PHYSIOLOGICAL ACTIVITIES OF STARFISH SAPONIN*

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In the previous paper¹⁾, the occurrence of saponin in starfish meal was reported, and the postulation was given that the saponin is responsible for the manifold toxicities of starfish extracts on other animals.

Since the saponin was found to be labile to heating in the course of investigation, the material was changed from sun-dried meal to the fresh starfish, *Asterias amurensis* LÜTKEN, and extraction procedure was also modified. These improvements enabled us to isolate saponin of high purity and to examine actions of this substance upon animals reported to be affected by starfish itself or starfish extracts.

The present paper deals with the results of experiments on activities of the purified starfish saponin upon the rabbit blood cells, killifish, house fly maggots, earthworms, oysters, and cats.

Experimental and Results

Preparation of starfish saponin

In the light of instability to heating, the starfish saponin was treated under 45°C throughout procedure. The starfish, *Asterias amurensis* LÜTKEN, collected at the coast of Hommoku of the Tokyo Bay in July 1962 and stored at -25°C, was used as materials. At first, isolation was attempted in a small batch as shown in Fig. 1. The smashed starfish (1 kg) was extracted with 3 l of methanol by stirring for 30 minutes at room temperature. The extraction was repeated twice by using each 3 l and 2 l portion of methanol. The combined extract was concentrated in vacuum to about 250 ml and diluted with equal volume of water to be defatted with benzene saturated with 50% methanol in a liquid-liquid extractor for 18 hours. After being freed from benzene, the aqueous layer was adjusted to pH 3.0 with dilute hydrochloric acid, neutralized with dilute sodium hydroxide solution, and then dialyzed through cellophane membrane against running tap water for 6 hours. The dialyzate, after being concentrated in vacuum to about 200 ml, was shaken with half volume of water-saturated n-butanol three times. The butanol layers were combined and concentrated to about 150 ml. By shaking the concentrate with three volumes of ether and a half volume of water, the saponin was transferred to aqueous layer. Water extraction was repeated again. When freeze-dried, the aqueous layer gave white amorphous saponin

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in a yield of nearly 0.1% as shown in Table 1.

Fifty kilograms of material was subsequently treated similarly and 72 g of saponin was obtained. This preparation was used for the following biological tests.

Hemolytic activity of the starfish saponin

The hemolytic activity was estimated by the method of FUJITA and NISHIMOTO²⁾ and expressed as hemolytic index (H.I.). On every estimation the sensitivity of rabbit blood cell suspension was checked with the standard saponin (E. Merck, A 6,

Fig. 1. Extraction of the starfish saponin.

Table 1. Yield of saponin from 1 kg of raw starfish in hemolytic index and weight of solids.

Fraction No.	Total solids (g)	H.I./g	Total H.I.	Recovery in H.I.(%)
I Aqueous layer after Bz extn.	28.884	3,400	98,200	100
II Dialyzates	5.940	15,000	89,100	90.7
III n-BuOH layer	1.534	24,200	37,100	37.8
IV Aqueous layer after n-BuOH extn.	3.654	1,400	5,120	5.2
V n-BuOH:Et ₂ O layer	0.442	1,000	442	0.5
VI Amorphous saponin	0.954	30,600	29,200	29.7

Darmstadt), which showed hemolytic index of 33,000 on average. This value was adopted as standard to calibrate all the observed values.

The hemolytic index of the first preparation is given in Table 1, and that of the saponin obtained from the 50 kg material to be used for biological tests was 27,000.

Dose-death-time relationship in killifish

The relationship between time required to kill killifish, *Oryzias latipes* TEMMINCK et SCHLEGEL, and concentration of starfish saponin was examined. The concentrations of the test solutions varied from 45 to 2,560 H.I./50 ml for the starfish saponin and from 340 to 2,560 H.I./50 ml for the standard saponin when expressed by hemolytic activity. Five individuals of adult fish were put in beaker containing 50 ml of test solution, the temperature of which was maintained at 25°C. When the fish did not show any response to being taken out from water with forceps, it was assumed to be dead and the time was recorded. The curves showing dose-death-time relationship were quite identical with those reported in the previous paper for both the starfish saponin and the standard saponin. Although no appreciable difference was observed on the symptoms of fish, which were characterized by occasional rush and loss of balance, the starfish saponin was considerably stronger in toxicity than the standard saponin on the level of same hemolytic index. This was also true for earthworms and fly maggots, as described below. The minimum lethal dose of starfish saponin to killifish was 45 H.I./50 ml and that of standard saponin 340 H.I./50 ml.

Dose-death-time relationship in earthworms

Test solutions were prepared by dissolving the saponin in distilled water making series of different concentrations from 110 to 380 H.I./50 ml in equal ratio ($\gamma=1.2$) for the starfish saponin and from 255 to 1,300 H.I./50 ml for the standard saponin. Five individuals of earthworm, *Eisenia foetida* SARIGNY, were put in a petridish containing 50 ml of the test solution, and the temperature was maintained at 17°C. Vigorous movement and liberation of yellow mucus before death were characteristic to all the worms affected by saponin, whereas none of symptoms was observed on the control group placed in distilled water. In the most severe case the worms died in about 15 minutes. Dose-death-time relations are shown in Fig. 2, in which the minimum lethal dose of starfish saponin to earthworms is seen at around 100 H.I./50 ml.

Activity on house flies

The effects of starfish saponin on eggs and maggots of house fly, *Musca vicina* MAQUART, were investigated, which were provided by courtesy of Nihon Tokushu Nōyaku Co. Ltd.

Effect on hatchability of fly eggs: Thirty pieces of egg on the third day from the initiation of spawning were placed in a petridish containing two sheets of filter paper soaked with 5 ml test solution. The dishes were allowed to stand overnight in an incubator (23°C) and the eggs were examined under microscope. When an egg was found to be empty, it was recorded as hatched. The control experiment was

carried out by using biological saline. As shown in Table 2, neither starfish saponin nor standard saponin exhibited any apparent depressing effect upon hatchability even on high concentrations ranging from 1 to 5%. It should be mentioned, however, that the most of worms hatched were found to be killed in each saponin solutions at the end of test.

Toxicity to fly maggots: According to the method of National Institute of Health³⁾,

Fig. 2. Dose-death time relationship in earthworms for starfish saponin and standard saponin.

Table 2. Effects on hatchability of fly maggots.

Concentrations	No. of tests	Hatchability (%)		
		Starfish saponin	Standard saponin	Control
1 %	1	84	80	92
	2	87	87	97
	3	62	86	90
3 %	1	97	97	96
	2	82	70	96
5 %	1	80	77	80

concentrations of the test solutions were divided into ten levels in equal ratio ($r=1.2$): from 250 to 1,300 H.I./5 ml for the starfish saponin and from 440 to 2,250 H.I./5 ml for the standard saponin. The maggots were used on the second day after hatching. The average body weight and body length of the maggots which were killed by dipping them in boiling water for 5 minutes were 22.3 mg and 12 mm, respectively. Test solution (5 ml) was poured into a bottle (9×9 cm) and 50 individuals of maggot were placed in it. The mortality was determined after 24 hours and the results are shown in Fig. 3.

In another experiment toxicity was examined by using solid media and the elder maggots on the fourth day. Test media were prepared as listed in Table 3 by adding different amounts of starfish saponin or standard saponin to the basal medium which consisted of yeast and wheat bran according to the prescription adopted in the laboratory of Nihon Tokushu Nōyaku Co. Ltd. Fifty individuals of maggot were placed on each test medium and kept at 28°C in an incubator until all the maggots

Fig. 3. Toxicity of starfish saponin and standard saponin to fly maggots.

○—○ Starfish saponin
●-----● Standard saponin

Table 3. Toxicity of starfish saponin and standard saponin contained in culture media to fly maggots.

Concentrations (%)	0.05	0.1	0.2	0.25	0.3
Starfish saponin	dead	dead	dead	dead	dead
Standard saponin	pupated	pupated	pupated	dead	dead

died or pupated. As shown in Table 3, maggots pupated normally within 2 days on the media containing the standard saponin at or less than 0.2%, but died before pupation at higher levels. In the case of starfish saponin, on the other hand, the maggots failed to pupate and died perfectly within 2 days at the concentration as low as 0.05%.

Toxicity of the starfish saponin and the stomach extracts of starfish on the oysters

Each 1 ml of sea water containing 80 mg of the starfish saponin was injected into the mantle cavity of three individuals of oyster, *Ostrea gigas* THUNBERG, through a hole at the edge of shell. The shellfish were then kept in an aquarium. After about one hour they opened the shells slightly, but responded sharply to stimuli by tightly closing the shell. Any sign of relaxation of adductor muscles was not recognized in the succeeding 6 hours.

The stomach extracts were prepared after the method of IMAI⁴⁾ who reported the presence of a paralytic action upon the adductor muscle of oysters in the stomach extracts of starfish, *Asterias amurensis*. The specimen was collected in February at Misaki Marine Biological Station. Five grams of the homogenized stomach was extracted with twice volumes of sea water by stirring for 20 minutes. The residue was centrifuged off and the solution was made up to 10 ml with sea water. To each of nine individuals of the oyster was introduced 1 ml of the test solution through a hole into the mantle cavity. The shellfish revealed neither abnormality nor recognizable paralysis of the adductor muscle.

Vomiting effect on cats

Two individuals of kitten weighing 495 g and 675 g respectively were given starfish saponin orally by means of capsule. The smaller one received 50 mg of the starfish saponin, and the larger one 100 mg. Both of them willingly took cat-food soon after the administration of capsule and showed no symptom in the first two hours. Then they became gradually weakend and vomited twice respectively. After about an hour they recovered without any ill effect and none of the severe intoxications reported by PARKER⁵⁾, such as paralysis, convulsion and death, was observed.

Discussion

The various activities of starfish on other animals have been observed and discussed so far by many authors^{4-18, 30, 31)}. Among them, SAITO⁶⁾ recognized in dried starfish meal the occurrence of an undialyzable water-soluble substance toxic to earthworms and round-worms. ANDO and others^{7, 8)} observed that maggots failed to become pupae on the culture medium containing starfish meal over 40%. The present study clearly demonstrated that these activities are explained unequivocally by the presence of saponin.

As for the toxicity of the starfish extracts to bivalves, there have been much

arguments⁹⁻¹²⁾, most of which are able to be reduced to two alternatives: (1) the toxin theory which proposes the secretion of some substance inducing relaxation of the adductor muscles of shellfish, and (2) the mechanical theory which believes in starfish the ability to pull open the shells of molluscs. Not only the stomach extracts prepared in the same concentration as had been used by IMAI⁴⁾ but also the isolated starfish saponin in the dose equivalent to a starfish of medium size failed to relax the valves of oysters. These results may support the assertion of LAVOIE⁹⁾ that a very minute opening between the valves is sufficient to permit the insinuation of the asteroid stomach, and that the starfish reveals an enough pulling power to make such an opening of shell. In addition, he also reported that all the attempts to extract the alleged toxin from the stomach did not succeed. Thus, it may not be the case that the starfish saponin is employed by the animals as toxin for predation of their victims.

Toxicity of starfish extracts injected to the warm-blooded animals will be explained by the powerful hemolytic action of the starfish saponin as the case of ordinary saponin. When the starfish saponin was orally administered to cats in the dose nearly equivalent to starfish of medium size, only vomiting was recognized. Investigation was not conducted as to whether starfish contains other toxin which, when ingested, causes cats or dogs lethal intoxication as was reported. As already discussed in the previous paper, in the utilization of starfish as feedstuff or fertilizer much attentions should be paid for the content of saponin, since it is a well known fact that saponin causes retardation of growth of both chicks and plants¹⁹⁻²¹⁾.

Of special interest is the resemblance in the physiological activities between the starfish saponin and holothurin isolated as the first saponin of animal origin from sea cucumbers by YAMANOUCHI¹⁴⁾ and NIGRELLI²²⁾, independently. By these authors the following activities were demonstrated on holothurin^{14, 23-29)}: toxicity to various animals, strong hemolytic action, vomiting effect on dogs and retarding action on pupation of fruit fly. Developing sea urchin eggs, root hair of water cress, and Krebs-2 ascites tumors were also reported to be affected. Recently, NAKAZAWA^{30, 31)} has reported that the starfish stomach extracts revealed toxic effects on sea urchin eggs, eggs of *Fucaceous* algae and animal cells. The close similarity of the starfish saponin to holothurin may indicate that these phenomena are also explained by the presence of saponin.

The chemical study on the starfish saponin is now under progress.

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References

- 1) HASHIMOTO Y. and T. YASUMOTO: *This Bull.*, **26**, 1132 (1960).
- 2) FUJITA M. and K. NISHIMOTO: *J. Pharm. Soc. Japan*, **72**, 1645 (1952).
- 3) YASUTOMI and INOUE: "*Eiseigaichu Kujo no Riron to Zissai*" Hokuryukan Tokyo (1957).
- 4) IMAI T., S. HATANAKA and S. OSHIMA: *Bull. Jap. Soc. Sci. Fish., Tohoku Branch*, **7**, No. 1~2, 5 (1956).
- 5) PARKER C. A.: *Zoologist*, **5**, (1881).
- 6) SAITO K. and T. UEDA: *Merories of the Faculty of Fisheries Kagoshima University*, **3**, 141 (1953).
- 7) ANDO Y. and O. HASEGAWA: *Rept. of Hokkaido Hygienic Institute, Ser. 7*, 67 (1956).
- 8) AKIYA R.: *Hokkaido Suishi Jigyo Jumbo*, No. 361, 10 (1937).
- 9) LAVOIE M. E.: *Biol. Bull.*, **111**, 114 (1956).
- 10) SAWANO E. and K. MITSUGI: *Sci. Rept. Tohoku Imp. Univ., Ser. 4 (Biol.)*, **7**, 79 (1932).
- 11) SCHIEMENZ P.: *Mittheilungen des Deutschen Seefischerievereins, Bd. 12*, 102 (1895).
- 12) ANDO Y.: *J. Hokkaido Fish. Sci. Institute, Ser. 11*, 29 (1954).
- 13) ASANO M.: *Unpublished data* (1949).
- 14) YAMANOUCHI T.: *Publ. Seto Mar. Biol. Lab.*, **IV** (2~3), 83 (1953).
- 15) WOLFF M.: *Virchows Archiv.*, **104**, 180 (1886).
- 16) FÜRTH O. V.: "*Vergleichende Chemische Physiologie der Niederen Tiere*" p. 306 (1903).
- 17) WHITSON D. and W. H. TITUS: *Bull. Bingham Oceanographic Collection*, **IX**, 24 (1945).
- 18) BAUGHMAN N. L.: *Technological Studies of the Starfish, Fish and Wildlife Service, U. S. Dep. Interior Fish Leaflet* (1951).
- 19) BERTON W. H. and H. R. BIRD: *Poultry Sci.*, **33**, 239 (1954).
- 20) ANDERSON J. O.: *Poultry Sci.*, **36** 873 (1957).
- 21) MISHSTIN E. N. and A. N. NAUVOVA: *Akad. Nauk. S.S.S.R. Ser. Biol. No. 6*, 3 (1955), **C.A.**, **50**, 3571.
- 22) NIGRELLI R. F.: *Zoologica*, **37**, 89 (1952).
- 23) NIGRELLI R. F. and P. A. ZAHL: *Proc. Soc. Exptl. Biol. Med.*, **81**, 379 (1952).
- 24) SULLIVAN T. D., K. J. LADUE and R. F. NIGRELLI: *Zoologica*, **40**, 49 (1955).
- 25) SULLIVAN T. D. and R. F. NIGRELLI: *Proc. Am. Assoc. Cancer Research*, **2**, 151 (1956).
- 26) RUGGERI G. D., S. J. and R. F. NIGRELLI: *Zoologica*, **45**, 1 (1960).
- 27) QUAGLIO N., S. F. NOLAN, A. M. VELTRI, P. M. MURRAY, S. JAKOWSKA and R. F. NIGRELLI: *Anat. Record*, **128**, 604 (1957).
- 28) NIGRELLI R. F. and S. JAKOWSKA: *Ann. N.Y. Acad. Sci.*, **90**, 884 (1960).
- 29) CHANLEY J. D., J. PERLSTEIN, R. F. NIGRELLI, and H. SOBOTKA: *Zoologica*, **40**, 47 (1955).
- 30) NAKAZAWA S.: *The Botanical Magazine, Tokyo* **74**, 1 (1961).
- 31) NAKAZAWA S.: *Bull. Yamagata Univ. Yamagata, Japan (Natural Science)*, **5**, 349 (1961).