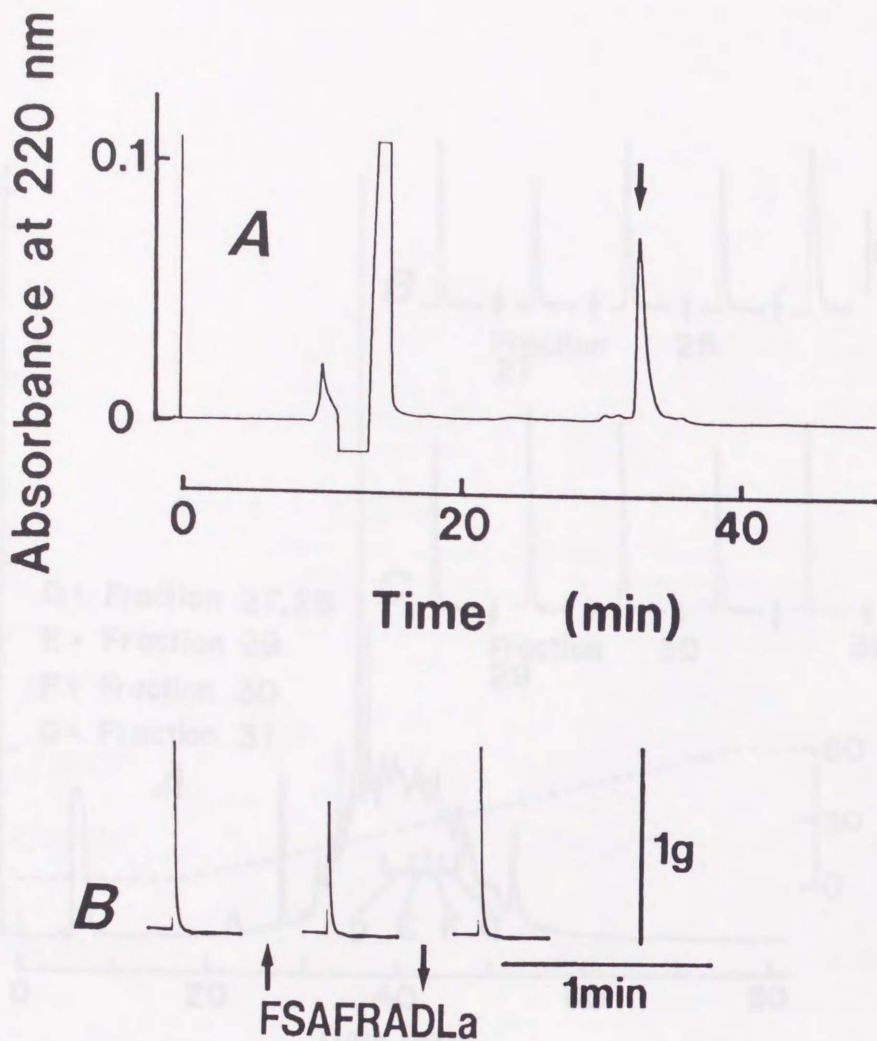
Fig. 5.6. Chromatograms of the third step (A) and the fourth (last) step of HPLC purification of UEP$_C$ and the contractile activity of the purified UEP$_C$ on the body-wall muscle of Urechis (C). The absorbance peaks indicated by arrows correspond to activity peaks. HPLC conditions are given in text.

<u>Fig. 5.7.</u>   Chromatogram of the last step of HPLC purification of
FSAFRADLamide (A) and inhibitory activity of the purified FSA-
FRADLamide on twitch contraction of the body-wall muscle of
<u>Urechis</u> (B).   The absorbance peak indicated by arrow corresponds
to activity peak.   HPLC conditions are given in text.   The twitch
contraction was elicited by an electrical pulse (15 V, 3 msec)
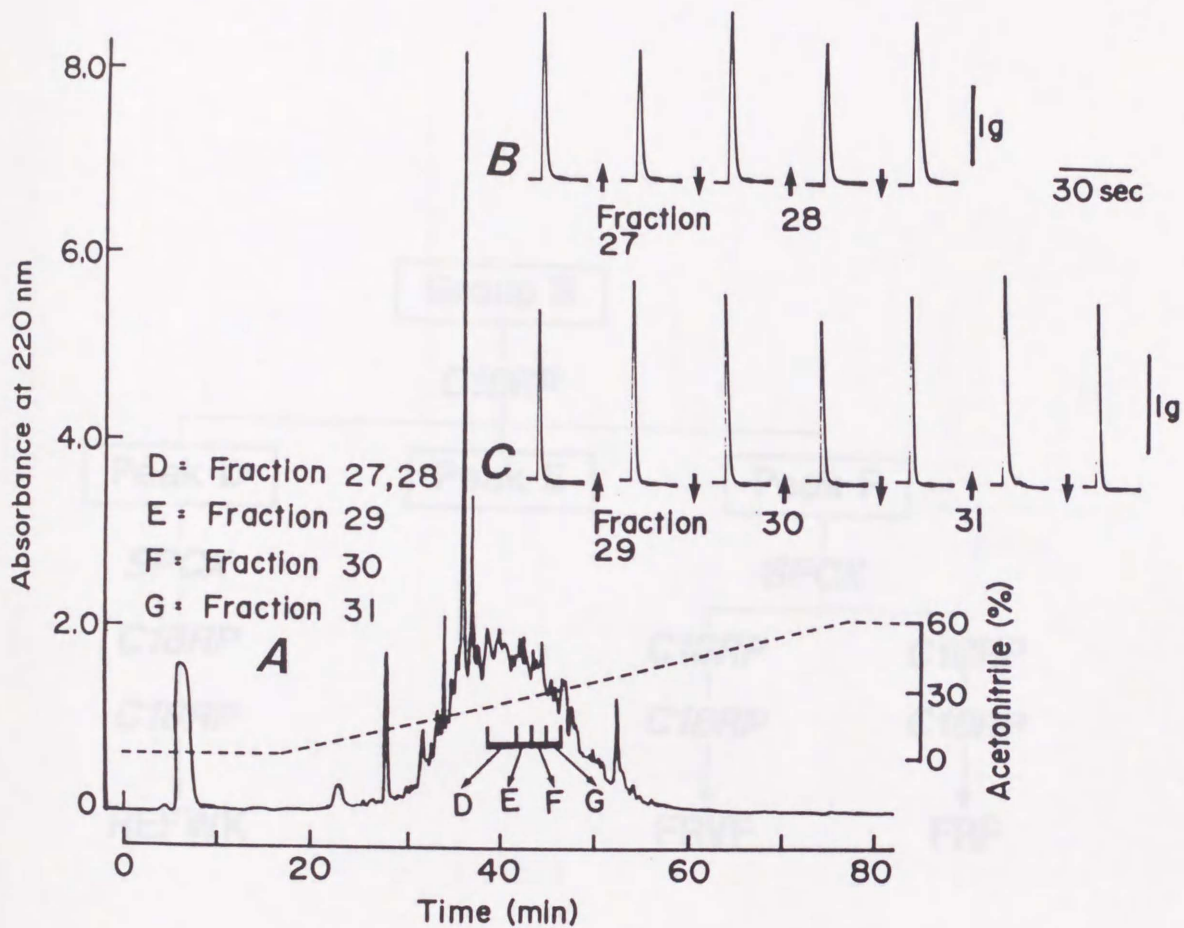applied at 10 min intervals.

Fig. 5.8. Chromatogram of the first HPLC separation step (A) of group B. The fractions of the three peaks showed modulating activities on twitch contraction of the body-wall muscle of Urechis. B: twitch-inhibiting activity of peak D (fraction 27,28) on the body-wall muscle of Urechis. C: twitch-potentiating activity of peak E (fraction 29) and G (fraction 31), and twitch-inhibiting activity of peak F (fraction 30) on the muscle. HPLC conditions are given in text. The procedures for recording the twitch contraction are the same as in Fig. 5.7.
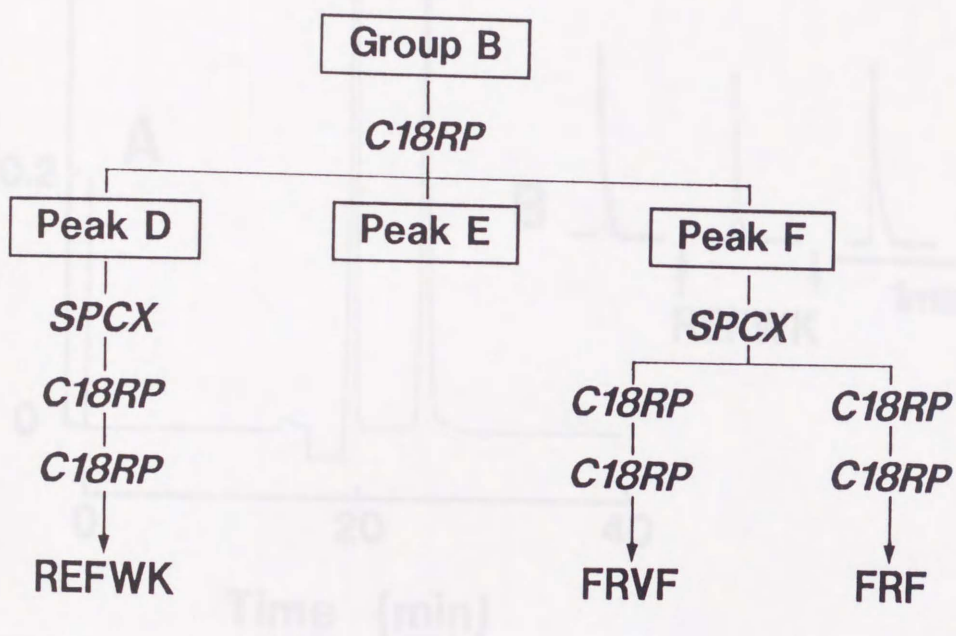
Fig. 5.9. HPLC purification procedures for REFWK, FRVF and FRF in group B. C18RP, C-18 reversed-phase column; SPCX, cation-exchange column.
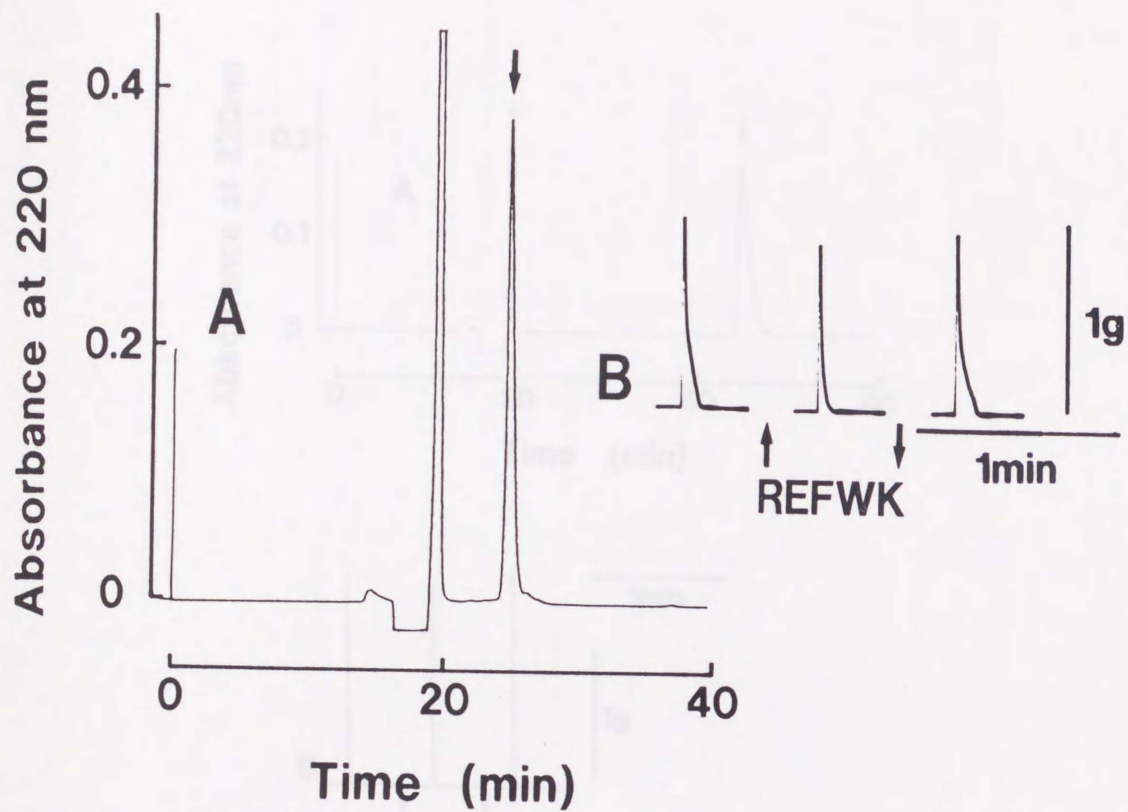
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Fig. 5.10.   Chromatogram of the last step of HPLC purification of REFWK (A) and inhibitory activity of the purified REFWFK on twitch contraction of the body-wall muscle of <u>Urechis</u> (B).   The absorbance peak indicated by arrow corresponds to activity peak. HPLC conditions are given in text.   The procedures for recording the twitch contraction are the same as in Fig. 5.7.
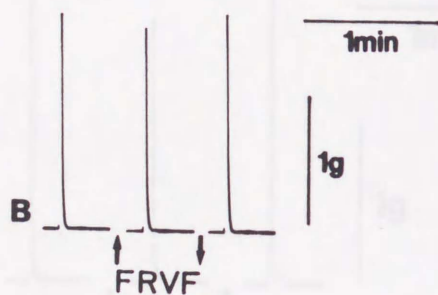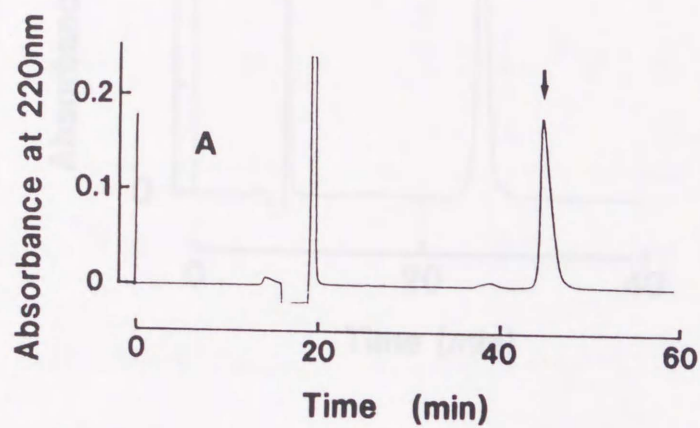
Fig. 5.11. Chromatogram of the last step of HPLC purification of FRVF (A) and inhibitory activity of the purified FRVF on twitch contraction of the body-wall muscle of <u>Urechis</u> (B). The absorbance peak indicated by arrow corresponds to activity peak. HPLC conditions are given in text. The procedures for recording the twitch contraction are the same as in Fig. 5.7.
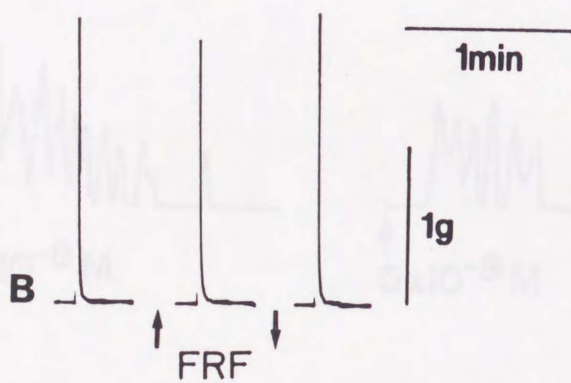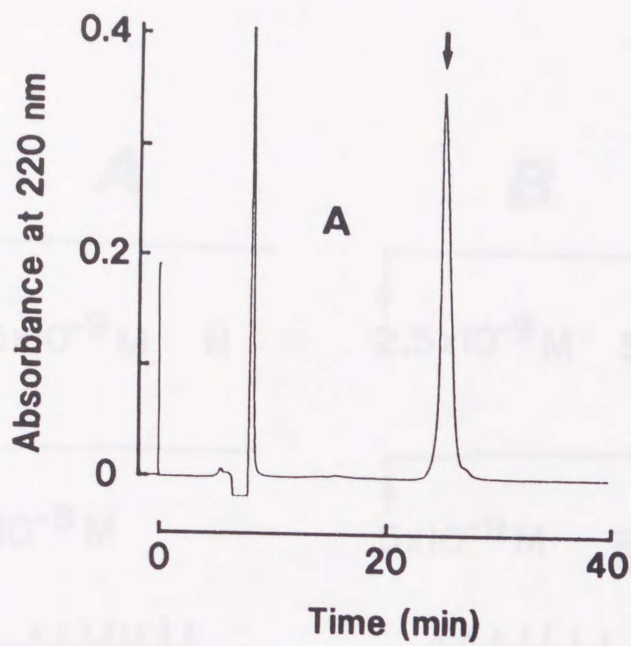
Fig. 5.12. Chromatogram of the last step of HPLC purification of FRF (A) and inhibitory activity of the purified FRF on twitch contraction of the body-wall muscle of Urechis (B). The absorbance peak indicated by arrow corresponds to activity peak. HPLC conditions are given in text. The procedures for recording the twitch contraction are the same as in Fig. 5.7.
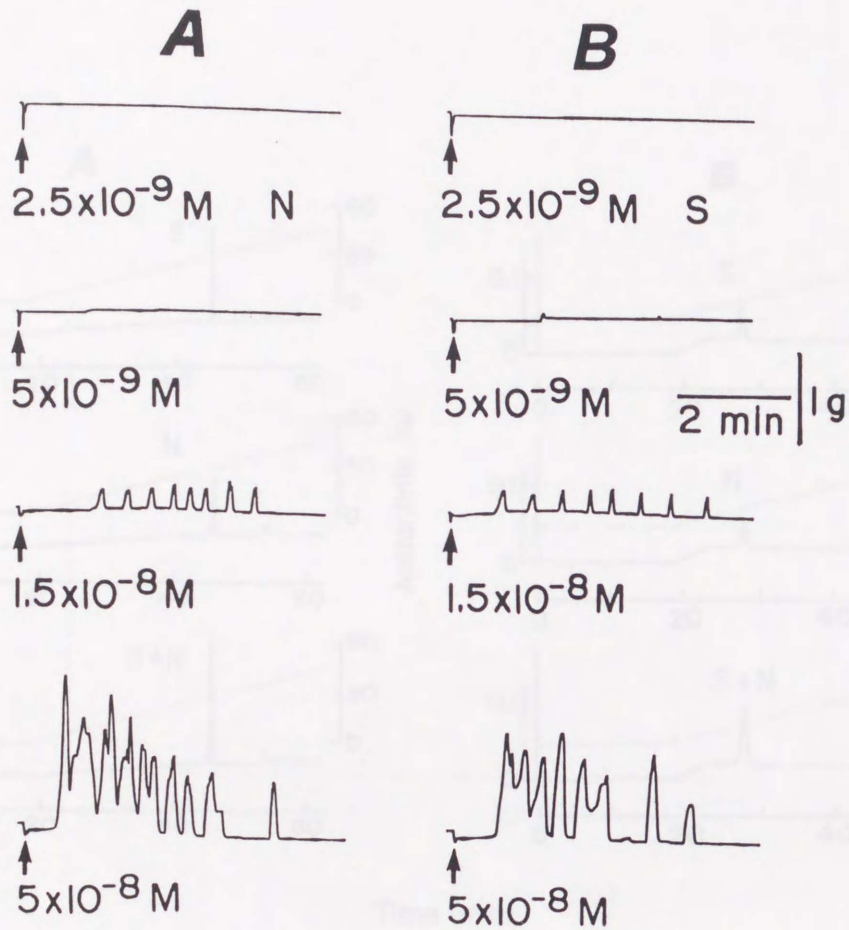
Fig. 5.13.   Comparison between contractile activities of the native (N) and synthetic (S) UEP$_C$ on the body-wall muscle of Urechis.   The concentrations of the native UEP$_C$ were estimated from the results of the amino acid analysis.

Fig. 5.14.   Comparison between HPLC behavior of the synthetic (S) and native (N) UEP$_C$.   A: C-18 reversed-phase HPLC.   The column was eluted with a 60-min linear gradient of 0-60% acetonitrile in 0.1% TFA (pH 2.2).   B: anion-exchange HPLC.   The column was eluted with a 32-min linear gradient of 0.04-0.2 M NaCl in 10 mM Tris-HCl buffer at pH 8.4.   S+N, a mixture of the synthetic and native UEP$_C$.

Fig. 5.15. Comparison between inhibitory effects of the synthetic (S) and native (N) REFWK on twitch contraction of the body-wall muscle of Urechis. The concentrations of the native REFWK were estimated from the results of the amino acid analysis. The procedures for recording the twitch contraction are the same as in Fig. 5.7.
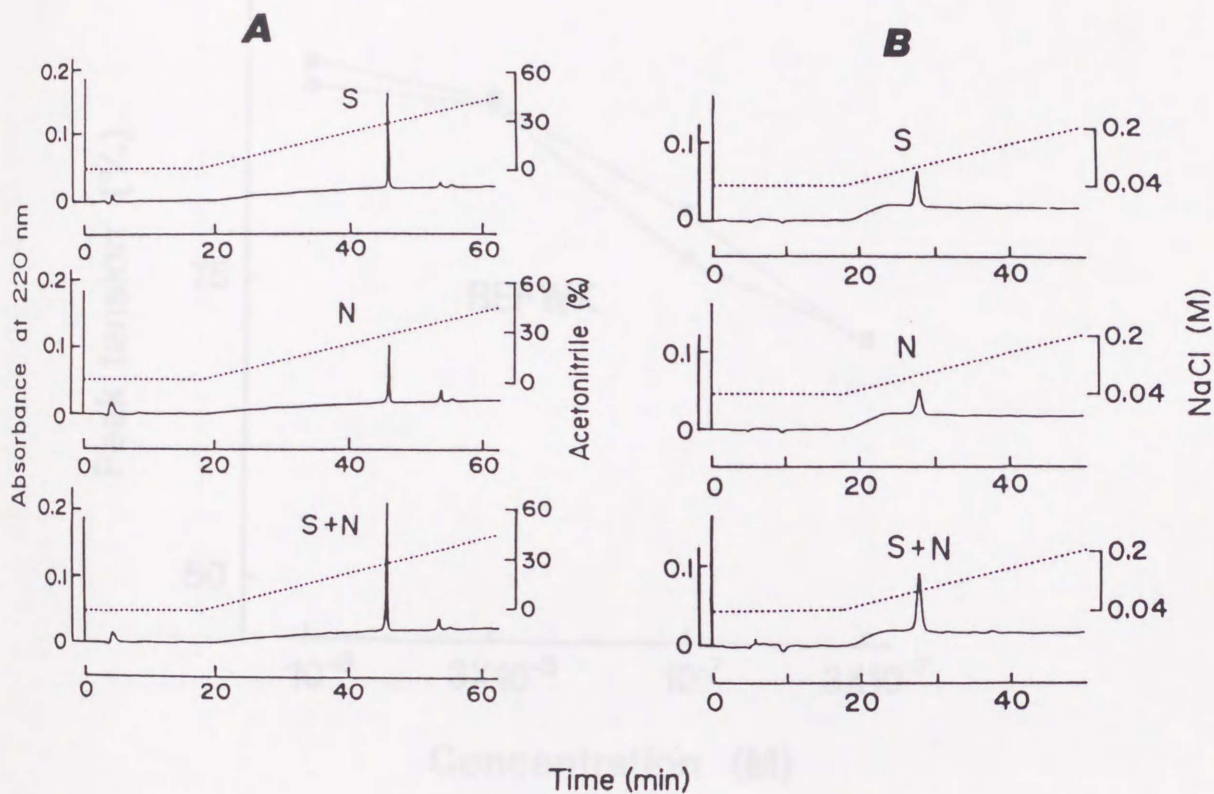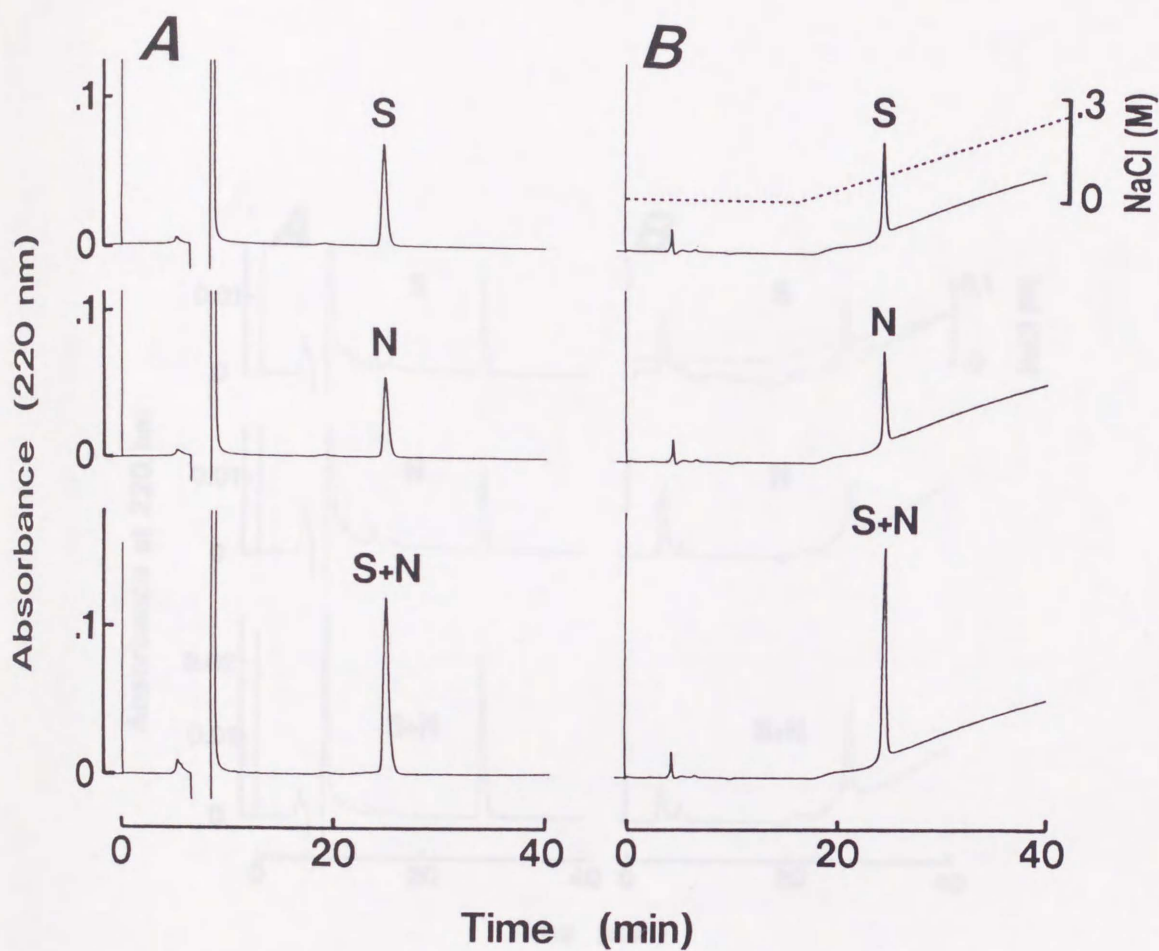
Fig. 5.16. Comparison between HPLC behavior of the synthetic (S) and native (N) REFWK. A: C-18 reversed-phase HPLC. The column was eluted isocratically with 17% acetonitrile in 0.1% TFA (pH 2.2). B: cation-exchange HPLC. The column was eluted with a 30-min linear gradient of 0-0.3 M NaCl in 10 mM phosphate buffer at pH 6.8. S+N, a mixture of the synthetic and native REFWK.
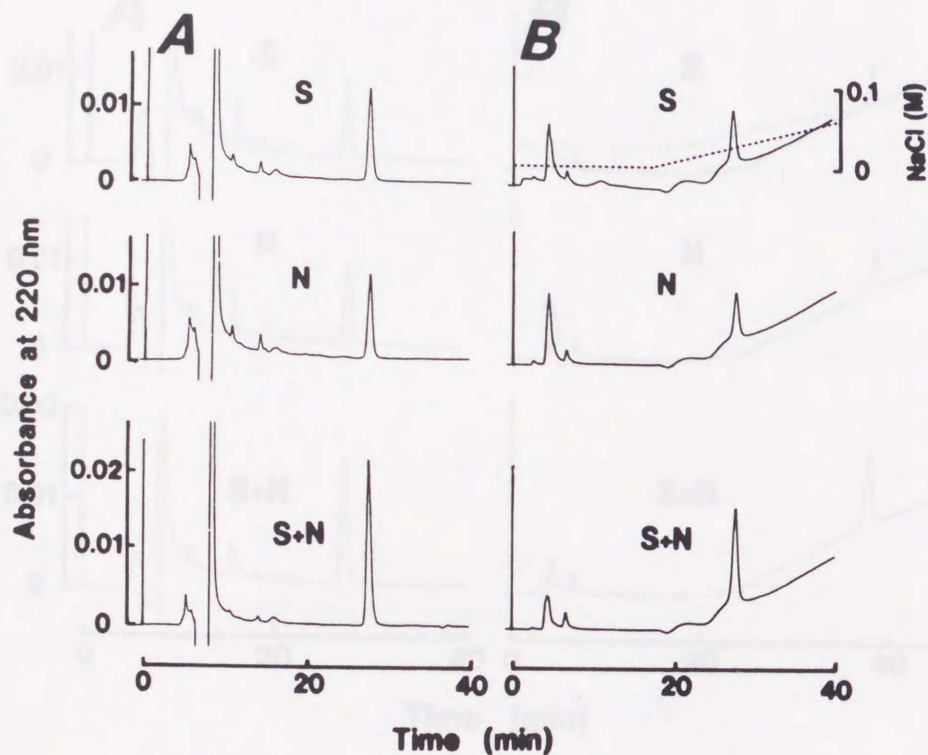
Fig. 5.17.   Comparison between HPLC behavior of the synthetic (N) and native (N) FRVF.   A: C-18 reversed-phase HPLC.   The column was eluted with 22.5% acetonitrile in 0.1% TFA (pH 2.2). B: cation-exchange HPLC.   The column was eluted with a 40-min linear gradient of 0-0.1 M NaCl in 10 mM phosphate buffer (pH 6.8).   S+N, a mixture of the synthetic and native FRVF.
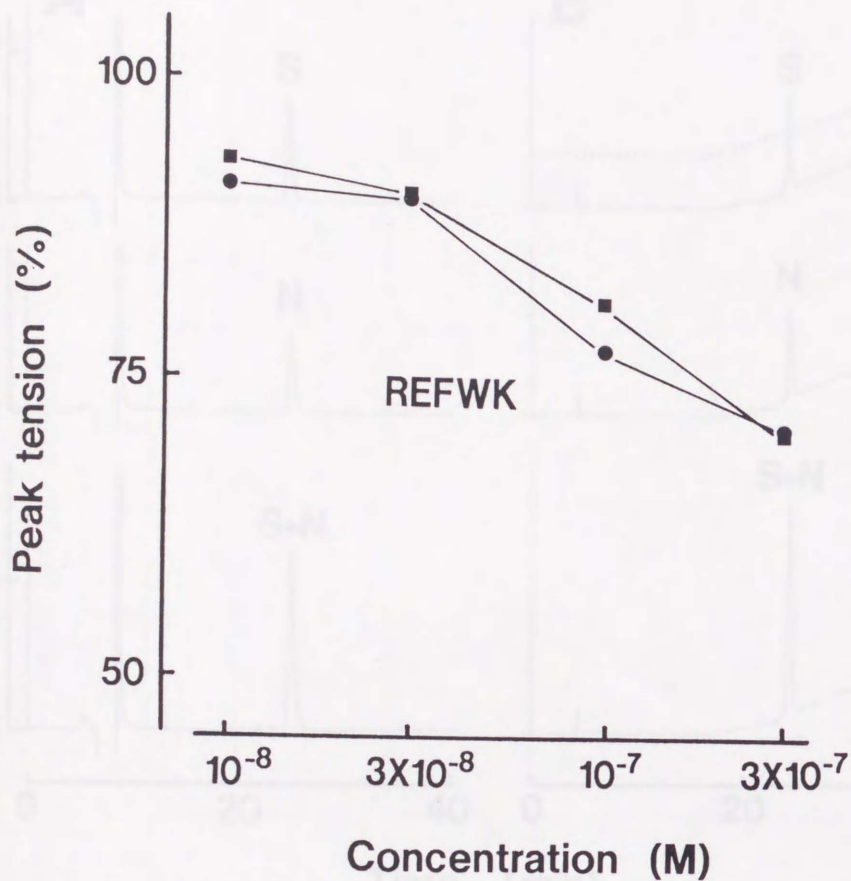
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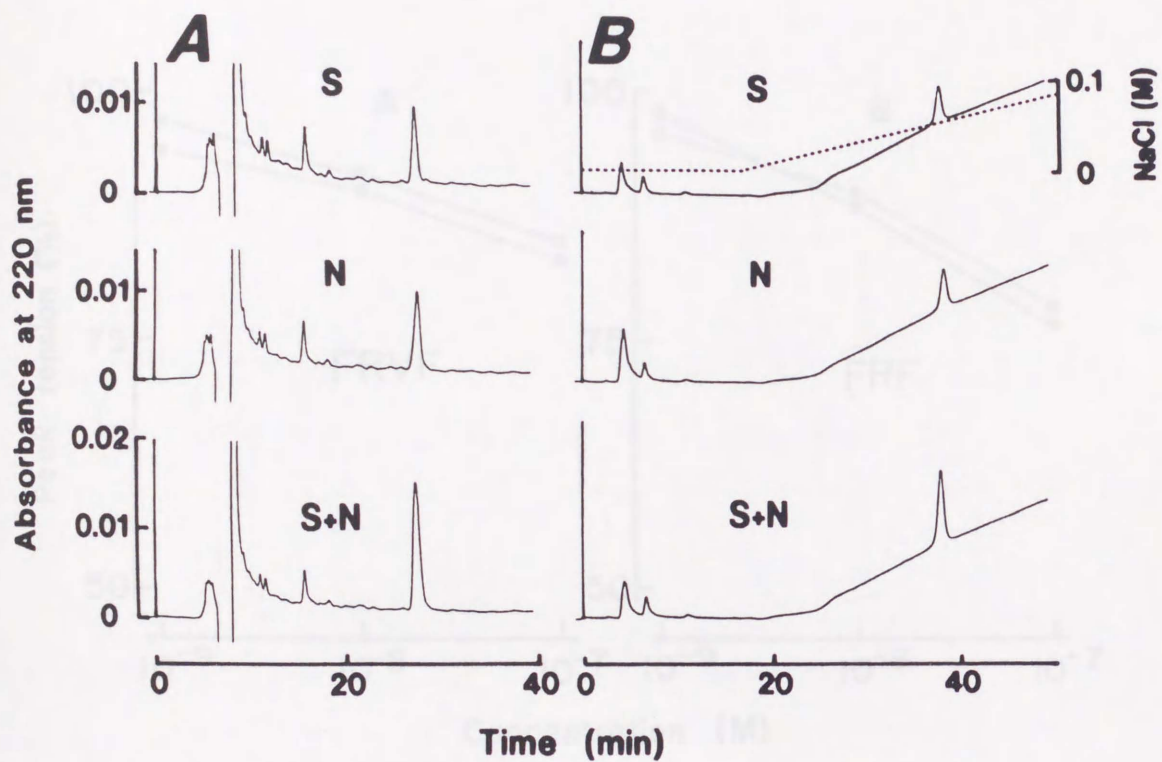
Fig. 5.18.   Comparison between HPLC behavior of the synthetic
(S) and native (N) FRF.   A: C-18 reversed-phase HPLC.   The column
was eluted with 21% acetonitrile in 0.1% TFA (pH 2.2).   B: ca-
tion-exchange HPLC.   The chromatographic conditions are the same
as in Fig. 5.16.   S+N, a mixture of the synthetic and native FRF.
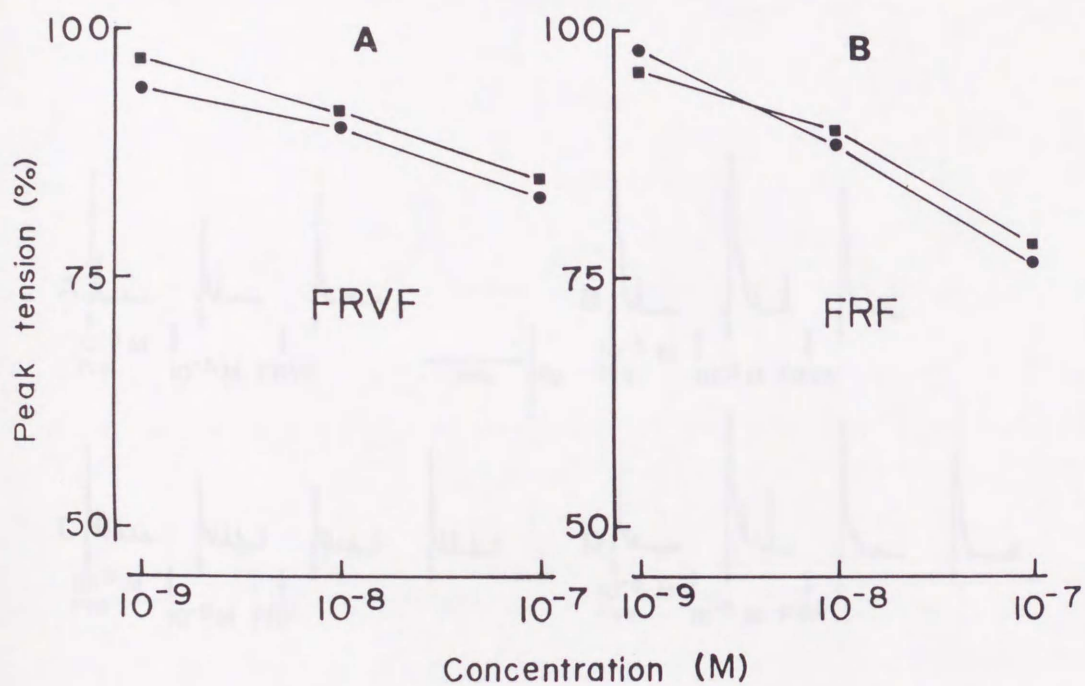
Fig. 5.19. Comparison between inhibitory effects of the syn-
thetic (closed circle) and native (closed square) FRVF (A) and
FRF (B), on twitch contraction of the body-wall muscle of
Urechis. The concentrations of the native peptides were estimat-
ed from the results of the amino acid analyses. The procedures
for recording twitch contraction are the same as in Fig. 5.7.

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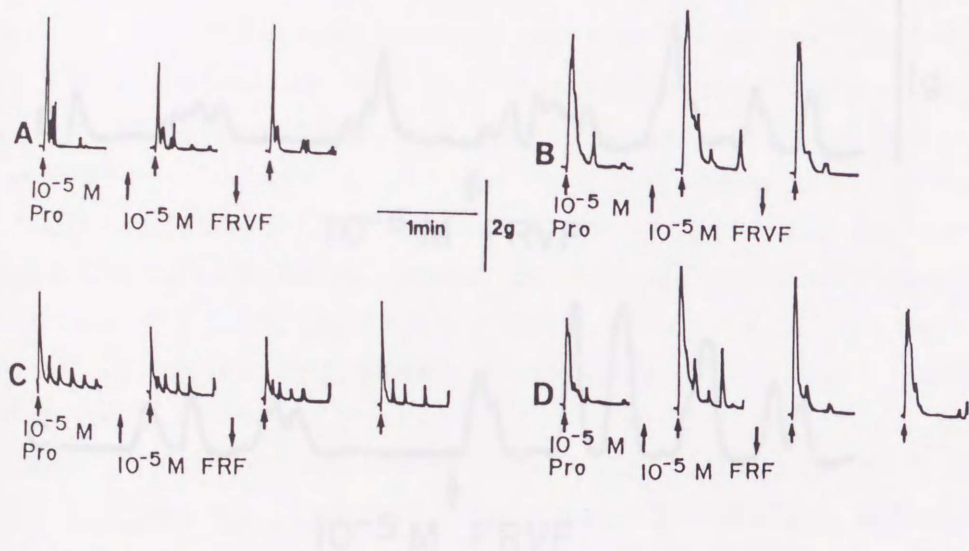
Fig. 5.20.   Effects of FRVF (A and B) and FRF (C and D) on
proline-induced contraction of the lateral (A and C) and longitu-
dinal (B and D) body-wall strips of Urechis. Proline (Pro) was
applied to the strips for 45 sec at 10 min intervals (short
upward arrows).   FRVF and FRF were introduced 8 min prior to the
proline contractions (long upward arrows) and washed out soon
after recording them (long downward arrows).

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10^{-7} M FRVF   2 min

10^{-6} M FRVF

10^{-5} M FRVF
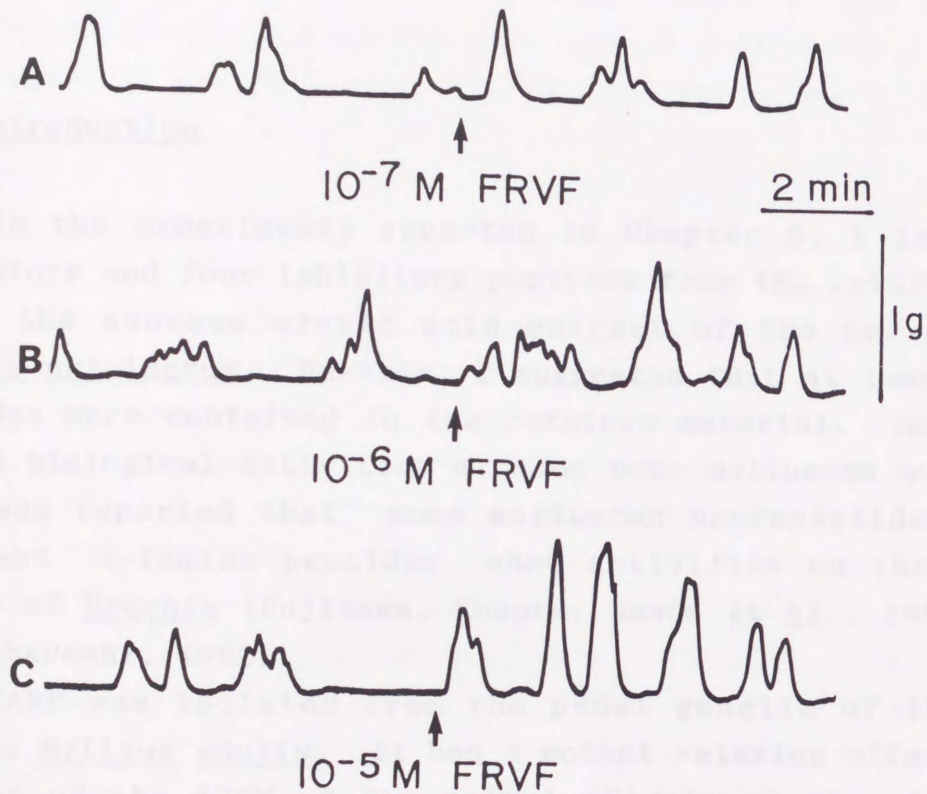
Fig. 5.21. Effect of FRVF on spontaneous contractions of the small intestine of Xenopus. Each dose of FRVF was introduced at each upward arrow. Between the records, the intestine was washed with normal saline for 10 min.

CHAPTER 6

BIOACTIVE PEPTIDES EXTRACTED WITH
ACETIC ACID-ETHANOL SYSTEM

6-1 Introduction

In the experiments reported in Chapter 5, I isolated two excitatory and four inhibitory peptides from the retained material of the aqueous acetic acid extract of the nerve cords of Urechis unicinctus. Further, I suggested that at least 19 other peptides were contained in the retained material. Some of them showed biological activities also on some molluscan muscles. It has been reported that some molluscan neuropeptides, such as CARP and S-Iamide peptides, show activities on the body-wall muscle of Urechis (Fujisawa, Kubota, Kanda et al., 1991; Muneoka and Kobayashi, 1992).

CARP was isolated from the pedal ganglia of the bivalve mollusc Mytilus edulis. It has a potent relaxing effect on catch tension of the ABRM of the animal (Hirata et al., 1987). The peptide also shows biological effects on muscles and neurons in many molluscs (Kiss, 1988, 1991; Hirata, Kubota, Imada, Muneoka and Kobayashi, 1989; Hirata, Kubota, Imada and Muneoka, 1989; Kobayashi and Muneoka, 1990; Mat et al., 1990; Moffett, 1991; Kiss and Osipenko, 1991). Several peptides related to CARP have been isolated from some molluscs (Cropper et al., 1987; Fujisawa, Kubota, Kanda et al., 1991). Furthermore, CARP has been shown to have a twitch-potentiating activity on the body-wall muscle of Urechis (Fujisawa, Kubota, Kanda et al., 1991).

A S-Iamide peptide (LSSFVRIamide) was isolated from the

ganglia of prosobranch mollusc *Fusinus ferrugineus* (Kuroki *et al*., 1992). The peptide shows contraction-modulating effects not only on various molluscan muscles but also on twitch contraction of the body-wall muscle of *Urechis* (Muneoka and Kobayashi, 1992).

In the present experiments, I further attempted to isolate bioactive peptides of the ventral nerve cords of *Urechis*, and to compare the structures of the peptides with the peptides of other phyla such as the Mollusca. In this experiments, I used ethanol for extraction of the peptides.

6-2 Materials and Methods

6-2-1 Extraction and Purification of Bioactive Peptides

The ventral nerve cords of *Urechis unicinctus* were excised from 2,000 specimens and immediately frozen on dry ice. The frozen nerve cords (84g) were immersed in ethanol-acetic acid solution (96:4), homogenized with a Polytron at 0 °C, and centrifuged at 28,000g for 40 min at 4 °C. After the pellet was re-extracted two times with the same solution, the supernatants (960 ml) were pooled and evaporated to dryness. The dried material was resolved in 30 ml of 0.1 N HCl and the solvent was again centrifuged at 28,000g for 40 min at 4°C. The supernatant was forced through five C-18 cartridges (Sep-pak C_{18}, Waters), in series. The retained material was eluted with 100% methanol in 0.1% TFA. The eluate was concentrated and subjected to HPLC (TRI ROTAR-VI, Jasco) purification.

The retained material was first applied to a C-18 reversed-phase column (Capcell Pak C_{18}, 10 X 250 mm, Shiseido). The column was eluted with a 120-min linear gradient of 0-60% aceto-nitrile in 0.1 % TFA (pH 2.2) at a flow rate of 1 ml/min. The bioactivities of the fractions were assayed on the inner circular body-wall muscle of *Urechis*. I obtained six bioactive peaks (peak 1-6) at this step (Fig. 6.1). By using an anion-exchange

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column (TSKgel DEAE-5PW, 7.5 X 75mm, Tosoh), a cation-exchange column (TSKgel SP-5PW, 7.5 X 75mm, Tosoh), a C-8 reversed-phase column (Finepak C$_8$5, 4.6 X 250 mm, Jasco) and a C-18 reversed-phase column (TSKgel ODS80T$_M$, 4.6 X 150 mm, Tosoh), I purified 20 bioactive peptides, and UEP$_A$ and UEP$_C$ from peak 4, 5 and 6 through several more steps of HPLC purification. The newly puri-fied peptides were provisionally termed P1 - P20.

The elution of the bioactive peptides from the anion-ex-change column was performed with a 40-min linear gradient of 0-0.4 M NaCl in 10 mM Tris-HCl at pH 9.5. The elution of the cation-exchange column was performed with a 60-min linear gradi-ent of 0-0.6 M NaCl in 10 mM phosphate buffer at pH 6.9. The elution of the reversed-phase columns was performed with acetoni-trile/TFA systems. The forgoing HPLC purification procedures of the bioactive peptides from peak 4, 5 and 6 are summarized in Fig. 6.2, 6.4 and 6.6, respectively.

6-2-2 Bioassay and Pharmacology

After each purification step, the biological activities of the fractions were assayed on twitch contraction of the inner circular body-wall muscle of Urechis. The twitch contraction was evoked by stimulating the muscle with an electrical pulse (15 V, 3 msec) at 10 min intervals. The actions of the purified pep-tides were also examined on phasic contractions of the ABRM of Mytilus edulis. The phasic contraction was evoked by stimulating the muscle with repetitive electrical pulses (15 V, 3 msec, 10 Hz, 50 pulses) at 10 min intervals. All the methods used in the bioassay and pharmacological experiments were basically the same as those described in Chapter 2.

The physiological saline for the muscles of the animals was ASW (see Chapter 2).

6-2-3 Structure Determination

The purified peptides were subjected to quantitative amino acid analysis (Hitachi L-8500) and amino acid sequence analysis by automated Edman degradation with a gas-phase sequencer (Applied Biosystems 477A) coupled with a PTH-amino acid analyzer (Applied Biosystems 120A) and fast atom bombardment mass spectrometric (FAB-MS) measurement (JEOL JMS HX-100). Thus, the probable structures of the purified peptides were determined. P1, P2, P3, P6 and P9 were synthesized, and their behavior on the reversed-phase HPLC and the cation-exchange HPLC were compared with those of the native peptides. Further, the relationships between dose and effect of the synthetic and native peptides were also compared by using the inner circular body-wall muscle of Urechis.

6-3 Results

6-3-1 Purification of Bioactive Peptides

The fractions of the first step of HPLC purification were examined on twitch contraction of the body-wall muscle of Urechis (Fig. 6.1). The fractions from 24 to 46 showed a twitch-potentiating, a twitch-inhibiting or a contractile activity on the muscle. I separated the fractions into six groups according to the peaks of the activities of them. The peak 1, 2, 3, 4, 5 and 6 consisted of fractions 23-26, 27-31, 32-35, 36-38, 39-42 and 43-47, respectively. In these experiments, I isolated 22 bioactive peptides from three peaks, peak 4, 5 and 6. Five peptides, P1, P2, P3, P4 and UEP$_A$, were isolated from peak 4 (Fig. 6.2). The chromatograms and the results of bioassays of the peptides, except UEP$_A$, obtained at the final purification steps were shown in Fig. 6.3. P1 showed a contractile action on the body-wall muscle of Urechis, while the other three peptides showed inhibitory action on twitch contraction of the muscle.

Eight peptides, P5, P6, P7, P8, P9, P10, P11 and P12, were purified from peak 5 (Fig. 6.4). The chromatograms and the

results of bioassays of the peptides obtained at the final purification steps were shown in Fig. 6.5. The peptides, P5-P10, showed an inhibitory action on the body-wall muscle (Fig. 6.5). The remaining peptide P11 showed a contractile action. Peak 6 was found to contain eight bioactive peptides and UEP_C (Fig. 6.6). P17 and P19 showed a contractile action and twitch-potentiating action on the muscle, respectively (Fig. 6.7). The other six peptides, P13, P14, P15, P16, P18 and P20, showed inhibitory action on twitch contraction of the muscle (Fig. 6.7).

The isolation experiments of the bioactive substances in the peak 1, 2 and 3, which were obtained at the first step, are now in progress.

6-3-2 Structures of Bioactive Peptides

Table 6.1 shows the results of the structure analyses carried out on the purified peptides, P1-P10. The results of the analyses of the purified peptides, P11-P20, are shown in Table 6.2. Based on these results, the structures of the purified peptides were proposed. The proposed structures are shown in Table 6.3. In the case of the peptide, P17, its C-terminus is not yet confirmed to be amidated or not.

Six peptides, which are P1 (AAPLPRLamide), P2 (ASSFVRIamide), P3 (PSSFVRIamide), P6 (VSSFVRIamide) and P9 (ARYFLamide), were synthesized. To confirm the structures of these peptides, each of the synthetic peptides compared with each corresponding native peptide on the reversed-phase HPLC and the cation-exchange HPLC. Further, concentration-action relationships of the synthetic and native peptides were compared by using the body-wall muscle of Urechis. As shown in Fig. 6.8, each of the synthetic peptides of ASSFVRIamide (P2), PSSFVRIamide (P3) and VSSFVRIamide (P6) showed the identical behavior with the corresponding native peptide on the C18 reversed-phase HPLC. Each mixture of the synthetic and native peptides showed single absorbance peak

on the HPLC. By using the cation-exchange column, I also obtained the results showing that each of the synthetic peptide has identical HPLC property with the corresponding native peptide (Fig. 6.9). Concentration-action relationships of each synthetic peptide and the corresponding native peptide for inhibiting twitch contraction of the body-wall muscle were also nearly identical (Fig. 6.10). The properties of synthetic AAPLPRLamide (P1) and ARYFLamide (P9) were also identical with the native peptides, respectively, on both a reversed-phase and a cation-exchange HPLC (Fig. 6.11, 12). Thus, the proposed structures of P1, P2, P3, P6 and P9 were confirmed.

6-3-3 The activities of the purified peptides on the ABRM of Mytilus.

The purified peptides were examined on phasic contraction of the ABRM of <u>Mytilus</u>. As shown in Table 6.4, five peptides, P1, P5, P7, P8 and P17, were shown to have potentiating activity on the phasic contraction of the muscle, while other six peptides, P2, P3, P4, P6, P9 and P20 showed inhibitory activity on the phasic contraction of the muscle.

6-4 Discussion

6-4-1 Structural Aspects of The Urechis Neuropeptides.

In this experiments, I attempted to isolate the bioactive peptides from acetic acid-ethanol extract of the ventral nerve cord of <u>Urechis</u>. In consequence, 20 novel peptides were purified. From their structures and activities on the body-wall muscle of the animal, 14 of the purified peptides can be grouped into some families (Table 6.5).

The inhibitory peptides, P2, P3 and P6, have -SSFVRIamide at their C-terminal portions as a common structure, and they show similar activity on both the body-wall muscle of <u>Urechis</u> and the

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ABRM of _Mytilus_ (Table 6.4). The three peptides are the members of a peptide family. The pentapeptides, P8, P9 and P18, can also be regarded as the members of a family. In these peptides, the second and third amino acid residues are not identical. However, the amino acid residues at these positions represnt conservative substitutions at the levels of gene and amino acid structure.

There is a resemblance in structure between P5 and P10. Furthermore, as shown in Table 6.6, P5 shares the C-terminal sequence (-FRADLamide) with the inhibitory peptide, FSAFRADLamide, isolated from aqueous acetic acid extract of the ventral nerve cord of _Urechis_ (see Chapter 5). All of these three peptides have inhibitory activity on twitch contraction of the body-wall muscle of _Urechis_. It is considered that the three peptides are the members of a peptide family.

The three inhibitory peptides, P14, P15 and P16, have the same sequence of the dipeptide P12 in their C-terminal portion (Table 6.5). These four peptides show an inhibitory activity on twitch contraction of the body-wall muscle of _Urechis_. I propose that the -FW-OH which is C-terminal common sequence of four peptides is an important structure to exhibit the inhibitory activity of these peptides. Therefore, I term these peptides FW peptides.

The sequences of the nonapeptides, P19 and P20, are homologous (50%). However, the activity of P19 on twitch contraction of the body-wall muscle of _Urechis_ is of excitation, while the activity of P20 is of inhibition. In some cases, the same neuropeptide show opposite actions on a muscle depending on its concentrations. For example, in the case of CARP, at low concentrations (3×10^{-10} – 5×10^{-9} M) the peptide potentiates phasic contraction of the ABRM of _Mytilus_, whereas at high concentrations (5×10^{-9} M or higher) it inhibits the contraction (Hirata et al., 1987; Hirata, Kubota, Imada and Muneoka, 1989). Each of the P19 and P20 might exhibit opposite actions on the body-wall

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muscle of Urechis depending on its concentrations.

In another case, two peptides having similar structures exhibit opposite actions on the same muscle. In 1986, the two decapeptides leucomyosuppressin (LMS) and leucosulfakinin-II (LSK-II) were isolated from head extracts of the cockroach, Leucophaea maderae (Holman et al., 1986d; Nachman et al., 1986b). Although the two peptides have a structural similarity (Table 6.7), they show opposite actions on the isolated hind gut of the animal. LMS shows an suppressing action on the spontaneous contractions of the isolated hind gut, while LMS-II shows an potentiating action on the contractions. It is required to study the structure-action relationships of the Urechis nonapeptides, P19 and P20, in detail.

The inhibitory peptide, P13, includes the sequence of ALT- in its N-terminal portion. ALT is the peptide isolated from the flowthrough of the ventral nerve cords of Urechis (see Chapter 4). ALT as well as P13 showed a potent inhibitory activity on contractions of the body-wall muscle. It is considered that P13 is a member of ALT family. It is very important to study the effect of P13 on AChE activity.

Furthermore, some of the purified neuropeptides have structural relations with the known neuropeptides isolated from other phyla, as described below.

6-4-2 SCP-related Peptides, Pyrokinins and PBANs.

As shown in Table 6.8, the structure of P1 has a structural relation with the molluscan neuropeptides, small cardioactive peptides (SCPs) and the peptides of two insect neuropeptide groups, pyrokinins and pheromone biosynthesis neuropeptides (PBANs).

SCP_A and SCP_B, have been isolated from the anterior portion of the gut of Aplysia californica (Mahon et al., 1985; Lloyd et al., 1987) and central nervous system of Aplysia brasiliana

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(Morris et al., 1982), respectively. The actions of the peptides, in particular those of SCP$_B$, have been examined in many molluscs. SCP$_B$ shows a potent excitatory activity on the heart of pulmonate Helix (Lloyd et al., 1985) and phasic contraction of ABRM of Mytilus (Muneoka and Saitoh, 1986; Muneoka et al., 1991). Recently, SCP-related peptides have been isolated from Helix (Price et al., 1990). I have also isolated three SCP-related peptides from two pulmonates, AChatina fulica and Helix pomatia (Ikeda, unpublished). Further, two SCP-related peptides have been isolated from the ABRM of Mytilus (Fujisawa, unpublished). All the SCP-related peptides and P1 have -PRXamide in their C-terminal portions as a common structure (Table 6.8).

The insect neuropeptide groups designated pyrokinins and PBANs have the common sequence -FXPRLamide in their C-terminal portions (Table 6.8). Leucopyrokinin (Lem-PK), which is the peptide first determined as a pyrokinin, was purified from the head extract of cockroach Leucophaea maderae (Holman et al., 1986c). Recently, three pyrokinins were isolated from the locust Locusta migratoria and termed locustapyrokinin (Lom-PK), locustamyotrophin (Lom-MT-I) and locustamyotrophin II (Lom-MT-II) (Schoofs, Holman, Hayes, Tips et al., 1990; Nachman and Holman, 1991). These pyrokinins have a potentiating action on spontaneous contractions of the isolated hind gut and the oviduct of the cockroach.

Three PBANs have been isolated from the corn earworm Heliothis zea (Raina et al., 1989) and the silk worm Bombyx mori (Kitamura et al., 1989, 1990). It is considered that the PBANs regulate pheromone production in the adult animals.

These insect peptides and P1 (AAPLPRLamide), as well as the Mytilus peptide APNFLAYPRLamide, have the identical fragment sequence, -PRLamide, at their C-terminal portions. It seems that the insect peptides, the SCP-related peptides and the Urechis peptide P1 are the members of a big peptide family in inverte-

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brates.

6-4-3 Tachykinins

The Urechis peptide P11 has a sequence homology with the insect tachykinins, locustatachykinin (lom-TK) I and II (Table 6.9). Tachykinins which are represented by substance P (Chang et al., 1971) are a family of multifunctional peptides in vertebrates. Further, eledoisin which has been isolated from the salivary grands of cephalopod mollusc, Eledone (Anastasi and Erspamer, 1963), as a peptidic toxin, shows a structural resemblance to substance P. Schoofs, Holman, Hayes, Nachman et al. (1990) isolated two insect tachykinins from the ganglion extracts of locust, Locusta migratoria and discussed that the peptides are another branch of the ancient superfamily of tachykinin-like hormones.

All of the vertebrate tachykinins and eledoisin have -Mamide structure as common sequence in their C-termini, while the insect tachykinin and P11 have -Ramide structure at their C-termini. Very recently, a peptide which is related to insect tachykinins was isolated from the ganglia of bivalve mollusc, Anodonta (Fujisawa unpublished), and it has -Ramide structure in its C-terminus. From these results, it is suggested that the -Ramide type tachykinin-related neuropeptides are widely distributed in invertebrates.

6-4-4 S-Iamide Peptides.

The novel neuropeptide, LSSFVRIamide, was isolated from the ganglia of the prosobranch mollusc, Fusinus ferrugineus (Kuroki et al 1992), and termed S-Iamide peptide (S-IaP) from its structure (Muneoka and Kobayashi, 1992). The peptide shows a potentiating action on twitch contraction of the radula retractor muscle of the animal at low doses, while at higher doses it shows an inhibitory action on the muscle. The peptide also shows contrac-

tion-potentiating or contraction-inhibiting effect on various muscles of other molluscs. Furthermore, the peptide shows a potent inhibitory effect on twitch contraction of the body-wall muscle of Urechis. The Urechis inhibitory peptides, P2, P3 and P6, also have -SSFVRIamide at their C-terminal portions as a common structure, and they also show an inhibitory activity on phasic contraction of the ABRM of Mytilus. Recently, some other S-IaP-related peptides were isolated from the central nervous systems of the pulmonates, Achatina fulica and Helix pomatia (Ikeda, Kuroki et al., 1992), and of the bivalve, Anodonta cygnea (Fujisawa, unpublished), by using the ABRM of Mytilus as the bioassay system. All of those peptides show an inhibitory action on phasic contraction of the ABRM. The structures of these S-IaP-related peptides are shown in Table 6.10.

Six out of the eight S-IaP-related peptides have -SSFVRI-amide at their C-terminal portions as a common structure. The other two peptides have -SNFIRIamide and SGFVRIamide, respectively. However, the differences in amino acid residues of these molluscan peptides represent conservative substitutions. Therefore, the peptide having -SNFIRIamide and SGFVRIamide can be regarded as members of the same family, i.e. S-Iamide peptide family.

In the present study, I showed that S-IaPs are distributed in both the Mollusca and the Echiura. It is very interesting that such closely related neuropeptides as S-IaPs are distributed interphyletically. The S-IaPs have been found to show biological actions on various muscles of invertebrates and vertebrates (Ikeda, unpublished). The peptide family might be distributed not only in the Mollusca and the Echiura but also in other animal groups.

6-4-4 MIP-Related peptides.

The three Urechis peptides , P8 (AKYFLamide), P9 (ARYFLa-

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mide) and P18 (AKFFLamide), have a structural similarity with molluscan neuropeptides, Mytilus inhibitory peptides (MIPs).

In 1988, Hirata et al. isolated two congeneric hexapeptides, GSPMFVamide and GAPMFVamide, from the pedal ganglia of Mytilus edulis, and termed them Mytilus inhibitory peptides (MIPs). The MIPs show a potent inhibitory effect on contractions of the ABRM of the animal. From the study of structure-action relationships of the MIPs, the essential structure in the MIPs for exhibition of the inhibitory action has been suggested to be -FVamide, and Pro^3 has been shown to be very important (Fujisawa, Kubota, Ikeda et al., 1991).

Fujisawa, Kubota, Ikeda et al. (1991) isolated five MIP-family peptides from the ABRMs themselves. Further, MIPs were shown to have inhibitory effect on muscles and neurons of other molluscs (Hirata, Kubota, Iwasawa et al., 1989; Yongsiri et al., 1989; Kiss, 1990). Later, 22 MIP-analogue peptides have been isolated from two pulmonates, Helix pomatia (Ikeda et al., 1991) and Achatina fulica (Ikeda, Yasuda-Kamatani et al., 1992). As shown in Table 6.11, the MIP-family peptides have the -PXFV(I)amide structure in their C-terminal portions as a common sequence. However, a MIP-related pentapeptide, MRYFVamide, which lacks the proline residue, was also isolated from the ABRM of Mytilus (Fujisawa, unpublished). This peptide shows a structural relation with three Urechis peptides, P8, P9 and P18, which were purified in the present experiments (Table 6.12).

The Urechis peptide ARYFLamide as well as the Mytilus peptide MRYFVamide, was found to have an inhibitory activity on phasic contraction of the ABRM of Mytilus. Chemical properties of Leu resemble to that of Val. Therefore, it is supposed that the inhibitory effect of ARYFLamide is mediated by MIP receptors at the neuromuscular junctions of the ABRM. One of the other Urechis peptides AKYFLamide, however, show a potentiating activity on the ABRM. Although both Arg and Lys have an positive

charge, AKYFLamide show an opposite activity to that of ARYFLamide on the ABRM. Further, another _Urechis_ peptide, AKFFLamide, does not show any activity on the ABRM. It is interesting that peptides having such a structural similarity show different activities on the same muscle.

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Table 6.1.   Results of structure analyses of the purified peptides, P1-P10.

P1   AAS (pmoles):   Ala - Ala - Pro - Leu - Pro - Arg - Leu
                      5382 5211 2608 2451 2200 694   569
     AAA (Leu=2.0):   Ala, Leu, Arg, Pro
                      2.0 2.0 1.0 2.0
     FAB-MS: 736.5 m/z (M+H)$^+$

P2   AAS (pmoles):   Ala - Ser - Ser - Phe - Val- Arg - Ile
                      405   23   26   411   378 48   77
     AAA (Phe=1.0):   Ser, Ala, Val, Ile, Phe, Arg
                      1.6 0.9 0.9 0.9 1.0 1.0

P3   AAS (pmoles):   Pro - Ser - Ser - Phe - Val - Arg - Ile
                      3121 514   441   1909 2752 1092 270
     AAA (Phe=1.0):   Ser, Val, Ile, Phe, Arg, Pro
                      1.7 1.0 1.0 1.0 1.0 1.0

P4   AAS (pmoles):   Ala-Ile-Ser-Ala-Gly-His-Arg-Tyr-Met-Gly-Leu
                      643 583 84 789 380 60 92 378 468 213 59
     AAA (Leu=1.0):   Ser, Gly, Ala, Met, Ile, Leu, Tyr, His, Arg
                      1.0 2.2 2.1 0.8 1.0 1.0 1.0 1.0 1.2
     FAB-MS: 1174.4 m/z (M+H)$^+$

P5   AAS (pmoles):   Ala - Met - Phe - Arg - Ala - Asp
                      879   430   173   57   16   3
     AAA (Leu=1.0):   Asp, Ala, Met, Leu, Phe, Arg
                      1.0 2.1 0.8 1.0 1.1 1.0
     FAB-MS: 822.1 m/z (M+H)$^+$

P6   AAS (pmoles):   Val - Ser - Ser - Phe - Val - Arg
                      872   99   62   50   7   2
     AAA (Phe=1.0):   Ser, Val, Ile, Phe, Arg
                      1.7 1.9 0.9 1.0 1.0
     FAB-MS: 806.3 m/z (M+H)$^+$

P7   AAS (pmoles):   Leu - Arg - Asn - Asn - Phe - Leu
                      1354 506   975   860   158   16
     AAA (Leu=2.0):   Asp, Leu, Phe, Arg
                      2.1 2.0 1.1 1.0
     FAB-MS: 775.3 m/z (M+H)$^+$

P8   AAS (pmoles):   Ala - Lys - Tyr - Phe - Leu
                      3913 628   649   57   +
     AAA (Leu=1.0):   Ala, Leu, Tyr, Phe, Lys
                      1.0 1.0 1.0 1.0 1.0
     FAB-MS: 640.3 m/z (M+H)$^+$

P9   AAS (pmoles):   Ala - Arg - Tyr - Phe - Leu
                      982   282   437   68   7
     AAA (Leu=1.0):   Ala, Leu, Tyr, Phe, Arg,
                      1.0 1.0 1.0 1.0 1.1
     FAB-MS: 668.2 m/z (M+H)$^+$

P10 AAS (pmoles):   Ala - Tyr - Phe - Arg - Tyr - Asp - Leu
                      678   442   270   214   323   269   6
     AAA (Leu=1.0)   Asp, Ala, Leu, Tyr, Phe, Arg
                      1.0 1.0 1.0 2.0 1.0 1.1
     FAB-MS: 946.3 m/z (M+H)$^+$

AAS, amino acid sequence analysis; AAA, amino acid analysis; FAB-MS, fast atom bombardment mass spectrometric measurement

Table 6.2.   Results of structure analyses of the purified peptides, P11-P20.

P11 AAS (pmoles):   Ala-Ala-Gly-Met-Gly-Phe-Phe-Gly-Ala-Arg
                      699 655 380 378 271 179 174 140 172 91
     AAA (Phe=2.0):   Gly, Ala, Met, Phe, Arg
                      3.0 3.0 0.8 2.0 1.0
     FAB-MS: 983.3 m/z (M+H)$^+$

P12 AAS (pmoles):   Phe - Trp
                      319   78
     AAA (Phe=1.0):   Phe, Trp
                      1.0 0.2
     FAB-MS: 352.2 m/z (M+H)$^+$

P13 AAS (pmoles):   Ala - Leu - Thr - Phe - Tyr
                      440   238   141   207   54
     AAA (Leu=1.0):   Thr, Ala, Leu, Tyr, Phe
                      1.0 1.0 1.0 1.0 1.1
     FAB-MS: 614.3 m/z (M+H)$^+$

P14 AAS (pmoles):   Ser-Ser-Gly-Pro-Asp-Ala-Ala-Phe-Trp
                      24 23 88 105 63 88 120 42   4
     AAA (Phe=1.0):   Asp, Ser, Gly, Ala, Phe, Pro
                      0.9 1.6 1.0 1.8 1.0 0.9
     FAB-MS: 937.3 m/z (M+H)$^+$

P15 AAS (pmoles):   Gly - Gly - Asp - Met - Ser - Glu - Phe
                      35   39   23   12   3   10   6
     FAB-MS: 928.4 m/z (M+H)$^+$

P16 AAS (pmoles):   Gly - Ser- Glu - Asp - Leu - Glu - Thr - Phe
                      50   10   11   9   7   12   6   4
     AAA (Leu=1.0):   Asp, Thr, Ser, Glu, Gly, Leu, Phe
                      1.1 1.0 1.1 2.1 1.4 1.0 1.2
     FAB-MS: 1083.3 m/z (M+H)$^+$

P17 AAS (pmoles):   Gly-(Asn)-Asn-Leu-Gly-Leu-Arg-Met-(Met)-(Ser)-
                      25   +   9   8   8   6   2   4   6   +
                      Glu-Phe-(Gly)-Pro
                      7   2   +   2
     AAA (Leu=2.0):   Asp, Glu, Gly, Met, Leu, Phe, His, Arg, Pro
                      2.1 1.0 2.1 0.9 2.0 2.2 0.8 1.8 +

P18 AAS (pmoles):   Ala - Lys - Phe - Phe - Leu
                      737   390   66   15   2
     AAA (Leu=1.0):   Ala, Leu, Phe, Lys
                      1.0 1.0 2.1 1.0
     FAB-MS: 624.1 m/z (M+H)$^+$

P19 AAS (pmoles):   Ala-Trp-Ala-Thr-Gly-Val-Gln-Arg-Val
                      66 26 60 17 34 30 20 20 14
     AAA (Arg=1.0):   Thr, Glu, Gly, Ala, Val, Arg
                      1.0 1.1 1.1 2.0 1.8 1.0
     FAB-MS: 1171.6 m/z (M+H)$^+$

P20 AAS (pmoles):   Arg-Trp-Gly-Asp-Asn-Val-Met-Arg-Val
                      49 93 132 160 71 98 63 19 62
     AAA (Arg=2.0):   Asp, Gly, Val, Met, Arg, Trp
                      2.0 1.1 1.9 1.0 2.0 0.3
     FAB-MS: 1317.9 m/z (M+H)$^+$

AAS, amino acid sequence analysis; AAA, amino acid analysis; FAB-MS, fast atom bombardment mass spectrometric measurement

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Table 6.3. Proposed structures of the purified peptides.

P1:	H-Ala-Ala-Pro-Leu-Pro-Arg-Leu-NH$_2$
P2:	H-Ala-Ser-Ser-Phe-Val-Arg-Ile-NH$_2$
P3:	H-Pro-Ser-Ser-Phe-Val-Arg-Ile-NH$_2$
P4:	H-Ala-Ile-Ser-Ala-Gly-His-Arg-Tyr-Met-Gly-Leu-NH$_2$
P5:	H-Ala-Met-Phe-Arg-Ala-Asp-Leu-NH$_2$
P6:	H-Val-Ser-Ser-Phe-Val-Arg-Ile-NH$_2$
P7:	H-Leu-Arg-Asn-Asn-Phe-Leu-NH$_2$
P8:	H-Ala-Lys-Tyr-Phe-Leu-NH$_2$
P9:	H-Ala-Arg-Tyr-Phe-Leu-NH$_2$
P10:	H-Ala-Tyr-Phe-Arg-Tyr-Asp-Leu-NH$_2$
P11:	H-Ala-Ala-Gly-Met-Gly-Phe-Phe-Gly-Ala-Arg-NH$_2$
P12:	H-Phe-Trp-OH
P13:	H-Ala-Leu-Thr-Phe-Tyr-OH
P14:	H-Ser-Ser-Gly-Pro-Asp-Ala-Ala-Phe-Trp-OH
P15:	H-Gly-Gly-Asp-Met-Ser-Glu-Phe-Trp-OH
P16:	H-Gly-Ser-Glu-Asp-Leu-Glu-Thr-Phe-Trp-OH
P17:	H-Gly-Asn-Asn-Leu-Gly-Leu-Arg-Met-His-Arg-Glu-Phe-Phe-Pro-*
P18:	H-Ala-Lys-Phe-Phe-Leu-NH$_2$
P19:	H-Ala-Trp-Ala-Thr-Gly-Val-Gln-Arg-Val-Trp-NH$_2$
P20:	H-Arg-Trp-Gly-Asp-Asn-Val-Met-Arg-Val-Trp-NH$_2$

*, the C-termini of the peptides are not yet determined to be amidated or not.

Table 6.4. Activities of the purified peptides on the body-wall muscle of Urechis and phasic contraction of the ABRM of Mytilus.

	Structure	Body-wall muscle of Urechis	ABRM of Mytilus
P1 :	AAPLPRLa	contraction	potentiation
P2 :	ASSFVRIa	inhibition	inhibition
P3 :	PSSFVRIa	inhibition	inhibition
P4 :	AISAGHRYMGLa	inhibition	inhibition
P5 :	AMFRADLa	inhibition	potentiation
P6 :	VSSFVRIa	inhibition	inhibition
P7 :	LRNNFLa	inhibition	potentiation
P8 :	AKYFLa	inhibition	potentiation
P9 :	ARYFLa	inhibition	inhibition
P10:	AYFRYDLa	inhibition	-
P11:	AAGMGFFGARa	contraction	-
P12:	FW	inhibition	potentiation
P13:	ALTFY	inhibition	-
P14:	SSGPDAAFW	inhibition	-
P15:	GGDMSEFW	inhibition	-
P16:	GSEDLETFW	inhibition	-
P17:	GNNLGLRMHREFFP*	contraction	potentiation
P18:	AKFFLa	inhibition	NA
P19:	AWATGVQRVWa	potentiation	NA
P20:	RWGDNVMRVWa	inhibition	inhibition

One-letter abbreviation is used to represent the amino acid residues. a, amide. *, the C-termini of the peptides are not yet determined to be amidated or not. -, not examined. NA, no activity.

<u>Table 6.5.</u>   Structural grouping
of the <u>Urechis</u> peptides.

P2	A	S	S	F	V	R	I	a		
P3	P	S	S	F	V	R	I	a		
P6	V	S	S	F	V	R	I	a		
P5		A	M	F	R	A	D	L	a	
P10		A	Y	F	R	Y	D	L	a	
P8			A	K	Y	F	L	a		
P9			A	R	Y	F	L	a		
P18			A	K	F	F	L	a		
P12							F	W		
P14	S	S	G	P	D	A	A	F	W	
P15		G	G	D	M	S	E	F	W	
P16		G	S	E	D	L	E	T	F	W
P19	A	W	A	T	G	V	Q	R	V	W	a
P20	R	W	G	D	N	V	M	R	V	W	a

One-letter abbreviation is used; a, amide;
*, the C-termini of the peptides are not yet
determined to be amidated or not.

Table 6.6. The peptides having
-FRXDLamide structure in their
C-terminal portions.

Leucosulfakinin	F	S A F R A D L a
P5		A M F R A D L a
P10		A Y F R Y D L a

One-letter abbreviation; a, amide.

Table 6.7. Structures of the cockroach peptides, leucomyosuppressin and leucosulfakinin

Leucomyosuppressin	pQ D V D H V F L R F a
Leucosulfakinin	pQ S D D Y G H M R F a
	$\overset{|}{SO_3H}$

One-letter abbreviation; pQ, pyroGlu; a, amide. References are shown in the text.

Table 6.8. Structures of a _Urechis_ peptide, molluscan SCP-related peptides, and insect peptides, pyrokinins and PBANs.

Phyla/Class	Animals		Structures	References
Mollusca	_Aplysia_	SCP$_A$	ARPGYLAFPRMa	1
		SCP$_B$	MNYLAFPRMa	2
	Achatina		SGYLAFPRMa	3
			YLAFPRMa	3
	Helix	SCP$_B$	MNYLAFPRMa	4
			SGYLAFPRMa	4
			QPPLPRYa	3
	Mytilus		APNFLAYPRLa	5
			LAYPRLa	5
Echiura	_Urechis_	P1	AAPLPRLa	
Insecta	_Leucophaea_	Lem-PK	pQTSFTPRLa	6
	Locusta	Lom-PK	-GWPQQPFVPRLa	7
		Lom-MT	GAVPAAQFSPRLa	8
		Lom-MT-II	EGDFTPRLa	7
	Heliothis	Hez-PBAN	-DSRTKYFSPRLa	9
	Bombyx	PBAN-I	-ESRTRYFSPRLa	10
		PBAN-II	-ESRTRYFSPRLa	11

One-letter abbreviation; pQ, pyroGlu; a, amide. The C-terminal undecapeptide fragments of Lom-PK, Hez-PBAN, PBAN-I and PBAN-II are shown. References: 1, Lloyd et al., 1987; 2, Morris et al., 1982; 3, Ikeda et al., unpublished; 4, Price et al., 1990; 5, Fujisawa et al., unpublished; 6, Holman et al., 1986C; 7, Nachman and Holman, 1991; 8, Schoofs, Holman, Hayes, Tips et al., 1990; 9, Rsina et al., 1989; 10, Kitamura et al., 1989; 11, Kitamira et al., 1990)

Table 6.9 Strucrures of a <u>Urechis</u> peptide, locutatachykinins, eledoisin and substance P.

Phyla/Class	Animals		structure
Insecta	<u>Locasta</u>	Lom-TK-I	GPSGFYGVRa
		Lom-TK-II	APLSGFYGVRa
Echiura	<u>Urechis</u>	P11	AAGMGFFGARa
Mollusca	<u>Anodonta</u>		pQYGFHAVRa
	<u>Eledone</u>	eledoisin	pQPSKDAFIGLMa
Mammalia		substance P	RPKPQQFFGLMa

One-letter abbreviation; pQ, pyroGlu; a, amide. References are shown in the text.

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Table 6.10. Structures of S-Iamide peptides.

Phyla	Animals		structures	References
Echiura	Urechis	P2:	ASSFVRIa	
		P3:	PSSFVRIa	
		P6:	VSSFVRIa	
Mollusca	Anodonta		SGFVRIa	Fujisawa, unpublished
	Fusinus		LSSFVRIa	Kuroki et al., 1992
	Helix		TSSFVRIa	Ikeda, Kuroki et al.,
	Achatina		SPSSFVRIa	1992
			APSNFIRIa	

One-letter abbreviation; a, amide

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Table 6.11.   Mytilus inhibitory peptides in molluscs.

Animals	structures		References
Mytilus	MIP1	GSPMFVa	Hirata et al.,
	MIP2	GAPMFVa	1988
	MIP3	DSPLFVa	and
	MIP4	YAPRFVa	Fujisawa, Kubota,
	MIP5	ASHIPRFVa	Ikeda et al., 1991
Helix	MIP-H1	GAPAFVa	Ikeda et al.,
	MIP-H3	AAPRFVa	1991
	MIP-H4a	GAPMFVa	
	MIP-H4b	GAPLFVa	
	MIP-H5	GSPYFVa	
	MIP-H6	GAPYFVa	
	MIP-H7	RAPYFVa	
	MIP-H8	SVPIFVa	
	MIP-H9	GVPYFVa	
	MIP-H10	GPPMFIa	
	MIP-H11	AAPFFVa	
	MIP-H12	RAPFFVa	
Achatina	MIP-A1	AAPKFVa	Ikeda, Yasuda-Kamatani
	MIP-A2	GAPKFVa	et al., 1992
	MIP-A3	GAPVFVa	
	MIP-A4	GAPYFVa	
	MIP-A5	AAPYFVa	
	MIP-A6	GPPMFVa	
	MIP-A7	GAPFFVa	
MIP-A8	DAPKFVGRRDPPYFVa		
MIP-A9	AAPKFVGRRGSPYFVa		
MIP-A10	AAPKFVGRRGAPYFVa		

One-letter abbreviation; a, amide.

Table 6.12. Structures and actions of some MIP-related peptides of _Mytilus_ and _Urechis_

Animals	Structures		Activity on the ABRM
Mytilus	MIP9	MRYFVa	−
Urechis	P9	ARYFLa	−
	P8	AKYFLa	+
	P18	AKFFLa	NA

One-letter abbreviation; a, amide. −, inhibition.
+, potentiation. NA, no activity.
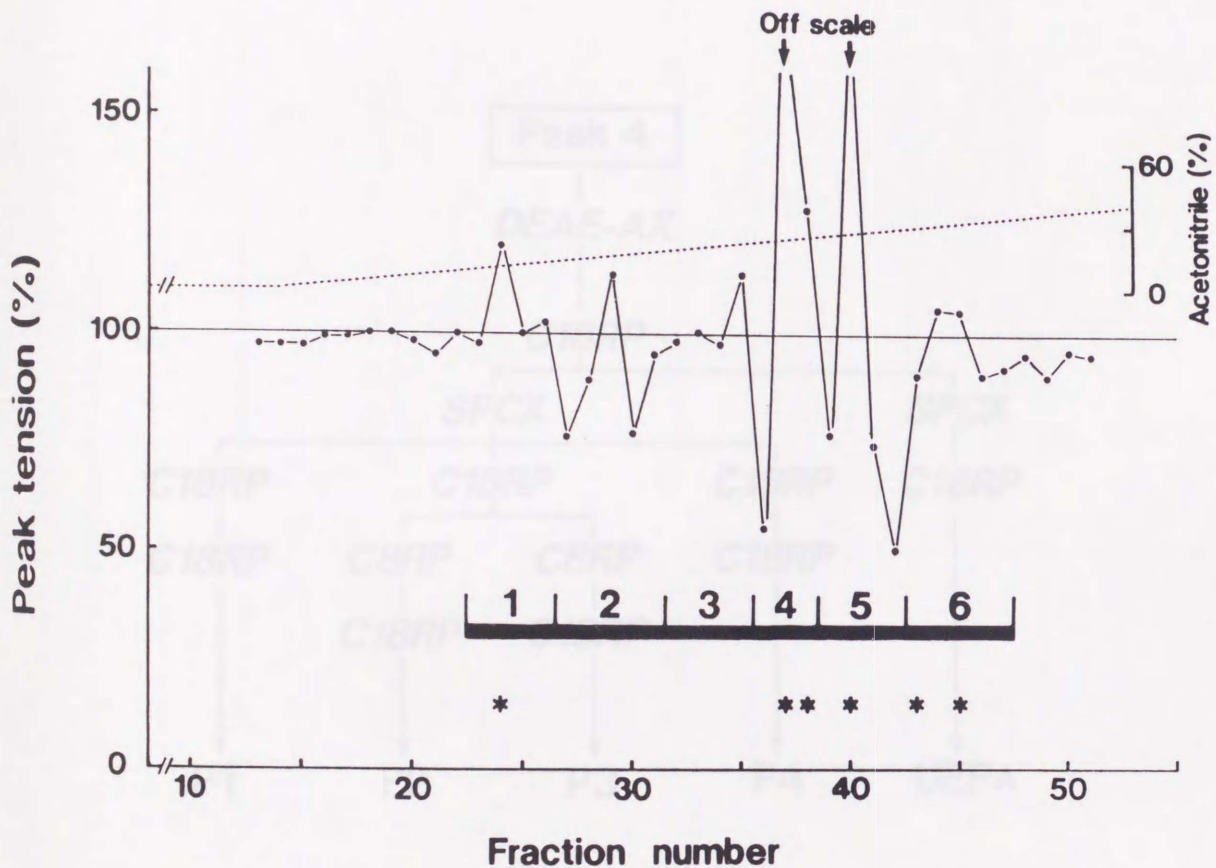MIP9: Fujisawa, unpublished

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Fig. 6.1. Biological activities of the fractions separated at the first step of HPLC purification on twitch contraction of the body-wall muscle of Urechis. Each peak tension was shown as a percentage of the control peak tension. Twitch contraction was evoked by applying an electrical pulse (15 V, 3 msec) of stimulation to the muscle at 10 min intervals. Each fraction was introduced 8 min prior to evoking the contraction in it and washed out soon after recording the contraction. Asterisks show contractile activity of the fractions on the muscle. Horizontal thick line show the fractions of active peaks 1-6. HPLC conditions are given in text.
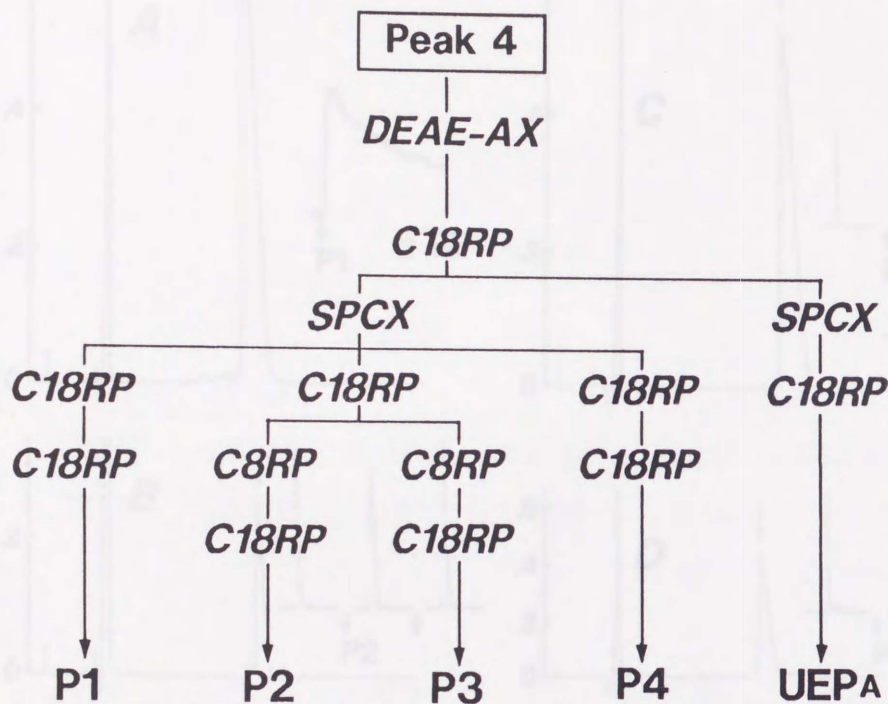
Fig. 6.2. Summarized procedures for HPLC purification of the peptides (P1-P4, and UEP$_A$) in peak 4 obtained at the first step of HPLC purification. DEAE-AX: anion-exchange column. SPCX: cation-exchange column. C18RP: C-18 reversed-phase column. C8RP: C-8 reversed-phase column.
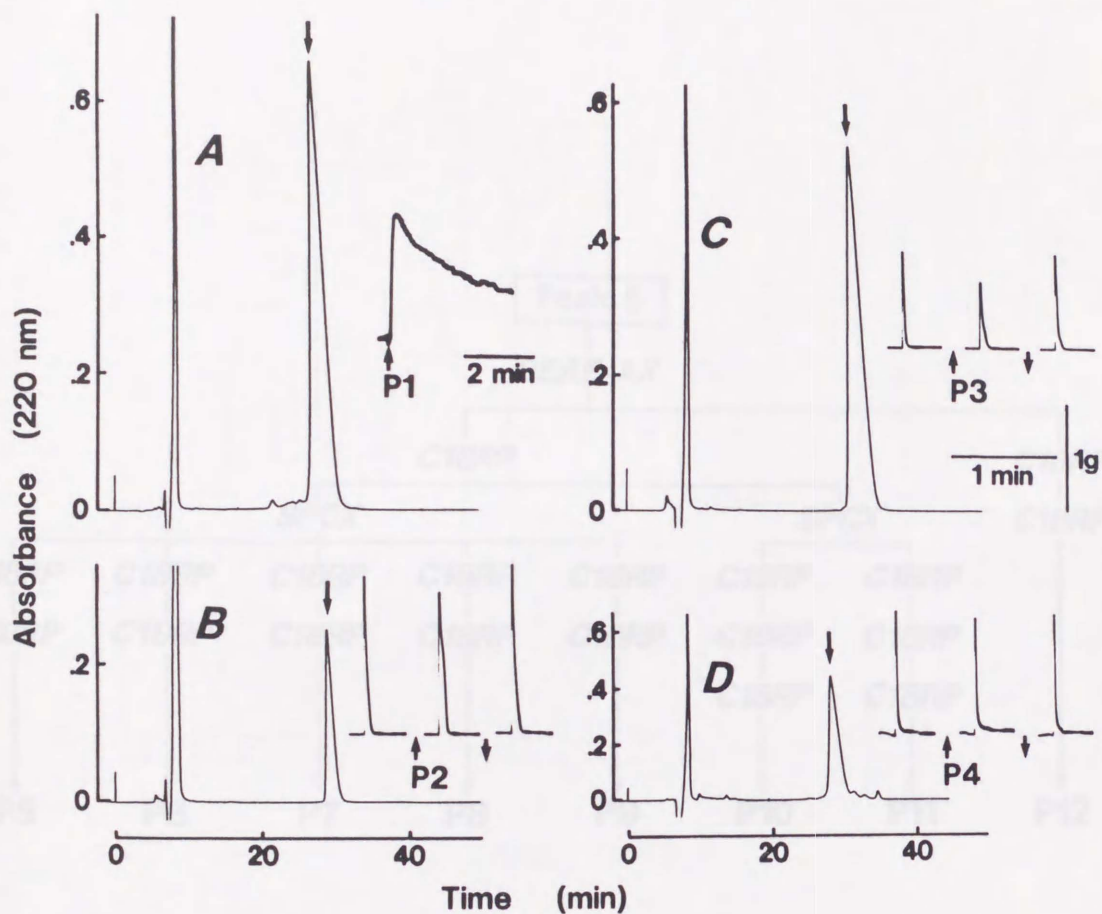
<u>Fig. 6.3.</u> Chromatograms of the final steps of HPLC purification of the peptides P1-P4 (A-D). Long downward arrows show absorbance peaks of the peptides. A C-18 reversed-phase column was used. Elution was performed with acetonitrile in 0.1% TFA (pH 2.2). Concentrations of acetonitrile were as follows: A,17.5%; B,17%; C,17%; D,17%. Inserts show the biological activity of the purified peptides on the body-wall muscle of <u>Urechis</u>. The peptides were applied at upward arrows and washed out at short downward arrows. Recording procedures of the contraction are the same as in Fig. 6.1.
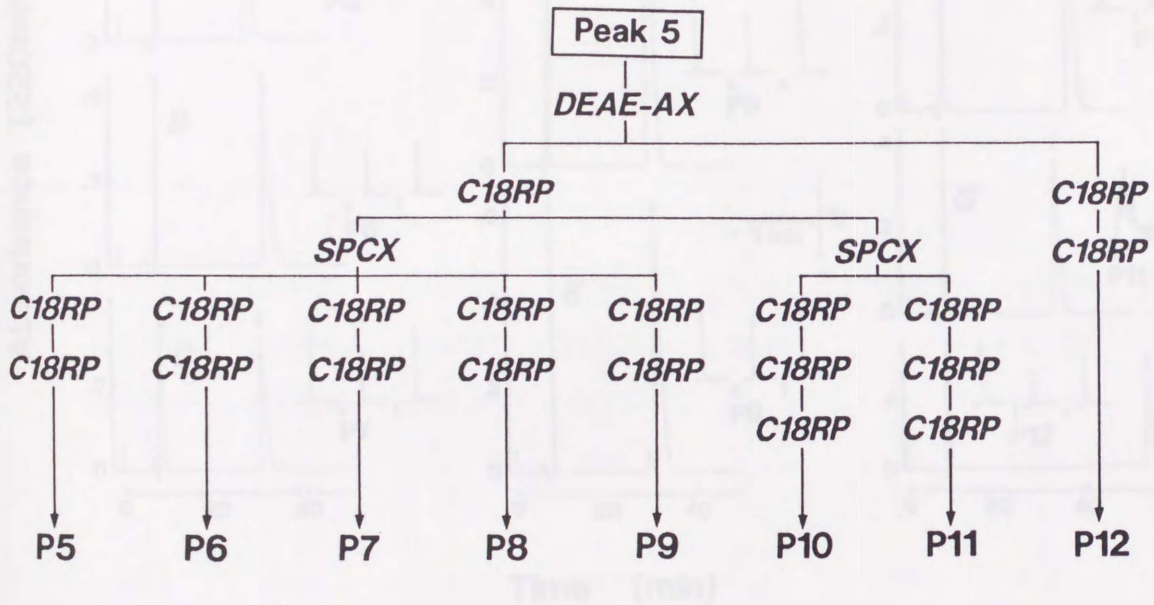
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Fig. 6.4. Summarized procedures for HPLC purification of the peptides (P5-P12) in peak 5 obtained at the first step of HPLC purification. See Fig. 6.2.
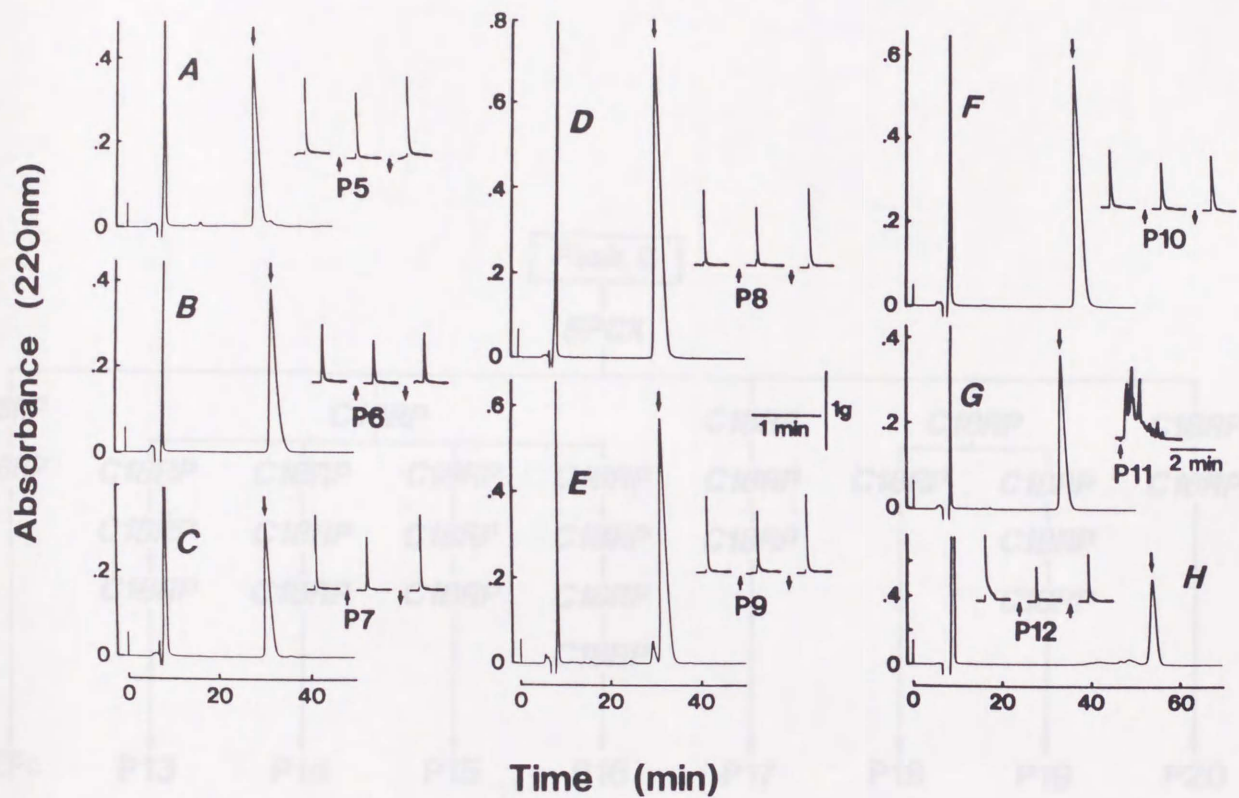
144

Fig. 6.5. Chromatograms of the final steps of HPLC purification of the peptides P5-P12 (A-H). A C-18 reversed-phase column was used. Elution was performed with acetonitrile in 0.1% TFA (pH 2.2). Concentrations of acetonitrile were as follows: A,18.5%; B,18%; C,17.5%; D,18%; E,18.5%; F,19.5%; G,19.5%; H,18%. The other procedures are the same in Fig. 6.3.

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Peak 6

SPCX

C18RP	C18RP	C18RP			C18RP	C18RP	C18RP

C18RP	C18RP	C18RP	C18RP	C18RP	C18RP	C18RP	C18RP	C18RP	C18RP

	C18RP	C18RP	C18RP	C18RP	C18RP		C18RP	

	C18RP	C18RP	C18RP	C18RP			C18RP	

				C18RP				

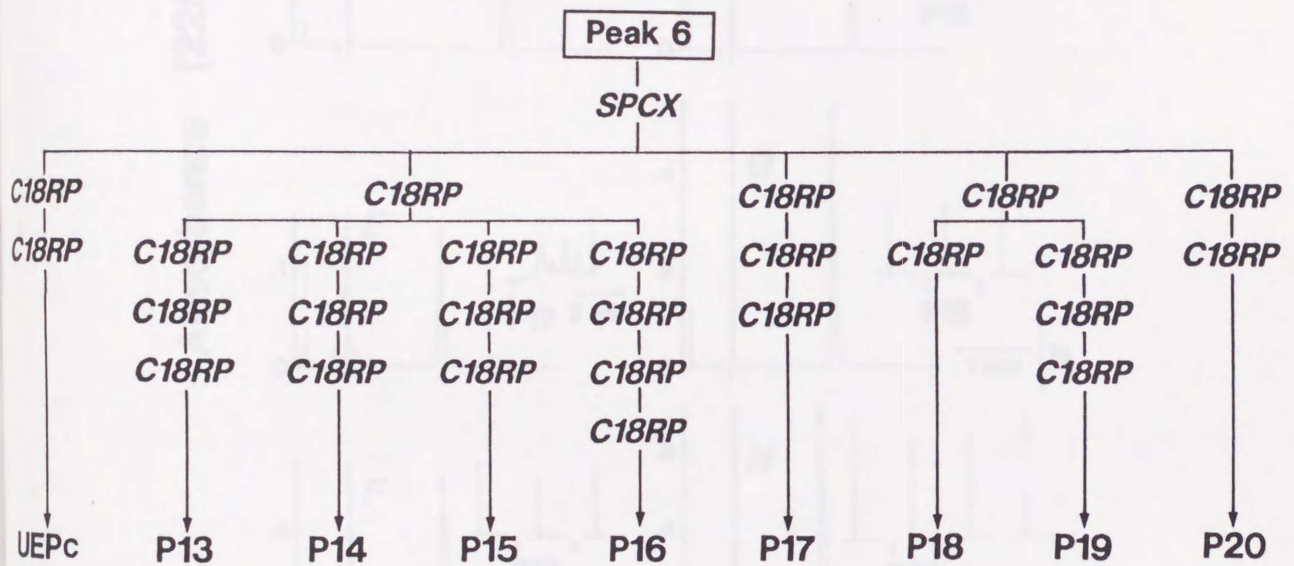| UEPc	P13	P14	P15	P16	P17	P18	P19	P20

Fig. 6.6. Summarized procedures for HPLC purification of the peptides (P13-P20, and UEP$_C$) in peak 6 obtained at the first step of HPLC purification. See Fig. 6.2.
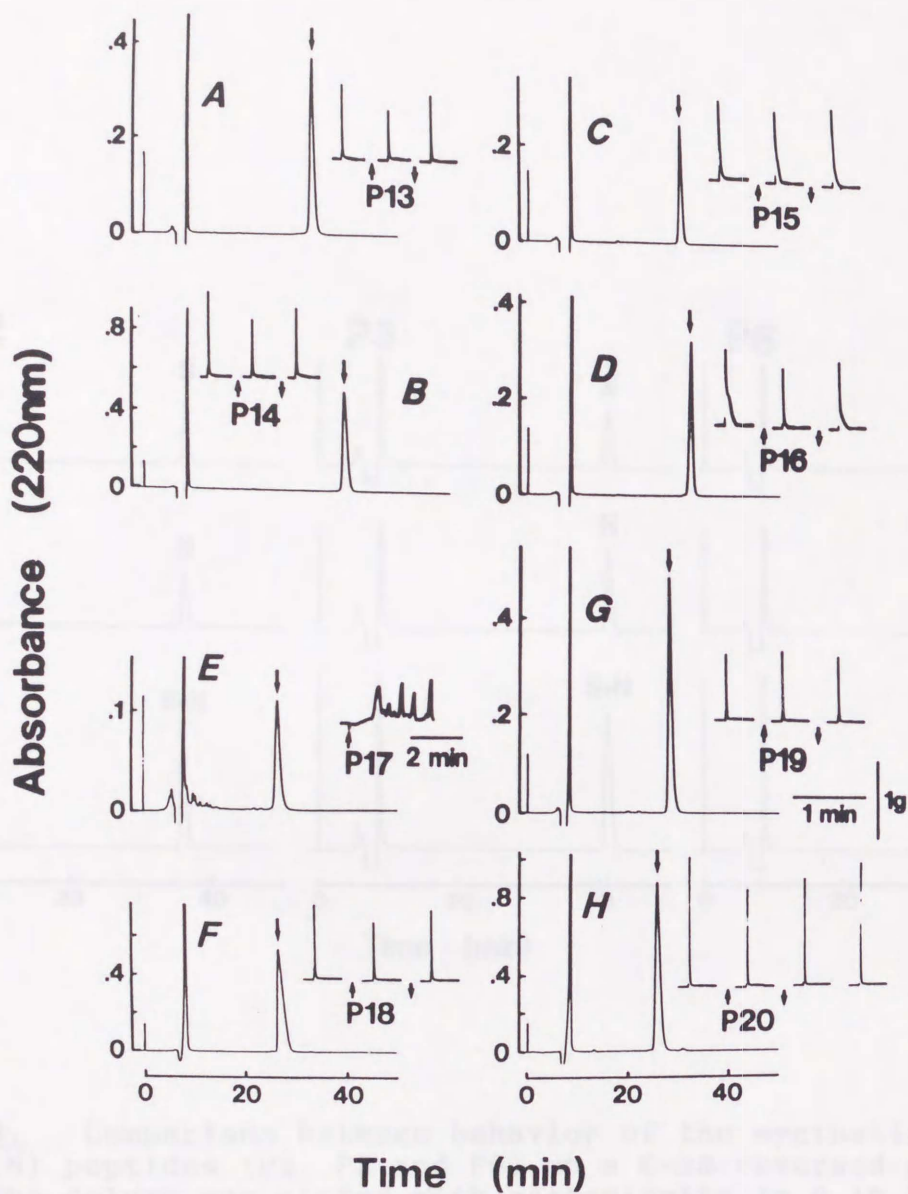
Fig. 6.7.   Chromatograms of the final steps of HPLC purification
of the peptides P13-P20 (A-H).   A C-18 reversed-phase column was
used.   Elution was performed with acetonitrile in o.1% TFA
(pH 2.2).   Concentrations of acetonitrile were as follows:
A,22.5%; B,22.5%; C,25%; D,26.5%; E,28%; F,22.5%; G,23%; H,24.5%.
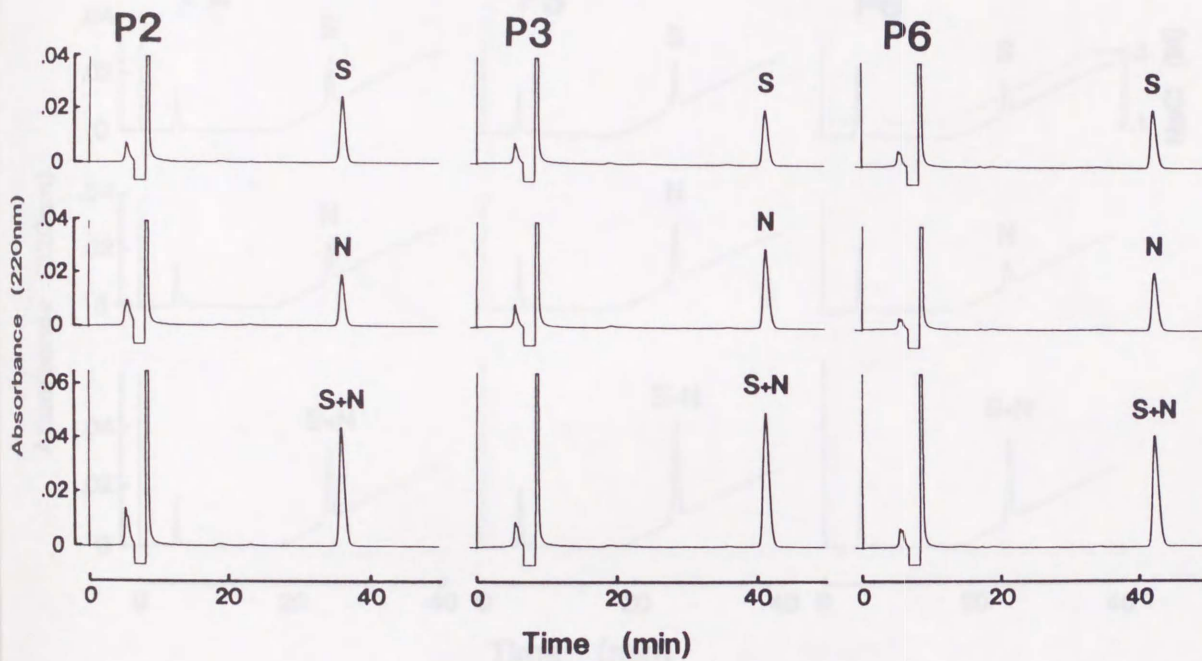The other procedures are the same in Fig. 6.3.

Fig. 6.8. Comparison between behavior of the synthetic (S) and native (N) peptides (P2, P3 and P6) on a C-18 reversed-phase HPLC. The column was eluted with acetonitrile in 0.1% TFA (pH 2.2). Concentration of acetonitrile: P1 and P3,17%; P6,18%. S+N, a mixture of the synthetic and native peptide.
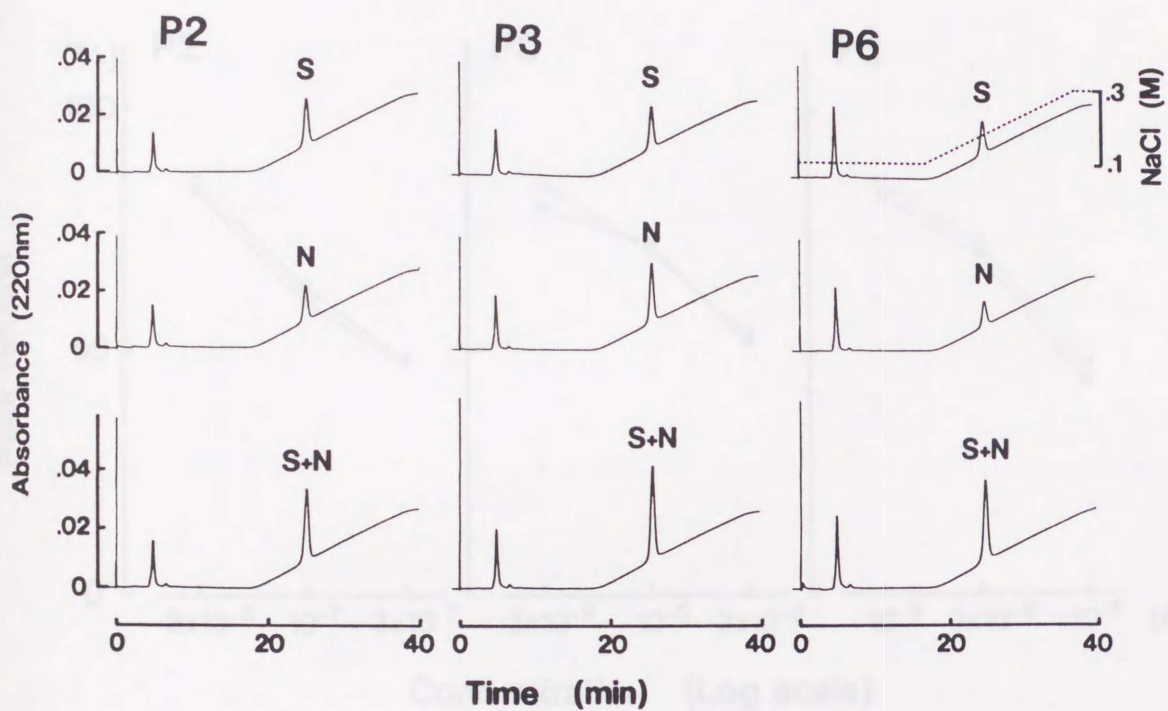
Fig. 6.9. Comparison between behavior of the synthetic (S) and native (N) peptides (P2, P3 and P6) on a cation-exchange HPLC. The column was eluted with a 20-min linear gradient of 0.1-0.3 M NaCl in 10 mM phosphate buffer (pH 6.9). S+N, a mixture of the synthetic and native peptide.
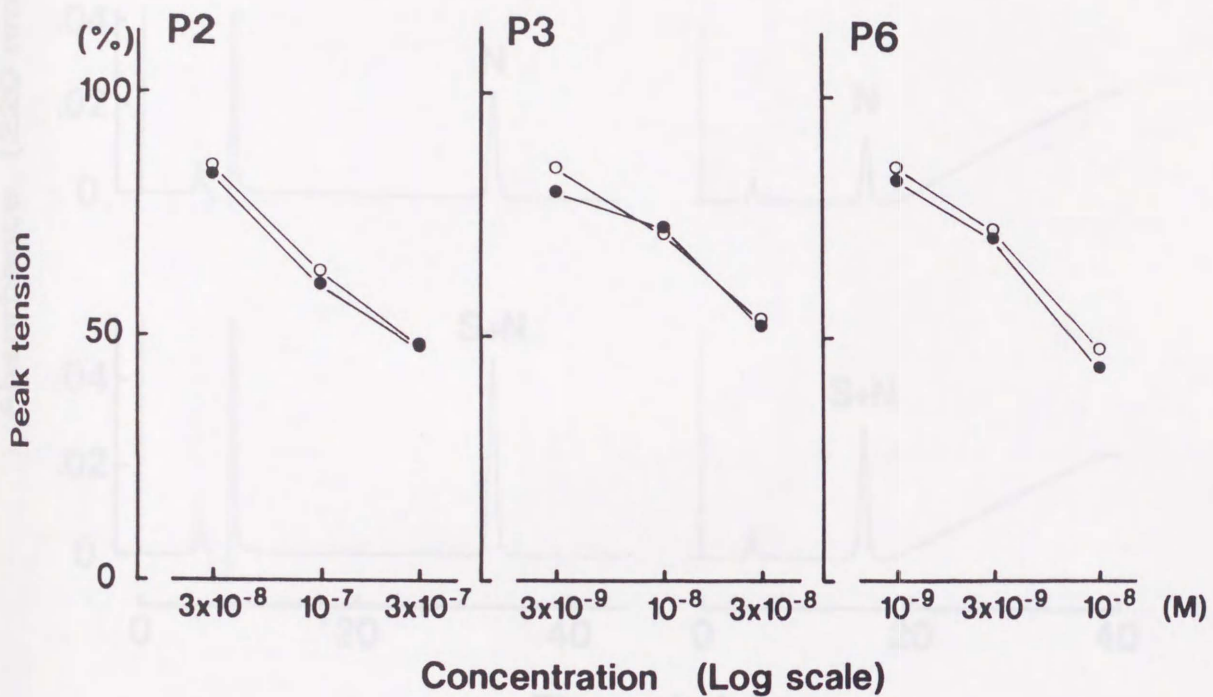
149

Fig. 6.10. Comparison between inhibitory effects of the synthetic (open circle) and native (closed circle) peptides (P2, P3 and P6) on twitch contraction of the body-wall muscle of Urechis. The procedures are the same as in Fig. 6.1. The concentrations of the native peptides were estimated from the results of the amino acid analyses.

<u>Fig. 6.11.</u> Comparison between behavior of the synthetic (S) and native (N) peptide (P1) on HPLC. A: C-18 reversed-phase HPLC. The column was eluted with 17.5% acetonitrile in 0.1% TFA (pH 2.2). B: cation-exchange HPLC. The procedures for the elution were the same as in Fig. 6.9. S+N, a mixture of the synthetic and native P1.

A

.04
.02
0

B

S

.4
.2

NaCl (M)

.04
.02
0

N

N

.04
.02
0

S+N

S+N

0   20   40   0   20   40
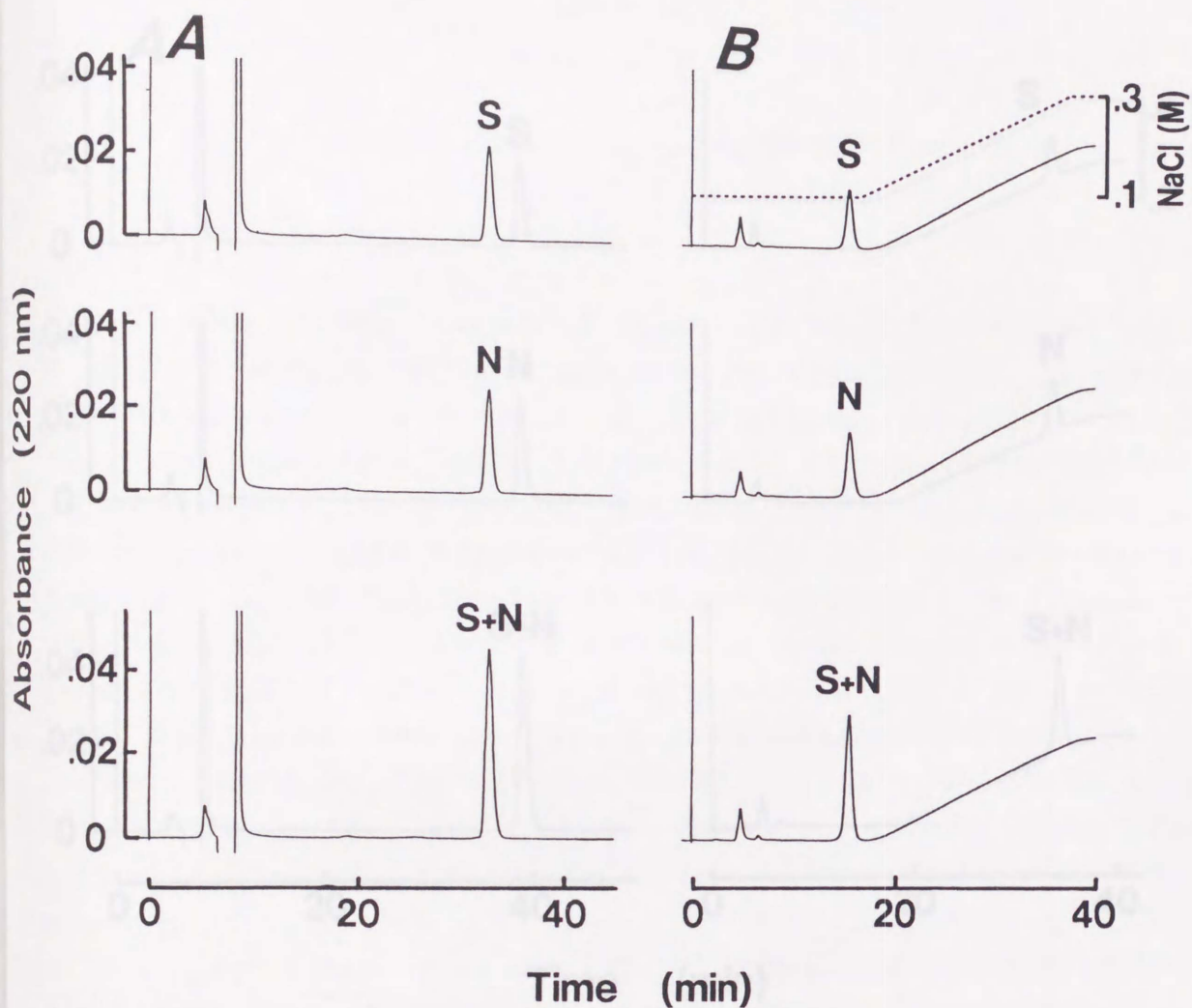
Absorbance (220 nm)

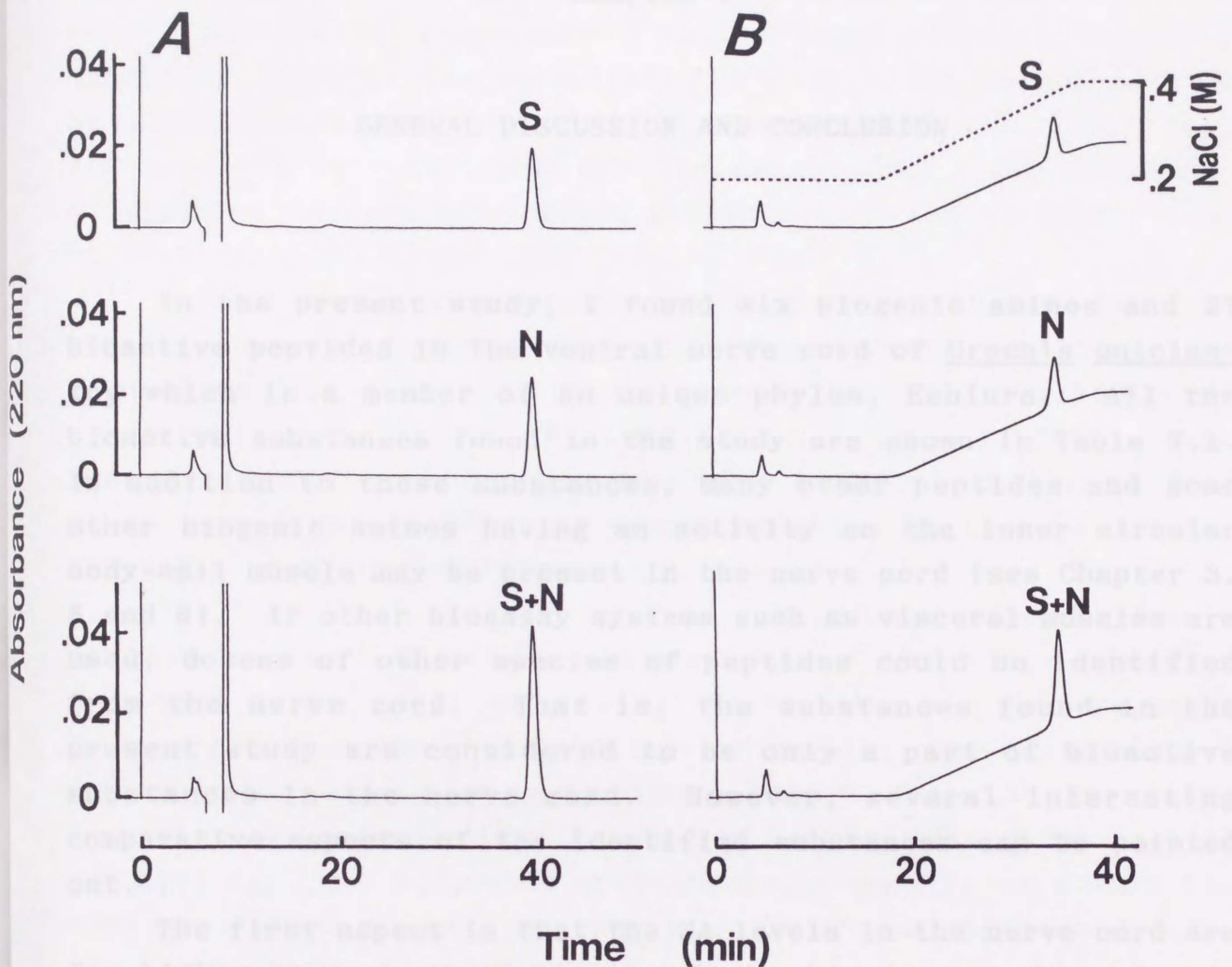Time   (min)

<u>Fig. 6.12.</u>   Comparison between behavior of the synthetic (S) and
native (N) peptide (P8) on HPLC. A: C-18 reversed-phase HPLC.
The column was eluted with 18% acetonitrile in 0.1% TFA (pH 2.2).
B: cation-exchange HPLC. The column was eluted with a 20-min
linear gradient of 0.2-0.4 M NaCl in 10 mM phosphate buffer (pH
6.9). S+N, a mixture of the synthetic and naive peptide P8.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

In the present study, I found six biogenic amines and 27 bioactive peptides in the ventral nerve cord of <u>Urechis</u> <u>unicinctus</u> which is a member of an unique phylum, Echiura. All the bioactive substances found in the study are shown in Table 7.1. In addition to these substances, many other peptides and some other biogenic amines having an activity on the inner circular body-wall muscle may be present in the nerve cord (see Chapter 3, 5 and 6). If other bioassay systems such as visceral muscles are used, dozens of other species of peptides could be identified from the nerve cord. That is, the substances found in the present study are considered to be only a part of bioactive substances in the nerve cord. However, several interesting comparative aspects of the identified substances can be pointed out.

The first aspect is that the NA levels in the nerve cord are far higher than those of OA, though <u>Urechis</u> is a member of protostomes in which OA levels are generally higher. In this respect, <u>Urechis</u> resembles deuterostomes. However, this may be a coincidence. The noradrenergic system of <u>Urechis</u> is supposed to have evolved separately from those of deuterostomes.

In <u>Urechis</u>, OA shows a potentiating effect on twitch contraction of the inner circular body-wall muscle, though the action of the amine is not potent. In contrast to OA, NA shows a potent inhibitory action on the contraction. This is the second interesting aspect. Because, in all of the tissues of other invertebrates examined so far, OA and NA have been shown to have

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qualitatively similar actions. Both of these actions of OA and NA are blocked by α-blockers. In the inner circular body-wall muscle of Urechis, the potentiating action of OA is not blocked by phentolamine, a typical α-blocker, while the inhibitory action of NA is effectively blocked by the drug. In the muscle of Urechis, the pharmacological properties of the NA system seem to be largely different from those of OA.

A large number of bioactive peptides have been isolated from nervous systems of vertebrates and invertebrates, and they have been classified into many groups according to the similarity of structure and action of the peptides. It is the third aspect that several of the Urechis peptides isolated in the present study were found to have structural relation to known neuropeptides isolated from other animal groups.

The inhibitory activity of FRVF, which is a member of the FR peptide family, on twitch contraction of the inner circular body-wall muscle of Urechis is not potent, but the peptide seems to be a neuromodulator controlling the muscle. The peptide shows an enhancing effect on spontaneous contractions of the small intestine of Xenopus. FRVF is clearly related with FRTF which is isolated from the pulmonate mollusc, Helix pomatia, and FRTF has also been shown to have an enhancing effect on spontaneous contractions of the Xenopus intestine.

The S-Iamide peptides are also found to be distributed in both the Mollusca and the Echiura. Furthermore, three Urechis peptides, ARYFLamide, AKYFLamide and AKFFLamide, are found to have a similar sequence with that of a molluscan neuropeptide, MRYFVamide, which is considered to be a member of Mytilus-inhibitory-peptide family. From these facts, it seems that the Echiura may be a phylum related to the Mollusca.

One of the other Urechis peptide AAPLPRLamide shows a structural similarity with the molluscan neuropeptides, small cardio-active peptides (SCPs), and two groups of insect neuropeptides,

pyrokinins and pheromone biosynthesis neuropeptides. Further, a
Urechis peptide, AAGMGFFGARamide, also shows a structural rela-
tion with both the molluscan neuropeptide, pQYGFHAVRamide, and
the insect tachykinins, GPSGFYGVRamide and APLSGFYGVRamide.   A
comparative pharmacological study on the actions of these pep-
tides on muscles of echiuroids, molluscs and insects are now in
progress.

The Echiura may be a phylum having similar characteristics
with those of the common ancestor of the Annelida, Mollusca and
Arthropoda. It is required to isolate neuropeptides of annelids
which are closely related to echiuroids, and to compare the
structures of them with those of echiuroid, molluscan and arthro-
pod peptides. Isolation of bioactive peptides in a polichaeta
annelid is now in progress in our laboratory.

Table 7.1. Bioactive substances found in the ventral nerve cords of _Urechis unicinctus_.

a). Biogenic amines

1.	Acetylcholine	4.	Dopamine
2.	Octopamine	5.	Noradrenaline
3.	Serotonin	6.	Adrenaline

b). Bioactive peptides

1.	AKCSGKWANSYLCAGAN	14.	FSAFRADLa
2.	TFCTIDLNC	15.	AMFRADLa
3.	REFWK	16.	AYFRYDLa
4.	FRVF	17.	ASSFVRIa
5.	FRF	18.	PSSFVRIa
6.	AAPLPRLa	19.	VSSFVRIa
7.	AISAGHRYMGLa	20.	LRNNFLa
8.	AAGMGFFGARa	21.	AKYFLa
9.	FW	22.	ARYFLa
10.	SSGPDAAFW	23.	AKFFLa
11.	GGDMSEFW	24.	ALT
12.	GSEDLETFW	25.	ALTFY
13.	GNNLGLRMHREFFP*	26.	AWATGVQRVWa
		27.	RWGDNVMRVWa

One-letter abbreviation is used to represent the amino acid residues of the peptides. a, amide. *, the C-termini of the peptides are not yet determined to be amidated or not.

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ACKNOWLEDGMENTS

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