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Isolation of Neosurugatoxin from the Japanese Ivory Shell, *Babylonia japonica*

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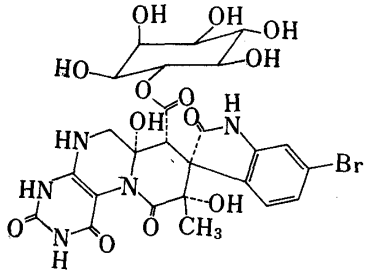
Isolation of neosurugatoxin, which is the causative agent of intoxication resulting from the ingestion of the toxic Japanese ivory shell, was accomplished by six steps of chromatography, to give 4 mg of the principle from 20 kg of the shellfish.

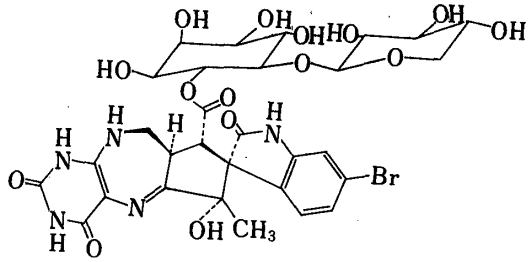
Keywords——surugatoxin; neosurugatoxin; Japanese ivory shell; *Babylonia japonica*; mydriasis-inducing activity; anti-nicotinic activity; intoxication

Intoxication as a result of eating shellfish often occurs without warning, and the toxicity of the shellfish usually disappears within several years. Thus, the causative toxin and the detailed process of intoxication should be clarified urgently. It is also interesting to note that many of the toxic compounds obtained from the sea show unique biological activities which could lead to the development of new drugs.

The Japanese ivory shell, *Babylonia japonica*, is commonly eaten in Japan, and is sometimes found to be poisonous.[1–5] In September 1965, the ivory shell collected in the Ganyudo area of Suruga Bay, Shizuoka Prefecture, caused intoxication in 26 cases; the victims complained of visual defects, including amblyopia and mydriasis, with thirst, numbness of lips, speech disorders, constipation and dysuria. In 1972, we reported the isolation and structure determination of surugatoxin[6] (**1**) and recently neosurugatoxin[7] (**2**) from the toxic ivory shell. In the course of further study on the toxin contained in toxic ivory shell, it was found that surugatoxin obtained by previously reported procedures[6] and subsequently purified by high performance liquid chromatography (HPLC) did not possess any toxicity. This paper describes the details of the isolation process of neosurugatoxin and some of its biological activity.



surugatoxin (**1**)



neosurugatoxin (**2**)

It was reported[8] that the toxicity of the ivory shell which was found in the Ganyudo area increased rapidly before 1969 and that after 1972 it decreased quickly. Finally, in 1978, the toxicity disappeared completely. However, during our survey in October 1981, it was observed that the toxicity in shellfish had increased again up to 2000 mouse unit. In this study, the shellfish collected at Ganyudo in 1973 and stored at −20°C were used as a source of material. Purification was followed by measuring the mydriasis-inducing activity in mice. Since it was reported[9] that the toxin is unstable to alkali and heat, all purification operations were carried out under weak acidic or neutral conditions in the cold (2—5°C). Chart 1 shows the isolation procedure for neosurugatoxin.

The mid-gut gland of ivory shell was homogenized in 1% AcOH, and the mixture was centrifuged. The supernatant was mixed with acetone and the precipitates formed were again removed by centrifugation. The supernatant was concentrated to 300 ml under reduced pressure, and defatted with ether. The concentrate thus obtained was applied to a Sephadex G-25 column in five batches, using water acidified to pH 3.9 with AcOH as the eluent. The column eluent was monitored by measurement of ultraviolet (UV) absorbance at 280 nm and the activity to evoke mydriasis in mice was tested. The profiles are shown in Fig. 1. The evoking activity emerged in the yellow fractions No. 65 to No. 90. The active effluents from five identical columns were combined and lyophilized (active fract. I).

This active material was successively fractionated on a CM-Sephadex ion exchange column in three batches of 1.2 g each. Fig. 2 shows the results of a typical run on this column. The evoking activity was found in two different groups of fractions (No. 40—90 and No. 100—150) from the CM-Sephadex column. Surugatoxin was isolated from the fractions eluted first (No. 40—90), and neosurugatoxin from the fractions eluted later (No. 100—150). On further purification of the first group of active fractions by several kinds of chromatographies, it was found that it contained a further two toxins besides neosurugatoxin. The isolation study which is in progress has shown that the activity of surugatoxin previously reported[6] is due to the contamination by these toxins. The active fractions in the second group from the three columns were combined and lyophilized (active fract. II).

The lyophilized material was then subjected to gel filtration on Bio-Gel P-2 in water acidified to pH 3.9 with AcOH. Fig. 3 shows the elution pattern of absorbancy at 280 nm and activity. The evoking activity was located in fractions No. 150 to No. 210 (active fract. III).

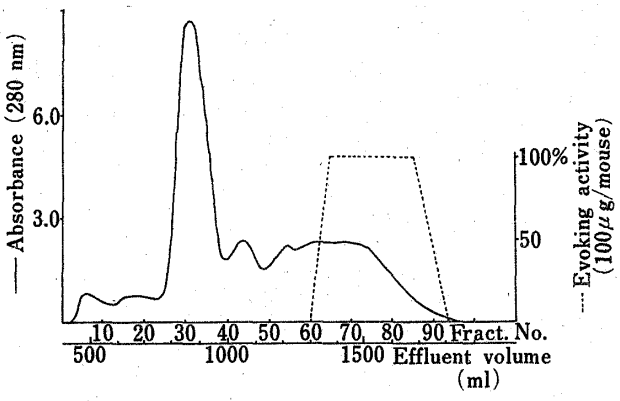


Fig. 1. Gel Filtration of 1% AcOH Extract on Sephadex G-25

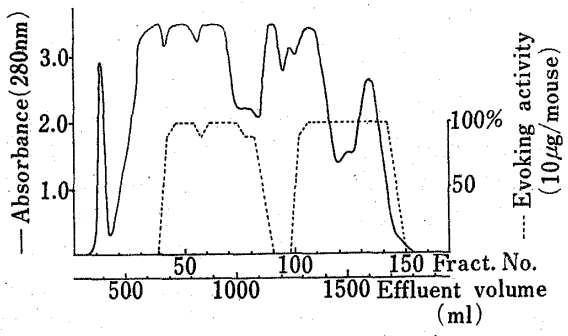


Fig. 2. Ion Exchange Chromatography of Active Fract. I on CM-Sephadex C-25

Japanese ivory shell, 20 kg
↓
mid-gut gland, 1.9 kg
↓ 1) extracted with 1% AcOH 2) added acetone 3) defatted with ether
extract
↓ Sephadex G-25
active fract. I, 3.7 g (>100%/100 μg)[a]
↓ CM-Sephadex C-25
later eluted active fract. II, 1.3 g (100%/10 μg)[a]
↓ Bio Gel P-2
active fract. III, 271 mg (80—100%/2.5 μg)[a]
↓ CM-Sephadex C-25
active fract. IV, 92 mg (100%/2 μg)[a]
↓ Sephadex G-15
active fract. V, 48 mg (100%/1 μg)[a]
↓ HPLC
active fract. VI, 8.2 mg (70—90%/0.2 μg)[a]
↓
neosurugatoxin

Chart 1. Isolation Procedure for Neosurugatoxin

a) Evoking activity in mice.

These active fractions were lyophilized and again purified by ion exchange chromatography on CM-Sephadex C-25 eluted with a linear gradient of 10 mM to 100 mM NH_4OAc at pH 5.0 (Fig. 4). The evoking activity appeared to be concentrated in fractions No. 31 to No. 34 (active fract. IV).

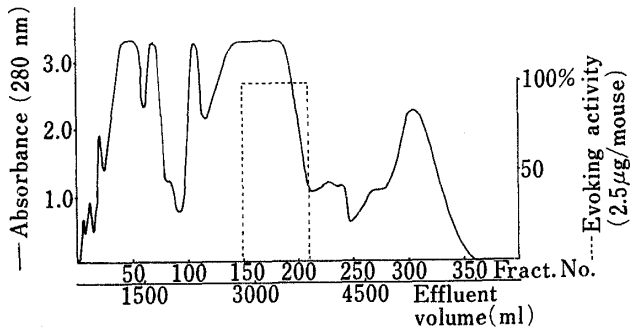


Fig. 3. Gel Filtration of Active Fract. II on Bio-Gel P-2

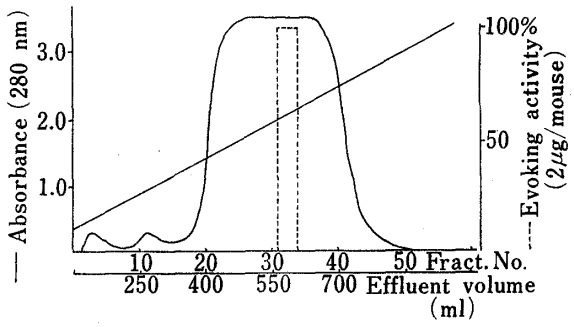


Fig. 4. Ion Exchange Chromatography of Active Fract. III on CM-Sephadex C-25

This active fract. IV was further purified by gel filtration on Sephadex G-15 in 10 mM NH_4OAc, pH 5.0 (Fig. 5). About 48 mg of active material colored pale yellow was obtained, and found to evoke 100% mydriasis in mice at a dose of 1 μg/mouse (active fract. V).

Final purification was achieved by successive application of preparative HPLC with a reverse phase column. Though many peaks were observed, as shown in Fig. 6, only one peak (retention time 25.17 min) showed evoking activity. The main active substance, obtained as a colorless powder, was crystallized from water and designated as neosurugatoxin. Approximately 4 mg of neosurugatoxin in the form of colorless prisms, mp 331—335°C dec., was obtained from 20 kg of shellfish. The details of the structure determination and physical data of neosurugatoxin have already been reported.[7]

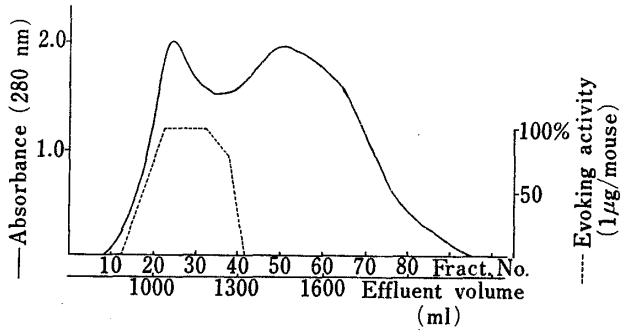


Fig. 5. Gel Filtration of Active Fract. IV on Sephadex G-15

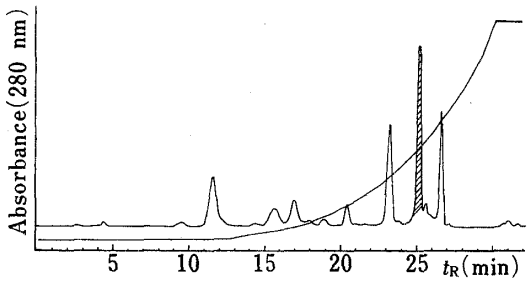


Fig. 6. Reverse Phase HPLC of Active Fract. V

Crystalline neosurugatoxin is practically insoluble in water and usual organic solvents except dimethylsulfoxide. The stability of neosurugatoxin was examined and the results are shown in Table I. Neosurugatoxin is extremely unstable in alkaline medium and is fairly heat-labile.

The evoking activity and anti-nicotinic activity of neosurugatoxin are shown in Fig. 7 and Fig. 8. Neosurugatoxin evoked mydriasis in mice at a minimum dose of 3 ng/g, and at a concentration of 1×10^{-9} g/ml inhibited the contractile response of isolated guinea pig ileum to 3×10^{-5} g/ml of nicotine.

Table I. Stability of Neosurugatoxin

Medium	Temperature (°C)	Time (h)	Activity (%)[a]
H_2O	40	3	100
	50	1	100
		2	70—80
		3	40—70
	60	1	50
		2	0
0.1 N AcOH	1	48	100
0.1 N HCl	1	3	100
1% NH_4OH	1	0.5	40—70
	1	1	20—40
	1	2	0

a) Activity to evoke mydriasis in mice (0.3 μg/mouse, *i.p.*).

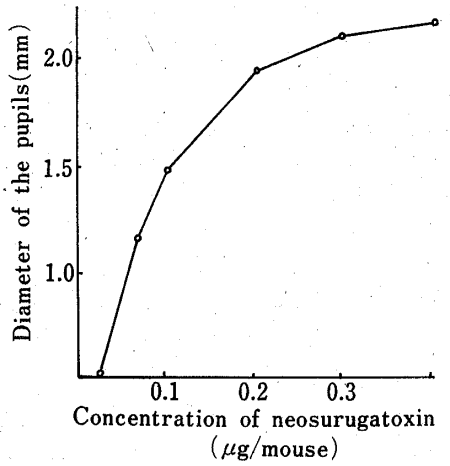


Fig. 7. Mydriatic Effect of Neosurugatoxin on Mice

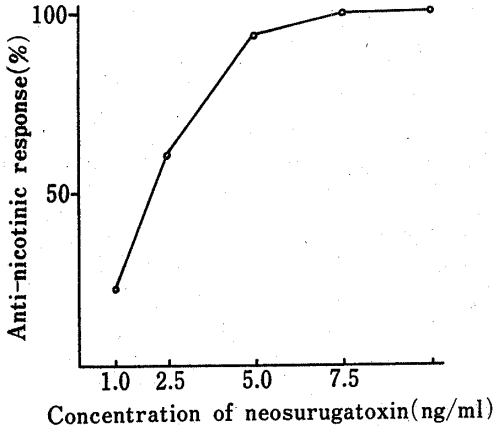


Fig. 8. Inhibitory Effects of Neosurugatoxin on the Contractile Response of Isolated Guinea Pig Ileum to Nicotine

Because the toxicity of the ivory shell from the Ganyudo area is increasing again, it is important to clarify the mechanisms of toxication, not only from the point of view of food hygiene and public health but also from that of marine conservation.

Experimental

Assay of Toxicity——Toxicity testing was carried out by a modification of the method of Pulewka[10] which was developed as an assay for atropine activity on mydriasis of mice. The test substance dissolved in water was given intraperitoneally to mice weighing approximately 20 g. After 40 min, the diameter of the pupil was measured. The evoking activity was rated at 100% (>2 mm diameter of pupil) to 0% (no change from before administration).

Extraction——The mid-gut gland (1.9 kg) which was separated from toxic ivory shell, was homogenized in 3 liters of 1% AcOH and the mixture was centrifuged at 3000 rpm for 20 min. The sludge was again extracted twice with 1% AcOH (1 l each) under stirring at 2°C for 5 h each time and the mixture was centrifuged under the same conditions. The three supernatants were combined and 5 l of cold acetone was added. The mixture was left overnight at 2°C and the resulting precipitates were removed by centrifugation. The supernatant was concentrated to 300 ml under reduced pressure at 30°C and defatted three times with 300 ml of ether to afford a brownish-yellow syrup.

Gel Filtration on Sephadex G-25 (Fig. 1)——The brownish-yellow concentrate was subjected to gel filtration on Sephadex G-25 (4.5×92 cm) in batches of 60 ml and the column was eluted with water acidified to pH 3.9 with AcOH. Fractions of 15 ml were collected. In total, 3.7 g of active yellow-colored material was obtained, which evoked >100% mydriasis in mice at a dose of 100 μg/mouse.

CM-Sephadex Chromatography (Fig. 2)——A portion (1.2 g) of the active material from Sephadex G-25 was dissolved in 15 ml of water acidified to pH 3.9 with AcOH and a small amount of insoluble material was removed by centrifugation. The supernatant was applied to a CM-Sephadex ion exchange column (2.5×88 cm). The column was eluted with water acidified to pH 3.9 with AcOH. The elution was monitored by measurements of UV absorbance at 280 nm and evoking activity. The fractions containing activity were pooled and lyophilized. In total, 1.3 g of powder (later eluted active fract. II) which evoked 100% mydriasis in mice at a dose of 10 μg/mouse was obtained from these three columns.

Bio-Gel P-2 (Fig. 3)——Active fract. II (1.3 g) was dissolved in 20 ml of water acidified to pH 3.9 with AcOH and subjected to gel filtration on Bio-Gel P-2 (3.6×97 cm). The active fractions were pooled and lyophilized. Active material (271 mg) which evoked 80—100% mydriasis in mice at a dose of 2.5 μg/mouse was obtained.

CM-Sephadex C-25 (Fig. 4)——A column (2.5×54 cm) of CM-Sephadex C-25, previously equilibrated with 10 mM NH_4OAc (pH 5.0), was used for purification of active fract. III. The column was developed with a 10—100 mM linear NH_4OAc gradient (pH 5.0). The active fractions were lyophilized and 92 mg of active material was obtained.

Sephadex G-15 (Fig. 5)——Active fract. IV (92 mg) was applied to a 1.6×91 cm column containing Sephadex G-15 and eluted with 10 mM NH_4OAc (pH 5.0).

HPLC (Fig. 6)——Active fract. V (48 mg) was dissolved in 5 ml of water and a 200 μl portion of sample solution was injected for each run. The conditions for HPLC were as follows: column, μBondapak C_{18} (Waters 7.8×300 mm); flow rate, 4.0 ml/min; eluent, 10—100% MeOH gradient. Column effluents were monitored by means of an absorbance detector at 280 nm. The peak (retention time 25.17 min) was collected. After removal of MeOH by careful evaporation at 20°C under reduced pressure, the concentrate was lyophilized to afford a white fluffy powder, yield 8.2 mg.

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