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Biological Activities of Ganglion Extracts from a Prosobranch Mollusc, *Fusinus ferrugineus*

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ABSTRACT

Acetone extract of the ganglia of *Fusinus ferrugineus* was applied to C-18 cartridges, and the flowthrough and retained materials were bioassayed on the radula retractor muscle of the animal. The flowthrough was found to have a potent contractile action and the retained material a potent contraction-inhibiting action in addition to a weak contractile action. The retained material was then applied to a column (2.6 × 40 cm) of Sephadex G-15. Fractions of 4 ml each were collected, and their activities were assayed on twitch contractions of the radula retractor. Three peaks (E_1, E_2 and E_3) of contraction-potentiating activity and one peak (I) of contraction-inhibiting activity were obtained. The maximum activities of peaks E_1, E_2 and E_3 were found at fractions 22, 44 and 50, respectively. The maximum activity of peak I was found at fractions 28—30. All of the activities of these peaks were destroyed by incubating the fractions with the peptidase subtilisin, suggesting that the active substances in the peaks are peptides. Using some kinds of molluscan muscles, the actions of the substances were examined in greater detail. The results suggest that peak E_1 may contain a novel excitatory peptide whose molecular weight is more than 1,000, and that peaks E_2 and E_3 may contain FMRFamide-related peptides. It is also suggested that peak I may contain two or more myomodulin-CARP-related inhibitory peptides.

Key words: *Bioactivity, Ganglion extract, Neuropeptide, Mollusc*

In 1977 Price and Greenberg[10] were the first to isolate the neuropeptide FMRFamide from the ganglia of the bivalve mollusc *Macrocallista nimbosa*. FMRFamide and related peptides have subsequently been found in many other animals. It is now well known that FMRFamide-related peptides (FaRPs) are widely distributed in not only molluscan but also non-molluscan animals and that they have diverse effects on muscles and neurones[2,9,11]. In the preliminary experiments of the present study, FMRFamide and some of its analogs were found to show a contractile effect on the redula retractor and protractor muscles of the prosobranch mollusc *Fusinus ferrugineus*. Therefore, it can be suspected that FaRPs might be present in this animal and might be involved in the regulation of the muscles as neuromediators.

In 1987, two novel heptapeptides whose structures are closely related to each other were isolated, one from the opisthobranch mollusc *Aplysia californica*[1] and the other from the bivalve mollusc *Mytilus edulis*[5]. The *Aplysia* peptide, which was termed myomodulin, potentiates contraction of the accessory radula closer muscle of the animal, and the *Mytilus* peptide, which was termed catch-relaxing peptide (CARP), relaxes catch tension in the anterior byssus retractor muscle (ABRM) of the bivalve. Later, CARP was found to have biological actions on not only *Mytilus* muscles but also on muscles of various other molluscs[4]. Thus, it is suspected that myomodulin-CARP-related peptides (MCRPs) may be widely distributed in molluscs. In the radula retractor and protractor muscles of *Fusinus*, CARP shows a potent inhibitory effect on their contractions[4]. MCRPs might also be physiologically involved in the regulation of the muscles.

In the present study, the biological activities of gel-filtrated acetone extracts of the ganglia of *Fusinus* on some kinds of molluscan muscles were examined. The results obtained suggest that FaRPs and MCRPs are present in the ganglia.

MATERIALS AND METHODS

Animals

Fresh living specimens of *Fusinus ferrugineus* 8-12 cm in length were obtained from a commercial source. The animals were kept in a cold box (4°C) and used for excision of the ganglion masses within 5 hrs.

For bioassay experiments, the radula retractor and proboscis retractor muscles of *Fusinus*, the heart of the clam *Meretrix lusoria* and the ABRM

of the mussel *Mytilus edulis* were used. *Fusinus* and *Meretrix* were obtained from the foregoing commercial source. *Mytilus* were collected from Hiroshima Bay. These animals were stored in aerated artificial seawater (ASW) in our laboratory at 20°C.

Ganglion extracts

The ganglion masses including buccal, cerebral, pedal, pleural and suboesophageal ganglia were excised from 400 specimens of *Fusinus*, immediately frozen on dry ice, and stored at -20°C. The frozen ganglia (about 10g) were steeped in 100% acetone (40 ml) and homogenized with Polytron. The homogenates were centrifuged (28000g for 30 min at 4°C). The pellet was re-extracted with 80% acetone (40 ml). The two acetone supernatants were pooled and evaporated to dryness. The dried material was taken up in 6 ml of 0.1 N hydrochloric acid and the fluid was again centrifuged (12000g for 10 min at 4°C). The supernatant was applied to two disposable C-18 cartridges (Waters Sep-Pak) in series.

The flowthrough from the cartridges was evaporated and used for the examination of its bioactivity. The retained material in the cartridges was eluted with methanol (8 ml). One eighth of the effluent was dried and used to examine its bioactivity. The rest of the effluent was also evaporated. The residue was taken up in 0.1 M acetic acid (0.5 ml), applied to a column (2.6 × 40 cm) of Sephadex G-15, and eluted with the same solvent. Fractions of 4 ml each were collected and lyophilized. Each lyophilized material was taken up in 0.7 ml of distilled water and stored at -20°C to examine its bioactivity.

Bioassays

The methods employed to dissect muscle bundles of the radula retractor of *Fusinus* and the ABRM of *Mytilus* were essentially the same as those described by Kobayashi[6] and Twarog[12], respectively. The muscle bundle of the proboscis retractor of *Fusinus* was made as follows. The muscle was isolated by cutting both ends and was carefully teased with a stainless needle under a dissecting binocular microscope to obtain a bundle of 1.5—1.8 mm in diameter and 14—17 mm in length.

The muscle bundles were mounted in an upright experimental chamber (2 ml). The methods of stimulating the muscles and recording the tension changes were essentially the same as those described by Muneoka and Twarog[8]. In the experiments of twitch contractions of the radula retractor muscle, a train of electrical pulses (20V, 2 msec, 0.2 Hz, 5 pulses) was applied to the muscle at 10 min intervals, and thus contraction trains, each consisting of 5 twitches, were evoked. Tetanic contraction of the proboscis retractor muscle was evoked by stimulating the muscle with repetitive electrical pulses (15 V, 1 msec, 10 Hz, for 5 sec) at 10 min intervals. Phasic contraction of the ABRM was also produced by stimulation with repetitive pulses (20 V, 3 msec, 10 Hz, for 5 sec) at 10 min intervals. Effects of the fractions on these contractions were examined by applying them to the muscles 8 mins prior to the contractions. The extracts were washed out soon after recording the contractions. Catch contraction of the ABRM was produced by applying 10^{-4} M ACh for 2 mins. Five minutes after washing-out ACh, extract was applied for another 5 mins to examine its relaxing effect on catch tension.

Each of the frozen fractions was thawed before the bioassay experiment and used as stock sample solution. An appropriate amount of the stock sample solution was removed, diluted in ASW and applied to the muscle preparation. Drugs were also used by diluting an appropriate amount of each stock solution with ASW. The method of application of the drugs to the muscles was the same as that in the extracts.

The heart preparation from *Meretrix* was made according to the methods of Welsh and Taub[13]. The whole heart was isolated, tied with cotton threads at the two auricular-ventricular regions of the ventricle, and suspended in an aerated organ bath (10 ml) filled with ASW. Thus, spontaneous cardiac activity was recorded on a pen recorder. The rectum was left in the ventricle. The effects of the extracts and drugs on the cardiac activity were examined by injecting their stock solutions into the bath.

The experiments were carried out in spring and early summer at room temperature (18—24°C).

Subtilisin treatment

After being adjusted at pH 7.8 by adding Tris-HCl (10 mM), the active fractions were incubated with subtilisin (10^{-5} g/ml) for 30 min at 36°C. The subtilisin-treated fractions were then boiled for 30 min. The control fractions were only boiled for 30 min after being adjusted at pH 7.8.

Physiological salines and drugs

The physiological saline for the proboscis retractor, the ABRM and the heart 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. For the saline of the radula retractor muscle, low-Mg^{2+} ASW (20 mM Mg^{2+} ASW) was used to obtain large twitch contractions. This saline was made by replacing a part of $MgCl_2$ in the normal ASW with osmotically equivalent NaCl.

CARP was synthesized by Drs. I. Kubota and N. Iwasawa (Suntory Institute, Osaka, Japan). The other drugs used were acetylcholine bromide (ACh, from Sigma), FMRFamide (from Peninsula Laboratories) and subtilisin (From Boehringer Mannheim Biochemicals).

RESULTS

The flowthrough material was found to show a potent contractile effect on the radula retractor muscle of *Fusinus* (Fig. 1A). In contrast, the re-

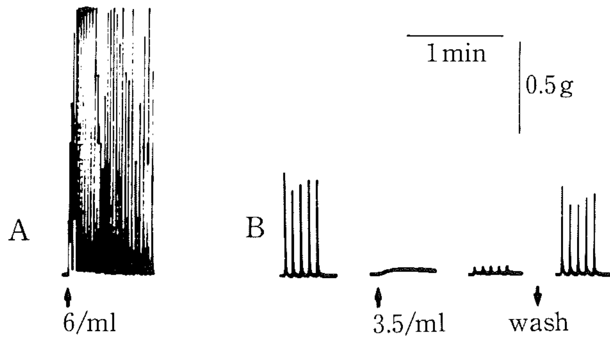
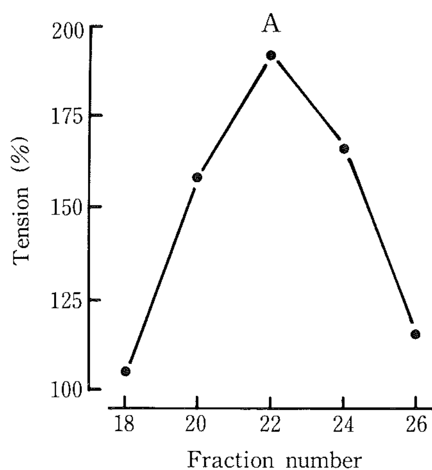


Fig. 1. Effects of the ganglion extracts which flowed through and was retained by C-18 cartridges on the radula retractor muscle of *Fusinus*. A: contractile effect of the flowthrough (6 ganglion masses/ml ASW). B: weak contractile effect and potent contraction-inhibiting effect of the retained material (3.5 ganglion masses/ml ASW). The retained material was applied 8 min prior to the second contraction train (upward arrow) and washed out soon after recording it (downward arrow).

ing activities of the fractions were observed when concentration of the extracts in the test solutions was relatively high (1.5 ganglion masses/ml ASW or more).

The peak I fractions were obtained soon after peak E_1. The maximum inhibitory activity on the contractions was found at fractions 28-30 (Figs. 3A and B). The inhibitory activities of the fractions were marked even when concentration of the extracts in the test solutions was very low (e.g., 0.1 ganglion mass/ml ASW). The peak I fractions also showed catch-relaxing activities on the ABRM of *Mytilus* (Figs. 3C and D). However, the catch-relaxing activities of the fractions did not completely correlate with their contraction-inhibiting activities. Peak I might contain more than one species of MCRP.

It has been shown that CARP inhibits cardiac activity of *Meretrix*[4]. Therefore, we examined the effect of fraction 30 on the cardiac activity. As shown in Fig. 4A, fraction 30 slightly potentiated



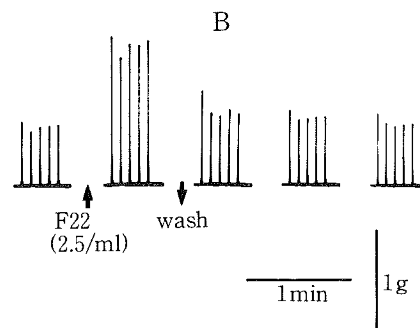


Fig. 2. Potentiating effect of peak E_1 fractions (2.5 ganglion masses/ml ASW) on twitch contractions of the radula retractor muscle of *Fusinus*. A: effect of fractions 18—26 on total tension of 5 twitches of contraction train. The total tension is indicated as percentage of the control total tension. B: effect of fraction 22 on twitches.

tained material displayed a potent inhibitory effect on twitch contractions of the muscle in addition to a weak contractile effect (Fig. 1B). The activities of the retained material were destroyed by treating it with subtilisin, though not destroyed by boiling only. Therefore, it can be expected that the retained material would contain both FaRP and MCRP.

The gel-filtrated fractions of the retained material showed four peaks of biological activity on twitch contractions of the radula retractor; three peaks (E_1, E_2 and E_3) of contraction-potentiating activity and one peak (I) of contraction-inhibiting activity.

The peak E_1 fractions were obtained soon after the void-volume fractions. The maximum potentiating activity of the peak on the twitch contractions was found at fraction 22 (Fig. 2). These potentiat-

the activity at a low concentration, but at a high concentration it first transiently inhibited and then accelerated the activity. These findings suggest that peak I may contain not only inhibitory but also excitatory substance.

At an appropriate concentration (2.5 ganglion masses/ml ASW), fraction 28 showed a potency almost identical with 10^{-9} M CARP in relaxation of catch tension in the ABRM (Fig. 4B). At the same concentrations, however, CARP showed only a slight inhibitory effect on twitch contractions of the radula retractor in contrast to fraction 28 which showed a marked inhibitory effect (Fig. 4C).

The maximum potentiating activities of peaks E_2 and E_3 on twitch contractions of the radula retractor were found at fractions 44 and 50, respectively (Figs. 5A and B). We also examined the ac-

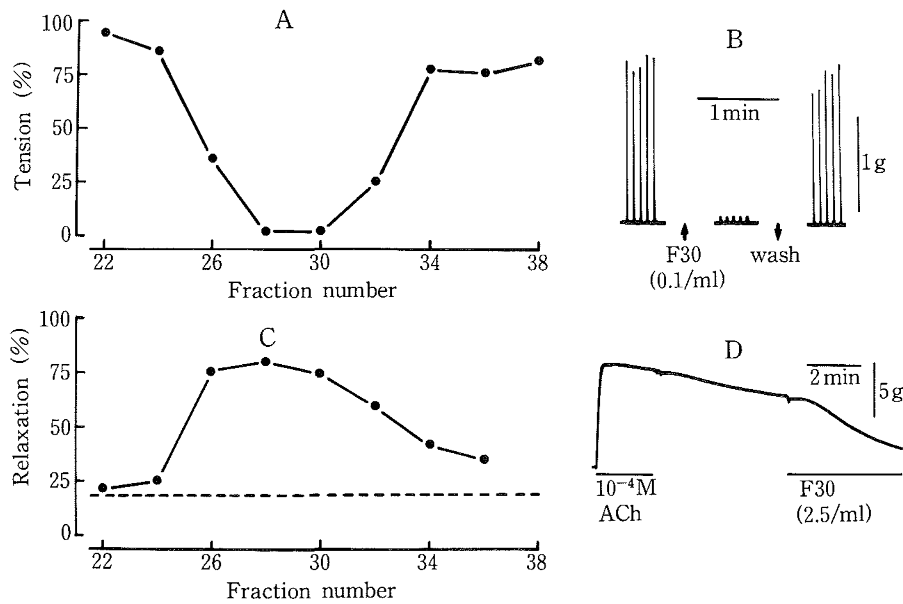


Fig. 3. Effects of peak I fractions on twitch contractions of the radula retractor muscle of *Fusinus* and catch tension of the ABRM of *Mytilus*. A: inhibitory effect of fractions 22—38 (0.1 ganglion mass/ml ASW) on total tension of 5 twitches of contraction train in the radula retractor. The total tension is indicated as percentage of the control total tension. Note that at this low dose fractions 22—26 show not potentiating but inhibitory action on twitches. B: inhibitory effect of fraction 30 on twitches. C: relaxing effect of fractions 22—36 (2.5 ganglion masses/ml ASW) on catch tension of the ABRM. Relaxation (tension relaxed in the presence of the fractions) was indicated as percentage of the tension just before application of the fractions. Broken line shows relaxation in normal ASW (control relaxation).

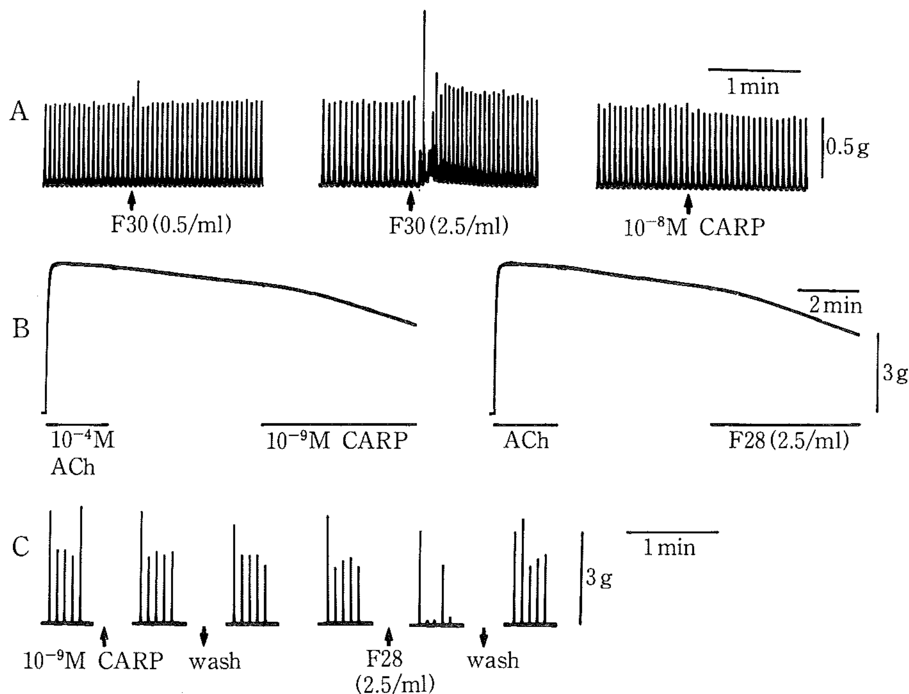


Fig. 4. Comparison between effects of peak I fractions and CARP on some molluscan muscles. A: effects of fraction 30 (0.5 and 2.5 ganglion masses/ml ASW) and CARP (10^{-8} M) on spontaneous cardiac activity of *Meretrix*. Between the records, the heart was washed with normal ASW for 10 min. B: effects of CARP (10^{-9} M) and fraction 28 (2.5 ganglion masses/ml ASW) on catch tension of the ABRM of *Mytilus*. C: effects of CARP (10^{-9} M) and fraction 28 (2.5 ganglion masses/ml ASW) on twitch contractions of the radula retractor muscle of *Fusinus*.

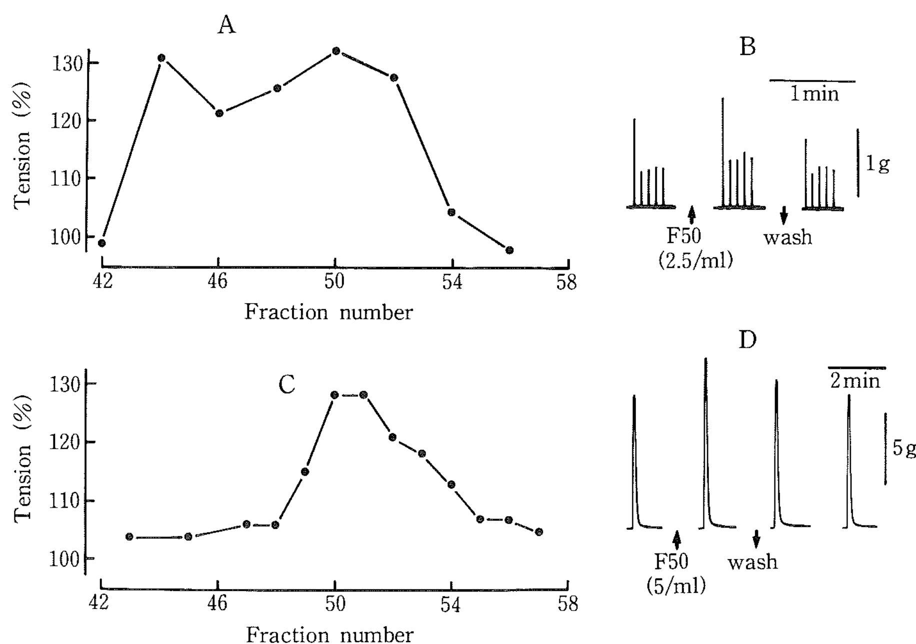


Fig. 5. Potentiating effects of peak E_2 and E_3 fractions on twitch contractions of the radula retractor muscle of *Fusinus* and phasic contraction of the ABRM of *Mytilus*. A: effect of fractions 42—56 (2.5 ganglion masses/ml ASW) on total tension of 5 twitches of contraction train in the radula retractor. The total tension is indicated as percentage of the control total tension. B: effect of fraction 50 on twitches. C: effect of fractions 43—57 (5 ganglion masses/ml ASW) on peak tension of phasic contraction of the ABRM. The peak tension is indicated as percentage of the control peak tension. D: effect of fraction 50 on phasic contraction.

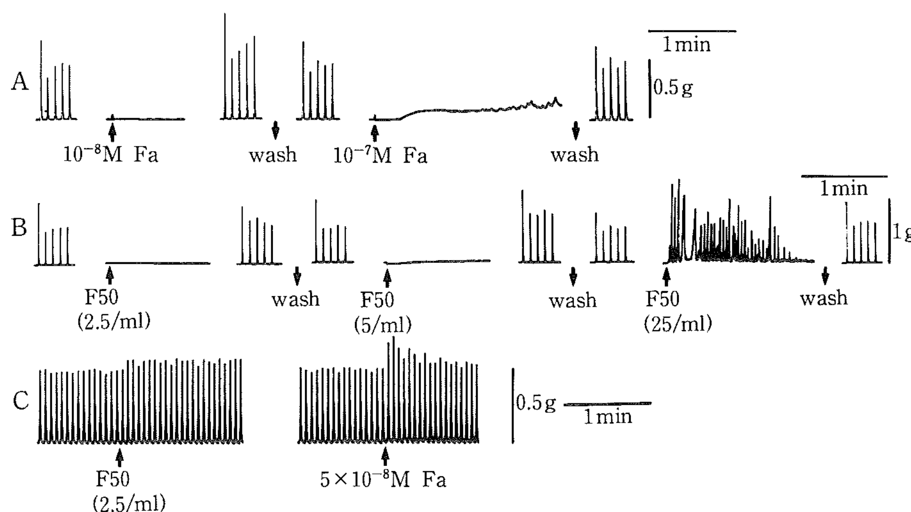


Fig. 6. Comparison between effects of FMRFamide and fraction 50 on some molluscan muscles. A: twitch potentiating effect of low dose (10^{-8} M) and contractile effect of high dose (10^{-7} M) of FMRFamide on the radula retractor muscle of *Fusinus*. B: twitch potentiating effect of low doses (2.5 and 5 ganglion masses/ml ASW) and contractile effect of high dose (25 ganglion masses/ml ASW) of fraction 50 on the radula retractor muscle of *Fusinus*. C: effects of fraction 50 (2.5 ganglion masses/ml ASW) and FMRFamide (5 $\times$ 10^{-8} M) on spontaneous cardiac activity of *Meretrix*.

tivities of fractions of peak E_2 and E_3 on phasic contraction of the ABRM in response to repetitive electrical pulses of stimulation. As shown in Figs. 5C and D, peak E_3 considerably potentiated the phasic contraction, whereas peak E_2 only slightly potentiated it. FMRFamide has been shown to potentiate the phasic contraction of the ABRM[7]. Therefore, it can be suggested from the results in Fig. 5 that peak E_3 may contain FaRP.

As shown in Fig. 6A, FMRFamide potentiated twitch contractions of the radula retractor at low concentrations (10^{-9}-10^{-8} M), and at high concentrations (10^{-7} M or higher), it produced contraction by itself. Similar potentiating and contractile responses

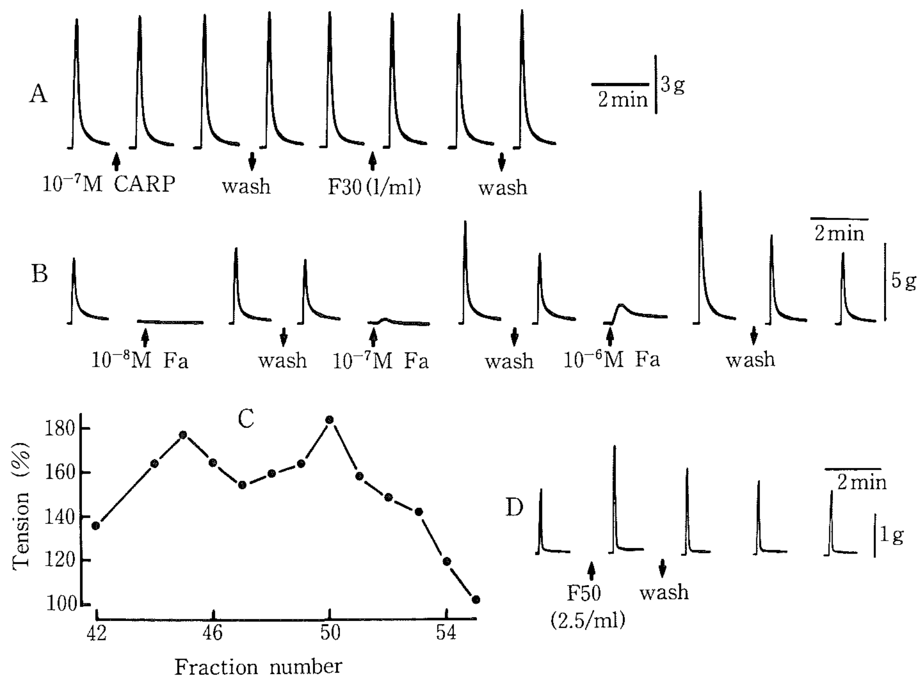


Fig. 7. Effects of CARP, fraction 30, FMRFamide and peak E_2 and E_3 fractions on tetanic contraction of the proboscis retractor muscle of *Fusinus*. A: effects of CARP (10^{-7} M) and fraction 30 (1 ganglion mass/ml ASW). B: effects of FMRFamide (10^{-8}-10^{-6} M). C: effect of fractions 42—55 (2.5 ganglion masses/ml ASW) on peak tension of the tetanic contraction. The peak tension is indicated as percentage of the control peak tension. D: effect of fraction 50.

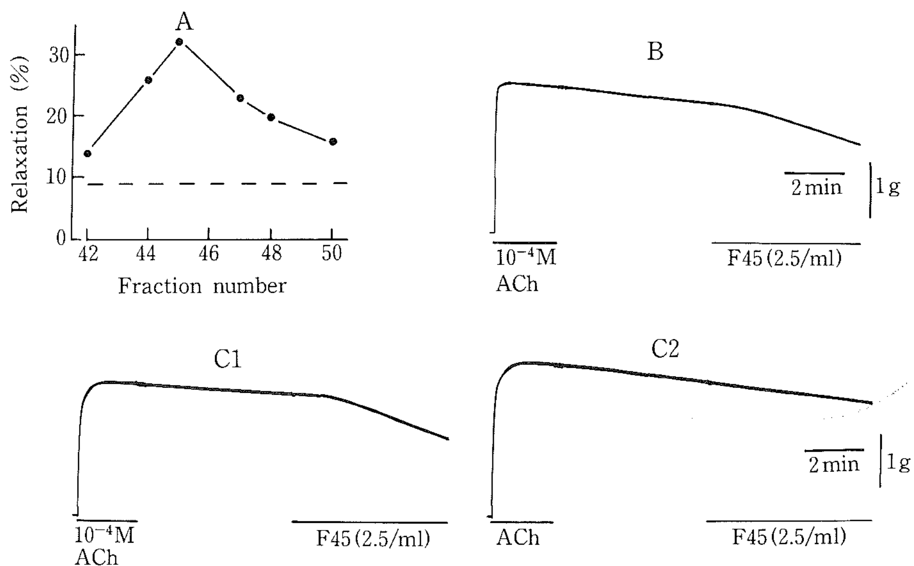


Fig. 8. Relaxing effect of peak E_2 fractions on catch tension of the ABRM of *Mytilus*. A: effect of fractions 42—50 (2.5 ganglion masses/ml ASW) on catch tension. Relaxation is indicated as in Fig. 3C. B: relaxation of catch tension by fraction 45 (2.5 ganglion masses/ml ASW). C: after-effect of high dose of FMRFamide on relaxation of catch tension in response to fraction 45 (2.5 ganglion masses/ml ASW). Between C_1 and C_2, the muscle was exposed to 10^{-5} M FMRFamide for 5 min.

of the muscle were observed when appropriate low and high concentrations of fraction 50 were applied, respectively (Fig. 6B). Further, both FMRFamide and fraction 50 were found to have an accelerating effect on the cardiac activity of *Meretrix* (Fig. 6C). These results support the notion that peak E_3 contains FaRP.

To examine the biological activities of peaks E_2 and E_3 in greater detail, the proboscis retractor muscle of *Fusinus* was used. This is because this muscle was found to be insensitive to CARP (Fig. 7A) but sensitive to FMRFamide (Fig. 7B), and hence the effect of FMRFamide can be detected in a test sample even if it contains both FMRFamide

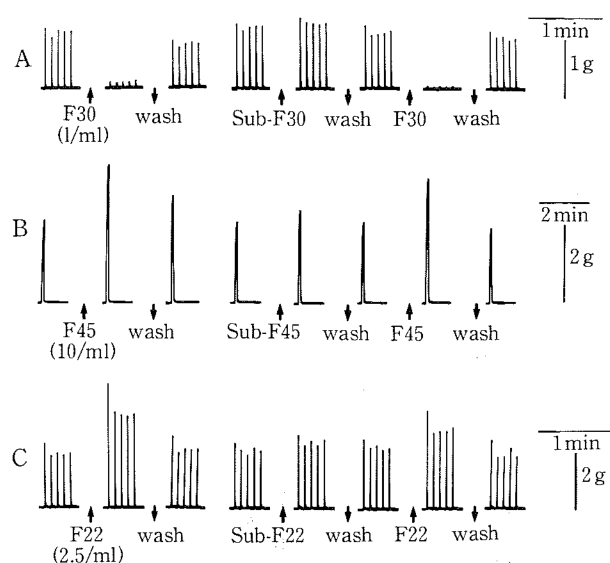


Fig. 9. Loss of bioactivities of fractions 30, 45 and 22 by incubating with the peptidase subtilisin (10^{-5} g/ml). A: loss of inhibitory activity of fraction 30 (1 ganglion mass/ml ASW) on twitch contractions of the radula retractor muscle of *Fusinus*. B: loss of potentiating activity of fraction 45 (10 ganglion masses/ml ASW) on tetanic contraction of the proboscis retractor muscle of *Fusinus*. C: loss of potentiating activity of fraction 22 (2.5 ganglion masses/ml ASW) on twitch contractions of the radula retractor muscle of *Fusinus*. The fractions treated with subtilisin are indicated as Sub-F30 (A), Sub-F45 (B) and Sub-F22 (C), respectively.

and CARP. As shown in Figs. 7C and D, the fractions of peaks E_2 and E_3, as well as FMRFamide, potentiatd contraction of the proboscis retractor in response to repetitive electrical pulses of stimulation. Two peaks of the potentiating activity were obtained and their maximum activities were observed at fractions 45 and 50. Thus, it can be suspected that peak E_2 might contain other species of FaRP than that in peak E_3.

It has been shown that low concentrations (10^{-8}-10^{-7} M) of FMRFamide relax catch tension in the ABRM and that this relaxing response is markedly depressed after the muscle has been briefly treated with high concentrations (10^{-6} M or higher) of FMRFamide[7]. As shown in Figs. 8A and B, peak E_2 relaxed catch tension of the ABRM. The maximum relaxing activity was found at fraction 45. The relaxing response to fraction 45 was not elicited after the muscle had been treated with 10^{-5} M FMRFamide for 5 min (Fig. 8C). These results support the notion that peak E_2 also has FaRP.

The inhibitory activity of fraction 30 on twitch contractions of the radula retractor was completely destroyed by treating the fraction with subtilisin (Fig. 9A). The potentiating activities of fractions 45 (Fig. 9B) and 50 on contraction of the proboscis retractor in response to repetitive electrical pulses of stimulation and the potentiating activity of fraction 22 on twitch contractions of the radula retractor (Fig. 9C) were also destroyed by treatment with subtilisin. Thus, it seems that all the active substances in peaks E_1, E_2, E_3 and I are peptides.

DISCUSSION

The peak E_1 fractions which showed contraction-potentiating activity on the radula retractor muscle were obtained soon after the void-volume fractions, and the peak I fractions which showed contraction-inhibiting activity were obtained soon after the peak E_1 fractions. It may be suggested from these findings that peak E_1 may have an elution volume which partially overlaps with the elution volume of peak I. Although the maximum potentiating activity of peak E_1 was found at fraction 22, the fraction which contains the maximum amount of the potentiating substance may not be fraction 22 but a fraction obtained slightly after it. Using the same gel-filtration system, Hirata et al[3] have shown that the maximum activity of CARP having molecular weight 829 is observed at fraction 29. Thus, it is reasonable to suppose that the molecular weight of the potentiating substance in peak E_1 is probably more than 1000, possibly more than 1500. The potentiating substance may be a novel excitatory peptide. Even if the substance is one of FaRPs, it would be a novel one. This is because FaRP having molecular weight more than 1000 has not been found in the Mollusca[9].

The maximum activity of peak I was observed at fractions 28–30; that is, peak I has an elution volume which is approximately identical with that of CARP. This finding and the results obtained in the examinations of the biological activities of peak I (Figs. 3 and 4) suggest that the peak may contain MCRPs of two or more species, though it is not clear whether it contains CARP or not. The result shown in Fig. 4A suggests that peak I may have an excitatory substance in addition to inhibitory substance. The excitatory substance might be a FaRP, because FMRFamide accelerates the cardiac activity of *Meretrix*. However, we have no further evidence that supports this possibility.

The properties of the biological actions of peaks E_2 and E_3 on molluscan muscles suggest that the active substances in the peaks are FaRPs. The maximum activities of peaks E_2 and E_3 were found at fractions 44–45 and 50, respectively. It can be supposed from such elution volumes that the substances in the peaks have molecular weight of 500—600. Thus, these substances might be FMRFamide and FLRFamide, because the molecular weight of the former is about 600 and that of the latter is about 580, and further, these are only two FaRPs so far isolated from prosobranch molluscs[9].

In summary, the present experimental results suggest that FaRPs and MCRPs are present in the ganglia of *Fusinus*. It seems probable that, in ad-

dition to these peptide, some other peptides exist in the ganglia which show biological actions on molluscan muscles. It is necessary to isolate these peptides and to determine their structures.

The results obtained from the experiments of the biological activity of the material which flowed through the C-18 cartridges suggest that an excitatory substance having a high hydrophilicity is present in the ganglia. In addition to this excitatory substance, other bioactive substances having high hydrophilicity might also exist. The bioactive substances in the flowthrough should also be identified.

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