[Chem. Pharm. Bull. 30(4)1381—1386(1982)]

Studies on Biological Activities of Melanin from Marine Animals. I.[1] Purification of Melanin from *Ommastrephes bartrami* Lesuel and Its Inhibitory Activity on Gastric Juice Secretion in Rats

Tsutomu Mimura,*,[a] Kazuhiro Maeda,[a] Hisayo Hariyama,[a] Shigeru Aonuma,[a] Mikio Satake,[b] and Takao Fujita[b]

Faculty of Pharmaceutical Science, Osaka University,[a] Yamadaoka 1-6, Suita, Osaka 565, Japan and The Central Research Laboratory of Nippon Suisan Co., Ltd.,[b] Kitanocho, Hachiohji, Tokyo 192, Japan

(Received September 2, 1981)

Crude squid melanin obtained from ink bags of *Ommastrephes bartrami* Lesuel was found to exhibit a strong inhibitory effect on gastric secretion in rats.

The crude melanin was separated into high molecular fraction SM I (mol. wt.>160000) and low molecular fraction SM II (average mol. wt. *ca.* 39000) by gel filtration on a Sephadex G-100 column, and Fr. SM II showed a stronger biological activity.

Fr. SM II contained a melanoprotein composed of melanin pigment 90%, protein 5.8% and carbohydrate 0.8%, and significantly decreased gastric secretion at the dosage of 1 mg/kg, *i.p.* or *i.v.* Fr. SM II also prevented ulcer formation in pylorus-ligated rats and aspirin-induced ulcer.

Keywords——*Ommastrephes bartrami* Lesuel; melanin; anti-ulcerogenic action; gastric secretion; pylorus-ligated rats; aspirin-induced ulcer; carrageenin-induced edema; body temperature

Melanin derived from microorganisms is chemically classified as allomelanin, but is also termed eatechol melanin due to the fact that it contains a catechol skeleton. On the other hand, eumelanin derived various animals, which is biosynthetically obtained from tyrosine as a starting material, is called indole melanin on the basis of its structure.

We have already reported that the gastric juice inhibitory substance obtained from the culture filtrate of *Streptomyces bottropensis* consisted of melanin pigment and protein such as melanoprotein.[2,3] This article describes the purification of melanin from ink bags of *Ommasterephes bartrami* Lesuel and shows that the gastric secretion inhibitory activity and other biological properties are expressed by squid melanin as well as bacterial melanin.

Materials and Methods

Material——Fresh ink bags of *Ommastrephes bartrami* Lesuel were frozen and dissolved before use.

Purification of Melanin——The ink bags were chopped and suspended in distilled water containing dil. NaOH (at pH 9.0). This suspension was homogenized in a Waring blender and extracted by adding dil. NaOH to the homogenate at pH 9.0. The extract was filtered through a double gauze layer and the filtrate was dialyzed against distilled water. Undialyzable fraction (crude melanin) was separated into high molecular fraction (SM I) and low molecular fraction (SM II) by gel filtration on a Sephadex G-100 column equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. Approximately 4.5 g (dry wt.) of Fr. SM I and 2.4 g (dry wt.) of Fr. SM II were routinely obtained from 234 g (wet wt.) of fresh squid ink bags.

Assay of Gastric Secretion Inhibitory Activity in Rats——Gastric secretion inhibitory activity in rats was assayed according to the method of Shay *et al.*[4] The rats were deprived of food but allowed free acess to water for 48 h before the experiment. Under ether anesthesia, the pylorus was ligated. After 4 h, the animals were sacrificed and the stomachs were removed. The gastric contents were centrifuged and gastric volume was measured. Total acid output was titrated with 0.02 N NaOH and total peptic activity was determined according to the method of Anson,[5] by using 0.6% casein as a substrate. They were expressed respectively, as μeq/100 g body wt. and mg as tyrosine/100 g body wt. Each sample, dissolved in saline, was administered intraperitoneally or intravenously immediately after pylorus ligation. As a control, saline alone was administered. Atropine sulfate J.P. was used as a positive control reagent.

Anti-ulcerogenic Activity——i) Gastric Ulceration in Pylorus-ligated Rats: Male Wistar rats weighing 150—200 g, previously fasted for 24 h and pylorus-ligated as described above, were used. After 16 h, the stomach was removed. The degree of gastric ulceration developed in the forestomach was estimated by the method of Narumi *et al.*[6]

ii) Aspirin-induced Gastric Lesions: Ulceration in the fundus was induced by the method of Okabe *et al.*[7] Male Wistar rats weighing 150—200 g were used as experimental animals. Each sample or metiamide (positive control reagent) dissolved in saline was administered intraperitoneally immediately after pylorus ligation.

Carrageenin-induced Paw Edema——According to the method of Winter *et al.*,[8] 0.1 ml of 1% carrageenin (Picnin A, Zushi Chem. Lab.) solution was injected subcutaneously into the foot pad of the hind paw of male Sprague-Dawley rats weighing about 150 g. The swelling rate of the edema was determined just after the injection and at 1 h intervals after 2 h. The sample of Fr. SM II was administered intraperitoneally 30 min before carrageenin injection.

Squirming and Capillary Permeability——According to the method of Whittle,[9] male mice (ddy strain), weighing 22 ± 1 g, were used. In the squirming test, we observed the number of squirmings for 10 min after the intraperitoneal administration of Fr. SM II. As a positive control, 0.7% acetic acid was used. In the permeability test, Fr. SM II was administered intraperitoneally to each animal 10 min after intravenous injection of 0.1 ml of a solution of pontamine sky blue 6BX.

Body Temperature——Rectal temperature of rats was measured by the method described in the previous paper.[2]

Hemolytic Activity——Hemolytic activity on sheep erythrocytes was measured by the method described in the previous paper.[3]

Contraction Test on Guinea-pig Small Intestine——A strip of small intestine from guinea-pig was suspended in a bath containing Tyrode's solution. The bath was kept at 28°C. The direct action of Fr. SM II and the influence of pretreatment with Fr. SM II on acetylcholine or electrical stimulation of the small intestine were observed.

Measurement of Molecular Weight by Gel Filtration——Molecular weight was measured according to the method of Andrews *et al.*[10] A sample was subjected to gel filtration on a Sephadex G-100 column (2.3×140 cm) with 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. For detection, the absorption of eluates was measured at 280 nm. As standards of molecular weight, we used γ-globulin (mol. wt. 160000), bovine serum albumin (mol. wt. 65000) and pepsin (mol. wt. 35000).

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis——The molecular weight of each sample was estimated by 7.5% of SDS polyacrylamide gel electrophoresis according to the method of Okuyama *et al.*[11]

Isoelectric Focusing——Isoelectric focusing was performed according to Matsuo *et al.*[12]

Chemical Analysis——The sample was hydrolyzed in 6 N HCl at 110°C for 24 h in evacuated, sealed tubes, and amino acid analysis was performed by using a Hitachi KLA-III analyzer. Pigment content was measured by weighing the residue after hydrolysis of a sample in 6 N HCl. Carbohydrates, after hydrolysis of a sample with 2 N trifluoroacetic acid in a sealed tube at 110°C for 2 h, were analyzed by using a Hitachi 034-2U liquid chromatography.

Stability Test——Stability was examined under the following conditions: i) heating at 100°C for 30 min in H_2O, ii) heating at 100°C for 30 min in 1 N NaOH, iii) heating at 100°C for 30 min in 1 N HCl. After heating, the pH of each sample was adjusted to 7.0 and it was subjected to bioassay.

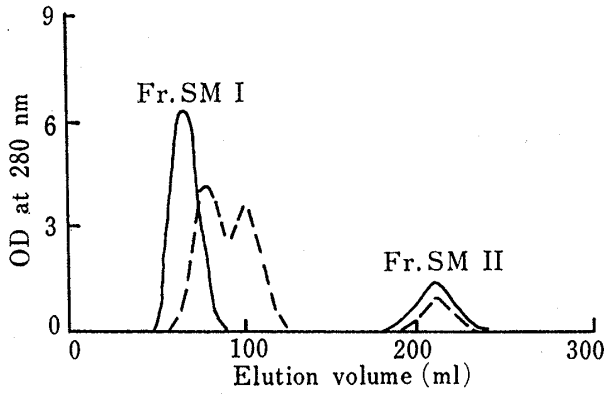

Fig. 1. Gel Filtration of SM on a Sephadex G-100 Column

Column size: 2.3×140 cm.
Solvent: 0.5 M NaCl–0.1 M phosphate buffer (pH 7.0).
——: SM.
----: (1) γ-globulin, (2) BSA, (3) pepsin.

Results

Crude melanin obtained from ink bags of *Ommastrephes bartrami* Lesuel was separated into high molecular fraction SM I (Fr. SM I) and low molecular fraction SM II (Fr. SM II) by gel filtration on a Sephadex G-100 column, as shown in Fig. 1.

Gastric Juice Secretion Inhibitory Activity of Fr. SM I and Fr. SM II

The inhibitory effects of Fr.SM I and Fr.SM II on gastric secretion are shown in Table I.

Table I. Effects of Fr. SM I and Fr. SM II on Gastric Secretion in Pylorus-ligated Rats (4 h)

Treatment	Dose (mg/kg)	No. of rats	Gastric volume (ml/100 g b.w.)	Total acid output (μeq/100 g b.w.)	Total peptic activity (mg as tyrosine/100 g b.w.)
Control[a]		8	2.36±0.16	233.4±21.9	186.6±10.9
Fr. SM I	5.0	8	0.67±0.08[d]	65.6± 5.8[d]	64.3± 4.9[d]
	2.5	8	0.76±0.10[d]	70.0± 9.7[d]	75.8± 6.4[d]
Fr. SM II	5.0	8	0.50±0.10[d]	44.4± 3.6[d]	51.9± 5.2[d]
	2.5	8	0.55±0.08[d]	45.1± 6.5[d]	63.9± 4.9[d]
	1.0	8	1.01±0.38[c]	55.8±27.2[d]	93.7±15.1[d]
	0.5	8	1.56±0.32[b]	181.6±39.0	128.7±21.7[b]
Control[a]		8	3.62±0.53	369.1±73.7	259.9±37.1
Atropine sulfate	5.0	8	0.36±0.07[d]	33.0± 5.6[d]	39.2± 5.6[d]
	1.0	8	1.04±0.20[d]	86.2±11.4[c]	87.9±12.2[d]

All values are means±S.E.
a) Saline.
Each sample was administered intraperitoneally after pylorus ligation.
Significantly different from the control group: *b*) $p<0.05$. *c*) $p<0.01$. *d*) $p<0.001$.

Table II. Effect of Fr. SM II on Gastric Secretion in Pylorus-ligated Rats (4 h)

Treatment	Dose (mg/kg)	No. of rats	Gastric volume (ml/100 g b.w.)	Total acid output (μeq/100 g b.w.)	Total peptic activity (mg as tyrosine/100 g b.w.)
Control[a]	—	8	3.07±0.32	338.2±37.9	241.8±29.3
Fr. SM II	5.00	6	1.47±0.18[c]	166.0±22.6[c]	118.2±14.6[c]
	1.00	6	1.61±0.18[c]	178.6±24.6[c]	134.0±12.6[b]
	0.25	8	2.48±0.28	302.3±39.4	202.0±21.8

All values are means±S.E.
a) Saline.
Each sample was administered intravenously immediately after pylorus ligation.
Significantly different from the control group: *b*) $p<0.05$, *c*) $p<0.01$.

Intraperitoneal administration of both fractions caused a significant reduction in gastric juice volume, total acid output and peptic activity. Atropine sulfate showed a significant inhibition of these parameters at the dose of 1 or 5 mg/kg, *i.p.*

We carried out further examination of the low molecular Fr.SM II, which showed stronger inhibitory activity. In the case of intravenous administration, Fr.SM II also showed significant inhibition of gastric secretion at doses of not less than 1 mg/kg as shown in Table II.

Anti-ulcerogenic Activity of Fr.SM II

In general, reagents that suppress gastric juice secretion are considered to be effective for preventing both ulcer formation in pylorus-ligated rats and aspirin-induced ulcer, and we therefore tested the effects of Fr.SM II on ulcer formation in pylorus-ligated rats and on aspirin-induced ulcer. Fr.SM II caused a significant reduction of the ulcer index in pylorus-ligated rats and also exhibited a suppressive effect on aspirin-induced ulcer, as shown in Tables III and IV. Metiamide, used as a positive control reagent, significantly decreased the ulcer index for aspirin-induced ulcer at dosages of 50 and 100 mg/kg.

Physicochemical Properties of Fr.SM II

i) **Molecular Weight**——The average molecular weight of Fr.SM II was calculated to be about 39000 by gel filtration (Sephadex G-100) in comparison with the elution peaks of standard substances; however, Fr.SM II showed a broad elution curve considered to be due to variation in its degree of polymerization. In SDS polyacrylamide gel electrophoresis, the molecular weight of Fr.SM II was also estimated to be 39000 on average (Fig. 2).

Table III. Effect of Fr. SM II on Gastric Ulceration in Pylorus-ligated Rats (16 h)

Treatment	Dose (mg/kg)	No. of rats	Ulcer index (mean±S.E.)	Inhibition (%)
Control[a]	—	10	3.5±0.5	—
Fr. SM II	25×2	10	0.2±0.1[c]	94.3
	10×2	10	0.9±0.4[c]	74.3
	5×2	10	1.2±0.4[b]	65.7
	1×2	10	2.4±0.3	31.4

a) Saline.
Each sample was administered intraperitoneally immediately and also at 8 h after pylorus ligation.
Significantly from the control group: b) $p<0.01$, c) $p<0.001$.

Table IV. Effect of Fr. SM II on Aspirin-induced Ulceration in Pylorus-ligated Rats (7 h)

Treatment	Dose (mg/kg)	No. of rats	Ulcer index (mean±S.E.)	Inhibition (%)
Control[a]	—	8	31.3±9.2	—
Fr. SM II	10	8	5.8±1.8[c]	81.5
	5	8	11.9±4.9	62.0
Control[a]	—	8	30.6±7.8	—
Metiamide	100	8	1.3±0.8[d]	96.7
	50	8	11.1±2.5[b]	63.7

a) Saline.
Each sample was administered intraperitoneally immediately after pylorus ligation.
Significantly different from the control: b) $p<0.05$, c) $p<0.01$, d) $p<0.001$.

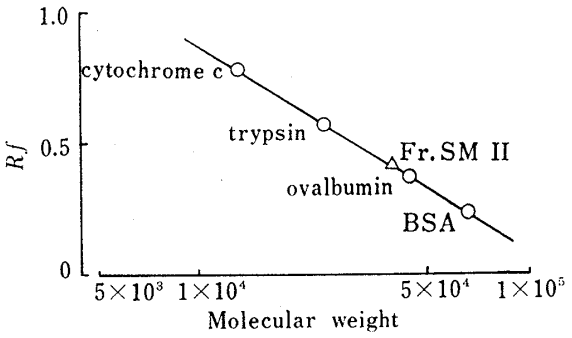

Fig. 2. Determination of the Molecular Weight of Fr. SM II by SDS-polyacrylamide Gel Electrophoresis

Electrophoresis was carried out in 7.5% polyacrylamide gel.

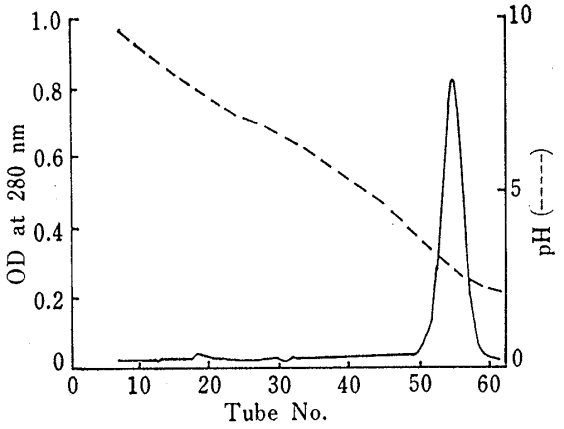

Fig. 3. Isoelectric Focusing of Fr. SM II on an Ampholine Column

ii) **Isoelectric Focusing**——The result of isoelectric focussing is shown in Fig. 3; Fr.SM II exhibited a single peak at 2.6.

Chemical Properties of Fr.SM II

Fr.SM II contained 5.8% protein and 0.8% sugar in addition to 90.0% melanin pigment as determined by chemical analysis. The protein in Fr.SM II was composed of 16 kinds of amino acids (tryptophan was not analyzed). Glycine, aspartic acid and glutamic acid were present in large amounts and there was a trace amount of a sulfur-containing amino acid (Table V).

Six kinds of carbohydrates were contained in Fr.SM II as shown in Table VI.

Table V. Amino Acid Composition of Fr. SM II

Amino acid	Molecular proportion
Lys	2.6
His	1.1
Arg	2.0
Asp	5.3
Thr	2.7
Ser	3.6
Glu	7.9
Pro	2.0
Gly	5.3
Ala	4.5
Val	2.1
Ile	1.7
Leu	3.3
Tyr	1.0
Phe	1.5
Met	Trace

Table VI. Carbohydrate Composition of Fr. SM II

Carbohydrate	Molecular proportion
Rib	4.7
Man	1.4
Fuc	1.0
Gal	Trace
Xyl	1.6
Glc	14.1

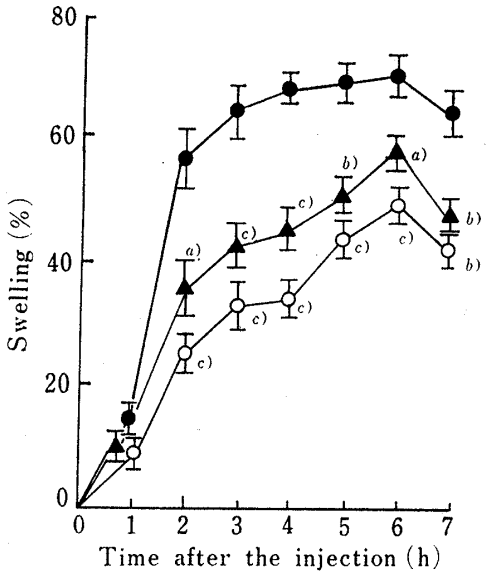

Fig. 4. Effect of Fr. SM II on Carrageenin-induced Edema of the Rat Hind Paw

—●—: control (n=12).
—○—: Fr. SM II 5 mg/kg, $i.p.$ (n=10).
—▲—: Fr. SM II 1 mg/kg, $i.p.$ (n=10).
Significantly different from the control group: *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

Biological Activities of Fr.SM II

i) Inhibitory Effect of Fr.SM II on Carrageenin-induced Paw Edema in Rats——The effect of Fr.SM II on edema of rat hind paw induced by carrageenin is shown in Fig. 4. Significant inhibition was observed in the groups administered 1 and 5 mg/kg ($i.p.$) of Fr.SM II.

ii) Effect of Fr.SM II on Squirming and Capillary Permeability——Intraperitoneal injection of Fr.SM II at doses less than 10 mg/kg did not induce squirming and did not stimulate peritoneal capillary permeability in mice.

iii) Effect of Fr.SM II on Body Temperature of Rats——Administration of Fr.SM II ($i.p.$ or $i.v.$) had no effect on the rectal temperature of rats through 4 h after the administration.

iv) Effect of Fr.SM II on Smooth Muscle——Fr.SM II had no direct action and no effect on contraction induced by acetylcholine or electric stimulation.

v) Effect of Fr.SM II on Hemolytic Activity——Addition of Fr.SM II exhibited no effect on hemolytic activity *in vitro* even at a high concentration, 200 μg/ml.

vi) Toxicity——Oral and intraperitoneal administration of Fr.SM II dissolved in saline at doses of 1 g/kg and 5 g/kg caused no toxic symptom in mice within 72 h.

Stability of Biological Activity of Fr.SM II

The biological activity of Fr.SM II was unaffected after treatment at 100°C for 30 min in distilled water, in 1 N NaOH or 1 N HCl.

Discussion

The chemical structure and the biological activities of melanin are not well known because of its low solubility in water. Fr.SM I and Fr.SM II, the high and low molecular

weight fractions, respectively, purified from ink bags of *Ommastrephes bartrami* LESUEL, contain melanin pigment as the major component, with protein and carbohydrate as minor components. Low molecular Fr.SM II has an indole skeleton, is relatively water-soluble, and has a mean molecular weight of about 39000 as determined by gel filtration on a Sephadex G-100 column and by SDS polyacrylamide gel electrophoresis. Fr.SM II consists of a melanin pigment complex with protein and carbohydrate, and its molecular weight appears to be distributed in the range of about 25000 to 50000. We have already reported that DOPA-melanin markedly reduced gastric juice secretion in rats, though it did not lower the normal body temperature of rats.[3] It can be considered that the pigment component in melanoprotein contributes mainly to the inhibition of gastric secretion. Details of the chemical structure of the melanin pigment contained in Fr.SM II must await further investigation.

We found significant inhibitory action of Fr.SM II on gastric secretion in pylorus-ligated rats at doses over 1 mg/kg (*i.p.* or *i.v.*). Since peptic ulcer is considered to be a result of imbalance between autolysis by gastric juice and the resistance of gastrointestinal mucosa, reagents which inhibit gastric juice secretion are expected to exhibit anti-ulcerogenic action. Therefore the effect of Fr.SM II on both ulcer formation in pylorus-ligated rats and aspirin-induced ulcer was examined. As expected, Fr.SM II inhibited these two kinds of ulcer formation. The inhibition mechanism of Fr.SM II on ulcer formation will be investigated in the near future by using a perfused stomach preparation.

It is well known that intraperitoneal administration of high molecular substances such as protein or polysaccharide inhibits ulcer formation, gastric secretion and the formation of edema in experimental animals. Although we studied the irritative action Fr.SM II by means of the squirming test and capillary permeability test in mice, it was clarified that Fr.SM II did not show any positive effect. Thus, we concluded that the biological activities of Fr.SM II were not based on irritative action.

Further investigations are in progress to clarify the inhibitory mechanism of Fr. SM II on gastric secretion through the examination of gastric mucosal enzymes, nerve systems and local circulation systems.

Acknowledgement The authors are grateful to Nissui Seiyaku Co., Ltd. for supplying materials.

References and Notes

1) This work was presented at the 30th Annual Meeting of the Kinki Branch of the Pharmaceutical Society of Japan, Matsubara, Nov. 1980.
2) T. Mimura, N. Muto, J. Tanaka, H. Oshita, N. Onishi, and S. Aonuma, *Chem. Pharm. Bull.*, **25**, 897 (1977).
3) T. Mimura, N. Muto, Y. Oda, N. Tanaka, and S. Aonuma, *Chem. Pharm. Bull.*, **26**, 998 (1978).
4) H. Shay, S.A. Komarov, S.S. Fels, D. Meranze, M. Gruentein, and M. Siplet, *Gastroenterology*, **5**, 43 (1945).
5) M.L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).
6) S. Narumi, T. Hirata, K. Gomaibashi, and M. Kano, *J. Takeda Res. Lab.*, **29**, 85 (1970).
7) S. Okabe, K. Takeuchi, K. Nakamura, and K. Takagi, *Japan. J. Pharmacol.*, **24**, 363 (1974).
8) C.A. Winter, E.A. Risley, and G.W. Nuss, *Proc. Soc. Exp. Biol. Med.*, **111**, 544 (1962).
9) B.A. Whittle, *Brit. J. Pharmacol.*, **22**, 246 (1964).
10) P. Andrews, *Biochem. J.* **91**, 222 (1964).
11) N. Okuyama and M. Kondo, "Protein, Nucleic acid and Enzyme, ISSN (seitaimaku jitsukenhou)," Kyoritsu Press, Tokyo, 1974, p. 155.
12) Y. Matsuo and T. Horio, *Protein, Nucleic acid and Enzyme*, **12**, 737 (1967).