STUDIES OF URCHI-TOXINS: SEPARATION, PURIFICATION AND PHARMACOLOGICAL ACTIONS OF TOXINIC SUBSTANCES

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Abstract—Urchi-toxins (crude Urchi-toxin F2 and Urchi-toxins F3, F4 and F5) derived from Toxopneustes pileolus were investigated physicochemically and pharmacologically. F2 was found to be a mixture of several substances (F3, F4 and F5) being basic peptides or proteins. F2 and F3 inhibited strongly and slightly the cardiac movement of experimental animals, respectively, while F4 and F5 rather stimulated the heart beat. F2 and F3 elicited remarkable contraction of the small intestine. F2, F3 and F5 stimulated contraction of the uterus. Actions of F2 on smooth muscles were inactivated by chymotrypsin.

While a considerable amount of research has recently been reported on animal toxins, there has been little information on toxins of marine organisms. Many marine organisms contain relatively potent toxins, which can result in critical conditions or occasionally death of humans and animals. Therefore, elucidation of their biochemical and pharmacological properties are of practical significance in establishing adequate measures for treatment of poisoning. Unknown biochemical and physiological functions may also be clarified through cellular and molecular level-investigations of reactions to such toxins. Tetrodotoxin, saxitoxin, erabutoxin and lacicotoxin have been given attention and investigations of these toxins have contributed greatly to advances in the study of the mechanisms of excitable membrane of autonomic nerves and to the physiological functions of neuro-muscular junctions at the synapse.

We have investigated a sea urchin (Toxopneustes pileolus) which belongs to Echinoderm and which contains relatively potent toxins. Alender (1) and colleagues (2), following the reports of Fujiwara (3) and Endean (4), have demonstrated that this toxin induces hemolysis, lowering in blood pressure and A-V blockade. Our experimental results have shown that the toxin is a complex mixture of several basic peptides or proteins having a kinin-like activity.

MATERIALS AND METHODS

1. Toxin

Crude mucous fluids were extracted and separated from the pedicellariae, poison gland of Toxopneustes pileolus belonging to Echinoderm. For some studies, the crude mucous fluid was further fractionated by passage through a column of Sephadex G-25. The procedure for extraction and separation is illustrated schematically as follows:
2. Physicochemical determination of toxinic substances

1) Determination of UV absorption and pH

An automatic recording spectrophotometer and a pH meter were used for determination of UV absorption spectra and pH values of F₂, F₃, F₄, and F₅, respectively.

2) Ultracentrifugal analysis

Sedimentation constants were obtained by ultracentrifugation of F₂ under conditions as indicated in Fig. 2 and approximate molecular weight of each toxin was calculated.

3) Thin-layer chromatography

Using a mixture of butanol-acetic acid-water (1:1:2) as the developing solvent and tert-butyl hypochlorite (Greig-Liebecq reagent) as the coloring agent, thin-layer chromatography was carried out for F₂, F₃, F₄, and F₅ according to routine method.

3. Pharmacological examination

1) Isolated heart

The pharmacological actions of each toxin on the isolated heart of frogs and isolated atrium of rabbits (male, weighing 2.8 kg) were examined by the methods of Yagi-Straub and of Magnus, respectively.

2) Peripheral blood vessels

The actions of toxins on peripheral blood vessels of an isolated rabbit ear were assessed by the method of Kravkov-Pissemski.

3) Isolated intestinal tract

The duodenum of male Wistar rats (weighing about 180 g), ileum of male rabbits (weighing about 2 kg) and male guinea pigs (weighing about 420 g) and colon of male Wistar rats (weighing about 180 g) were employed as sources for isolated intestinal preparations. The actions of toxins on these preparation were examined by the method of Magnus.

4) Isolated uterus

The action of each toxinic substance on the isolated uterine horn of female Wistar rats
(weighing about 180 g) about 2 weeks after bilateral removal of the ovaries was examined by the method of Magnus.

5) **Permeability of peripheral blood vessels**

The actions of each toxin on the capillary permeability were evaluated in accordance with the method of Judah and Willoughby (5), viz., trypan blue, 80 mg/kg, was injected i.v. immediately after intradermal injection of saline solution in a dose of 0.1 ml and, subsequently, the dye which permeated to the skin from the peripheral blood vessel was extracted 30 min later. The permeated dye was determined colorimetrically to serve as the control value. The actions of each toxin in a dose of 0.1 ml were then determined in the same manner. Male Wistar rats weighing about 200 g were employed.

**RESULTS**

1. **Crude Urchi-toxin F2**

   1) **Physicochemical properties of crude Urchi-toxin F2**

   **UV absorption spectrum:** The crude Urchi-toxin F2 solution exhibited absorption maxima in the ultraviolet at 260, 280 and 330 m\(\mu\) (Fig. 1).

   **Ultracentrifugal analysis:** The result obtained by the ultracentrifugal analysis of crude Urchi-toxin F2 indicated the presence of two peaks having sedimentation constants of 2.1 and 4.7 respectively. The approximate molecular weights of both peak substances were 30,000~40,000 and 70,000~80,000 respectively (Fig. 2).

   **Thin-layer chromatography:** The thin-layer chromatographic examination of F2 revealed one spot with long tailing which was suggestive of incomplete purification and separation of this toxin (Fig. 3).

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**Fig. 1. Ultraviolet absorption of Urchi-toxin F2 derived from male and female Toxopneustes pileolus**
pH Value: The pH values of a 1% solution of F₂ were between 8.5 and 9.2 indicating a basic substance.

2) Pharmacological actions of Urchi-toxin F₂

Action on isolated heart (frog and rabbit): Crude Urchi-toxin F₂ inhibited the movement of isolated frog heart and isolated rabbit atrium, viz., at the dose level of \(7.5 \times 10^{-3}\) g/ml, this toxin elicited a marked decrease in the cardiac movement and irregular rhythm resembling extrasystole. The inhibitory action was not blocked by atropine (Fig. 4).

Action on peripheral blood vessels: At the dose levels of \(10^{-3}\) and \(3 \times 10^{-3}\) g/ml, crude Urchi-toxin F₂ caused a transient constriction of peripheral blood vessels of the rabbit ear (Fig. 5).
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**Fig. 6.** Action of Urchi-toxin F2 on isolated rabbit ileum

**Fig. 5.** Action of Urchi-toxin F2 on peripheral blood vessel of extirpated rabbit ear (Doses: $10^{-4} \text{ g}, 3 \times 10^{-4} \text{ g}$)

**Fig. 7.** Actions of Urchi-toxin F2, histamine, bradykinin, and angiotensin on isolated guinea pig ileum

**Action on isolated intestinal tracts:** Crude Urchi-toxoin F2 provoked transiently a marked increase in motility of the isolated rabbit ileum and intensification of its tonus at the dose level of $10^{-4} \text{ g/ml}$ (Fig. 6). This action was not blocked by $10^{-8} \text{ g/ml}$ of atropine.

Crude Urchi-toxin F2 in a dose of $2 \times 10^{-5} \text{ g/ml}$ produced a contraction of isolated guinea pig duodeno-ileum. The percent contraction of the preparation after addition of F2 was nearly equal to that after $10^{-8} \text{ g/ml}$ of histamine, $10^{-9} \text{ g/ml}$ of bradykinin and $10^{-10} \text{ g/ml}$ of angiotensin. The contraction occurred slowly as in the case after addition of such peptides as bradykinin, angiotensin, etc. (Fig. 7).

Crude Urchi-toxin F2 at a dose level of $10^{-5} \text{ g/ml}$ caused a relatively strong contraction of both ascending and descending colons isolated from rats. The contraction-inducing activity of F2 in the concentration of $3 \times 10^{-4} \text{ g/ml}$ on the isolated colon was similar to that of angiotensin in the concentration of $10^{-7} \text{ g/ml}$. In contrast, the toxin at dose levels over $2 \times 10^{-8} \text{ g/ml}$ exerted a dilating action on the colon rather than contracting action, i.e.,
crude Urchi-toxin F₂ induced two different actions depending upon the concentrations (Fig. 8).

**Action on isolated rat uterus:** Like bradykinin and angiotensin, crude Urchi-toxin F₂ induced contraction of the isolated rat uterus. The activity at a dose level of $5 \times 10^{-4} \text{g/ml}$ was nearly equal to that of bradykinin at a dose level of $10^{-8} \text{g/ml}$ and angiotensin in a dose of $10^{-7} \text{g/ml}$ (Fig. 9).

**Permeability of peripheral blood vessels:** Crude Urchi-toxin F₂ stimulated the increase in permeability of peripheral blood vessels, and its action was inhibited moderately by diphenhydramine.

**Inactivation by chymotrypsin:** The behavior of crude Urchi-toxin F₂ on various smooth muscular organs and the physicochemical properties of the toxin suggested that it may be a basic polypeptide or protein. We therefore investigated whether or not the toxic substance could be inactivated by a peptidase “chymotrypsin”. It was found that the con-
tracting action of the toxin at a dose level of $5 \times 10^{-5}$ g/ml on isolated rat uterus was inactivated completely by $10^{-6}$ g/ml of chymotrypsin after incubation at 37°C for 15 min (Fig. 10).

The above results suggest that in crude Urchi-toxin $F_2$ there exists at least one toxic substance with a kinin-like action or an active peptide-like action. Thus, crude Urchi-toxin $F_2$ was further extracted and separated by chromatography on Sephadex G-25, thin-layer chromatography and other procedures.

2. Fractions of crude Urchi-toxin $F_2$

1) Separation by Sephadex G-25

Crude Urchi-toxin $F_2$ was separated by column chromatography using Sephadex G-25 (medium). The fractions in tube numbers 12~17, 22~29 and 30~34 showed absorption maxima at 280 m$\mu$. The fraction

![Fig. 10. Effect of Urchi-toxin $F_2$ on isolated rat uterus after incubation with chymotrypsin at 37°C for 15 min](image)

![Fig. 11. Centrifugal analyses of Urchi-toxins $F_3$, $F_4$ and $F_5$](image)
in Nos. 18-30 showed very high absorption maxima at 330 \text{m}_\mu. Each fraction which showed absorption maxima at 280 \text{m}_\mu and 330 \text{m}_\mu was designated as Urchi-toxins F_3, F_4 and F_5. Ultracentrifugal results of each fraction are given in Fig. 11. Only F_3 and F_5 exerted biological activities (Fig. 12).

2) \textit{Thin-layer chromatography for each fraction}

The thin-layer chromatographic results of F_3, F_4 and F_5 are given below.

| Urachi-toxins | Rf values          |
|---------------|--------------------|
| F_2           | 0.19 with tailing  |
| F_3           | 0                  |
| F_4           | 0.62 and 0.73      |
| F_5           | 0.68 and 0.84      |

Two spots each of F_4 and F_5 are attributed to the overlapping of both fractions with the fraction which showed absorption maxima at 330 \text{m}_\mu. Although each fraction could not be entirely separated from crude Urchi-toxin F_2 except for F_3, the biological activities of all fractions were tested.

3) \textit{pH Value}

One percent solutions of Urchi-toxins F_3, F_4 and F_5 were found to show the following pH values:

- Urchi-toxin F_3: 6.5-7.0
- Urchi-toxin F_4: 7.2-8.5
- Urchi-toxin F_5: 7.5-8.0
4) Biological activities of F3, F4 and F5

Action on isolated heart: The actions of Urchi-toxins F3, F4 and F5 on the isolated frog heart and isolated rabbit atrium were examined in the same manner. Unlike F2, these fractions had no inhibitory action on cardiac movement in the concentration equivalent to that of Urchi-toxin F2 which elicited strong inhibition of cardiac movement. Urchi-toxin F3 did however, inhibit the movement of the heart slightly. The cardiac movement was rather increased by Urchi-toxins F4 and F5 (Fig. 13).

Action on isolated intestinal tract and uterus: The isolated guinea pig ileum was employed as the smooth muscle organ. Urchi-toxins F3 and F5 produced a contraction of the ileum, and in particular Urchi-toxin F3 did induce a stronger contraction than Urchi-toxin F5.

The uterine horns were isolated 1 week after bilateral ovariectomy. In particular, Urchi-toxin F3 elicited a marked contraction of the isolated uterus much in the same way as its action on the isolated guinea pig ileum. Assuming the percent contraction-inducing activity of $10^{-8}$ g/ml of bradykinin on the isolated rat uterus to be 100%, the activities of Urchi-toxins F3, F4 and F5 at the same dose levels were compared. The contraction-inducing activities of F3 and F2 proved to be about 70% and 30-40% respectively (Fig. 14).

![Fig. 13. Actions of Urchi-toxin F2 and F5 on isolated frog heart.](image)

![Fig. 14. Comparison of biological activities of Urchi-toxins with that of bradykinin.](image)
Action on permeability of peripheral blood vessels: The permeability of peripheral blood vessels of rats was increased after injection of F3. The increasing action of Urchi-toxin F3 on the permeability was stronger than that of crude Urchi-toxin F2. However, the permeability of the blood vessel was only slightly increased after injection of Urchi-toxins F4 and F5. Diphenhydramine antagonized to a moderate degree the Urchi-toxin F3-induced increase in the permeability (Table 1).

| Item        | Urchi-toxin injected intradermally (μg) | Trypan blue extracted from skin (μg) | Capillary permeability inhibited by diphenhydramine (1 mg/kg, l.p.) (μg)* | No. of rats |
|-------------|----------------------------------------|--------------------------------------|-----------------------------------------------------------------------|-------------|
| Control (Saline) | /                                      | 1.08±0.29                            | /                                                                     | 6           |
| F2          | 10                                     | 8.10±0.34**                          | 7.42±0.31                                                             | 6           |
|             | 20                                     | 12.28±0.59                           | 10.37±0.45                                                            | 6           |
| F3          | 10                                     | 11.30±0.52**                          | 3.18±0.39                                                             | 6           |
|             | 20                                     | 14.88±0.64                           | 10.63±0.98                                                            | 6           |
| F4          | 10                                     | 1.02±0.15                             | —                                                                    | 6           |
|             | 20                                     | 1.17±0.19                             | —                                                                    | 6           |
| F5          | 10                                     | 1.05±0.10                             | —                                                                    | 6           |
|             | 20                                     | 1.12±0.13                             | —                                                                    | 6           |

* The inhibited capillary permeability by diphenhydramine is expressed as the amount of trypan blue recovered from the skin.

** Significantly different from the control (p<0.01).

DISCUSSION

As studies to elucidate the toxins of marine organisms have only begun in recent years, the pharmacological actions of such toxins have yet to be elucidated. The outstanding serial study in this field is the investigation on tetrodotoxin (toxin of globe fish). Although molecular weights differ, the majority of toxins present in Actinia equina, Condystis gigantea, Physalia physalis and Euthydra schistosa are believed to be basic peptides or proteinous substances. *Echinoderms* toxin, viz., holothrian, etc. derived from the Cuvier’s tube of sea cucumber, has chemically been confirmed as a glycoside having a steroid ring.

In the present work, the authors attempted to study the physicochemical and pharmacological properties of toxinic substances obtainable from the pedicellariae of sea urchin (*Toxopneustes pileolus*) having a considerably potent toxicity among *Echinoderms*.

The UV spectrophotometric examination of a toxinic substance, crude Urchi-toxin F2 isolated from this organism, suggested the presence of other basic toxinic substances with absorption maxima at 280 mμ and 330 mμ. According to calculation from ultracentrifugal sedimentation constants, crude Urchi-toxin F2 was found to contain a minimum of two substances with considerably large molecular weights. Thin-layer chromatographic separation of this crude toxin revealed that basic peptides or proteinous substances apparently exist in
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this toxin. The crude toxin was also proved to be not a single substance and by column chromatography was shown to contain at least three fractions (Urchi-toxins F3, F4 and F3). These results suggested that crude Urchi-toxin F3 is a complex toxin. As based on a UV absorption maximum of 280 m\(\mu\) along with positive reaction to Greig-Liebecq reagent and other physicochemical properties, we estimated that this complex toxin consists of several toxinic basic polypeptides.

Pharmacologically, crude Urchi-toxin F2 induced contraction of smooth muscles, increase in capillary permeability and inhibition of cardiac movement. Unlike the quick contraction of smooth muscles after administration of histamine or acetylcholine, smooth muscles contracted slowly after crude Urchi-toxin F2. The biological activity of F2 was inactivated completely by chymotrypsin and the stimulating action on the contraction of smooth muscles disappeared simultaneously. An anticholinergic agent such as atropine did not in any way antagonize the contraction-inducing action on the ileum, however, an antihistaminic drug such as diphenhydramine did slightly antagonize the action. Mendes, Abbud and Umiji (6) reported that the action of a toxinic substance similar to our crude Urchi-toxin F2 was cholinergic. However, our experimental results indicate that crude Urchi-toxin F2 is not a cholinergic substance. It is of interest that bradykinin caused only contraction of rat colon, whereas crude Urchi-toxin F2 exerted two different actions on the colon (contraction and relaxation). However, it remains obscure whether the dual action on the colon can be attributed to differences in concentrations of the test solutions and whether the indirect action of noradrenaline released by the tyramine-like action of angiotensin (7) underlies the smooth muscle relaxing action of crude Urchi-toxin F2. Urchi-toxin F3 stimulated the motility and tonus of smooth muscles more strongly than crude Urchi-toxin F2. It is reasonable to assume that after separation and purification, biological activities of F3 increase more than those of F2. F3 was found to show absorption maximum of 280 m\(\mu\). However, further study is required to determine the nature of the substance which has an absorption maximum at 330 m\(\mu\) and the relationship between the biological activities and this particular substance.

Crude Urchi-toxin F4 and Urchi-toxin F5 constricted peripheral blood vessels and increased the capillary permeability, though the actions were not as potent as those of bradykinin. As these actions are partially blocked by diphenhydramine, the increasing action of both toxins on the capillary permeability may be attributed to dilation of capillaries secondary to constriction of the peripheral vein like the action mechanism of bradykinin (8).

It is well known that bradykinin stimulates cardiac movement. Crude Urchi-toxin F2 strongly inhibited the movement of the heart and only F3 inhibited the cardiac movement slightly. Urchi-toxins F4 and F5 stimulated the heart, the action resembling that of bradykinin. It would thus appear that another fraction having an inhibitory action on the cardiac movement exists in crude Urchi-toxin F2. Such a fraction was not however obtained experimentally.

Viewing various physicochemical properties of the complex toxin and the pharmacological actions which are inactivated by chymotrypsin, it is obvious that basic polypeptides
having kinin-like actions exist as the constituents in the complex toxin. Utilizing different methods, separation and purification of the biologically active substances and fractions of Urchi-toxins (especially those of F3 and F5) are now underway. Investigations into the correlation between toxinic polypeptides and mechanisms of actions, which may throw some light on detoxification procedures, are also in progress.

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