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Studies on Biological Activities of Melanin from Marine Animals. II.[1] Purification of Melanin from *Octopus vulgaris* Cuvier and Its Inhibitory Activity on Gastric Juice Secretion in Rats

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Octopus melanin obtained from ink bags of *Octopus vulgaris* Cuvier was found to inhibit gastric secretion in rats in the same way as that from *Ommastrephes bartrami* Lesuel. The molecular weight of this melanin fraction (which has an indole skeleton) was estimated to be over 200000 by gel filtration on Sephadex G-200, and the Octopus melanin fraction (Fr. OM) released several kinds of proteins in the presence of SDS. The chemical composition of Fr. OM was melanin pigment 79%, protein 17.5% and sugar 1.7%, so Fr. OM was considered to be a melanoprotein. Fr. OM significantly reduced gastric secretion in rats at the dose of 1 mg/kg, *i.p.*, and also prevented both ulcer formation in pylorus-ligated rats and aspirin-induced ulcer.

Keywords——*Octopus vulgaris* Cuvier; melanin; gastric secretion; pylorus-ligated rats; aspirin-induced ulcer; anti-ulcerogenic action

We have already reported that the melanin fractions derived from ink juice of *Ommastrephes bartrami* Lesuel as well as that of a microorganism[2] strongly inhibited gastric juice secretion in rats. The former was separated into high molecular fraction SM I (mol. wt.>160000) and low molecular fraction SM II (mol. wt. *ca.* 39000).[3]

In this paper, we examined the melanin fraction (Fr. OM) obtained from ink juice of *Octopus vulgaris* Cuvier for physicochemical and biological activities.

Materials and Methods

Material——Fresh ink bags of *Octopus vulgaris* Cuvier 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 the homogenate was filtered through a double gauze layer. The filtrate was dialyzed against distilled water. The undialyzable fraction was subjected to gel filtration on a Sephadex G-200 column equilibrated with 0.1 M Na_2HPO_4 (pH 8.5) containing 0.5 M NaCl.

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] Male Wistar rats weighing 150—200 g, previously fasted for 48 h, were used as experimental animals. 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] A sample dissolved in saline was administered intraperitoneally immediately after pylorus ligation. As a control, only saline was administered. Atropine sulfate J.P. was used a positive control reagent.

Perfused Stomach Preparation——Male Wistar rats weighing 150 to 200 g were fasted for 24 h and used (under anesthesia) to obtain the perfused rat stomach preparation according to a modification by Sawada of the method of Ghosh and Schild.[6] Histamine·2HCl (Wako Pure Chemical Ind.) and tetragastrin (Nissui Seiyaku Co.) were used as stimulants. The sample was administered intraperitoneally.

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.*[7]

ii) Aspirin-induced Gastric Lesions: This ulceration in the fundus was induced by the method of Okabe *et al.*[8] Male Wistar rats weighing 150—200 g were used as experimental animals. Metiamide was used as a positive control reagent.

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

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

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis——Electrophoresis was performed on 7.5% acrylamide gel by the method described by Okuyama *et al.*[10]

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. Sugar analysis was performed by using a Hitachi 034-2U liquid chromatograph after hydrolysis of a sample in 2 N trifluoroacetic acid at 100°C for 2 h.

Isoelectric Focusing——Isoelectric focusing was carried out according to Matsuo *et al.*[11]

Results

Crude melanin obtained from the juice of *Octopus vulgaris* Cuvier was eluted as a single peak by filtration on a Sephadex G-200 column with 0.1 M Na_2HPO_4 (pH 8.5) containing 0.5 M NaCl (Fig. 1). This fraction was termed Fr. OM (Octopus melanin) and lyophilized. It was examined in the following experiments.

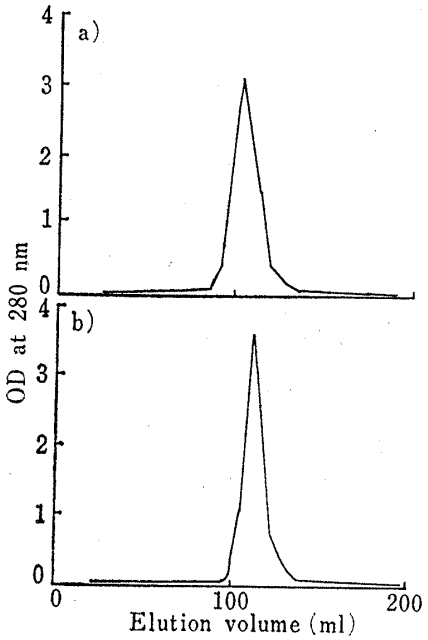

Fig. 1. Gel Filtration of Crude Octopus Melanin on a Sephadex G-100 or G-200 Column

a) Crude octopus melanin was applied to a column (1.6×128.0 cm) of Sephadex G-100.
b) The same sample was applied to a column (2.0×88.5 cm) of Sephadex G-200.
Each column was developed with 0.5 M NaCl–Na_2HPO_4 (pH 8.5).

Gastric Juice Secretion Inhibitory Activity of Fr. OM in Rats

Table I shows the inhibitory effect of Fr. OM on gastric juice secretion in pylorus-ligated rats. Fr. OM exhibited significant inhibition of the amount of gastric juice, total acid output and total peptic activity, at doses of 1 mg/kg *i.p.* and over. Atropine sulfate showed a significant inhibition of these parameters at the dose of 1 or 5 mg/kg, *i.p.*

Effect of Fr. OM on the Perfused Stomach Preparation

Fr. OM at doses of less than 10 mg/kg, administered intraperitoneally, showed no effect on acid output stimulated by tetragastrin (10 μg/kg, *i.v.*) or histamine·2HCl (1 mg/kg, *i.v.*) in the perfused stomach preparation of normal rats.

Anti-ulcerogenic Activity of Fr.OM

In general, substances that inhibit gastric secretion are considered to be effective for preventing both ulcer formation in pylorus-ligated rats and aspirin-induced ulcer. Therefore, Fr. OM, which significantly decreased the gastric juice volume, was examined for its preventive effect. As shown in Tables II and III, Fr. OM significantly decreased the ulcer index, in a dose-dependent manner, in both experimental models. Metiamide significantly decreased the ulcer index for aspirin-induced ulcer at doses of 50 and 100 mg/kg, *i.p.*

Table I. Effect of Fr. OM 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±0.19	297.3±36.8	205.4±11.7
Fr. OM	100	7	0.51±0.03[d]	28.7± 2.6[d]	35.2± 2.7[d]
	50	8	0.86±0.20[d]	54.2±14.9[d]	51.4± 9.0[d]
	25	7	1.05±0.24[d]	77.6±14.1[d]	70.3±11.0[d]
	10	6	1.34±0.25[d]	112.5±38.1[c]	91.4±23.1[d]
	5	8	1.69±0.32[d]	146.8±30.7[b]	125.0±21.5[b]
Control[a]	—	8	2.63±0.30	227.6±30.3	202.2±18.2
Fr. OM	1.0	8	1.90±0.10[b]	170.5±20.4	143.2±19.3[b]
	0.5	8	2.35±0.41	216.4±20.2	176.6±19.6
	0.25	8	2.53±0.22	232.0±24.8	200.0±19.2
Control[a]	—	8	3.57±0.53	369.1±73.7	249.9±37.1
Atropine sulfate	5	8	0.31±0.07[d]	33.0± 5.6[d]	29.2±5.6[d]
	1	8	0.99±0.20[d]	86.2±11.4[c]	77.9±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. OM on Gastric Ulceration in Pylorus-ligated Rats (16 h)

Treatment	Dose (mg/kg)	No. of rats	Ulcer index (mean±S.E.)	Inhibition (%)
Control[a]	—	9	3.1±0.5	—
Fr. OM	25×2	10	0.5±0.2[c]	83.9
	10×2	10	1.8±0.4[b]	41.9
	5×2	10	2.6±0.5	16.1

a) Saline.
Each sample was administered intraperitoneally immediately and also 8 h after pylorus ligation.
Significantly different from the control group: *b*) $p<0.05$, *c*) $p<0.001$.

Table III. Effect of Fr. OM 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]	—	9	25.4±6.0	—
Fr. OM	25	8	8.8±3.0[b]	65.4
	10	8	9.6±3.8[b]	62.5
	5	8	11.0±4.8	56.6
Control	—	8	30.6±7.8	—
Metiamide	100	8	1.3±0.8[c]	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*) $p<0.001$.

Chemical Properties of Fr. OM

Fr. OM was eluted in the flow-through fraction upon gel filtration on Sephadex G-100 or G-200, and was separated into three peaks by gel filtration on a Sephadex G-100 column equilibrated with 0.1 M phosphate buffer, pH 7.5, in the presence of 40 mM SDS and 0.5 M NaCl, as shown in Fig. 2.

These three fractions were designated as Fr. OM I, Fr. OM II and Fr. OM III, respectively. Figure 3 shows the ultraviolet (UV) absorption spectra of these fractions. Fr. OM I showed a slight absorption at 280 nm and therefore was considered to be composed mainly of melanin

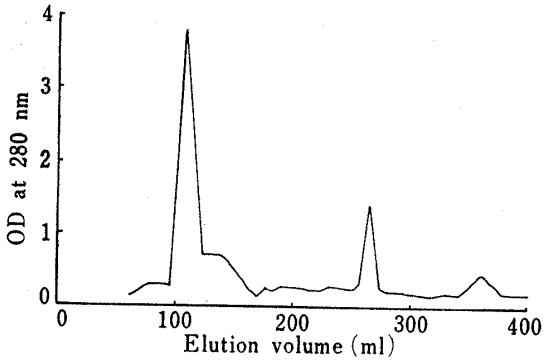

Fig. 2. Gel Filtration of Fr. OM on a Sephadex G-100 Column

Column size: 1.6 × 117.0 cm.
Solvent: 40 mM SDS–0.5 M NaCl–0.1 M phosphate buffer (pH 7.5).

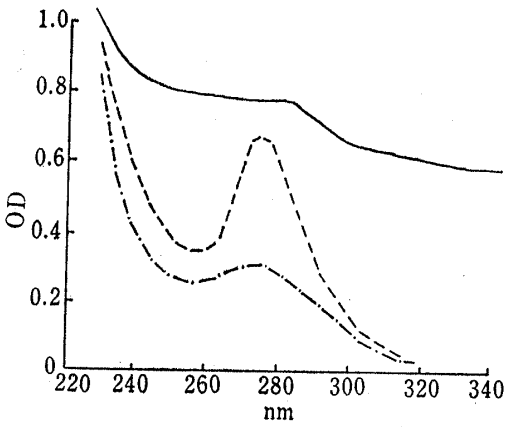

Fig. 3. Absorption Spectra of the Fractions

———: Fr. OM I.
--------: Fr. OM II.
-·-·-·-: Fr. OM III.

pigment. Fr.OM II and Fr. OM III were light brown in color and exhibited strong absorption at 280 nm, and these fractions were considered to consist of proteins which were released from Fr. OM by gel filtration in the presence of SDS.

In SDS polyacrylamide gel electrophoresis, Fr.OM gave at least eight bands of proteins and the melanin pigment of Fr. OM was not electrophoresed. Hence Fr. OM was anticipated to be a high molecular melanoprotein composed of melanin pigment, protein and sugar. The chemical composition of Fr. OM was melanin pigment 79.0%, protein 17.5% (calculated from the result of amino acid analysis) and sugar 1.7%. Fr. OM also exhibited a single peak at pH 1.8 in isoelectric focusing as shown in Fig. 4.

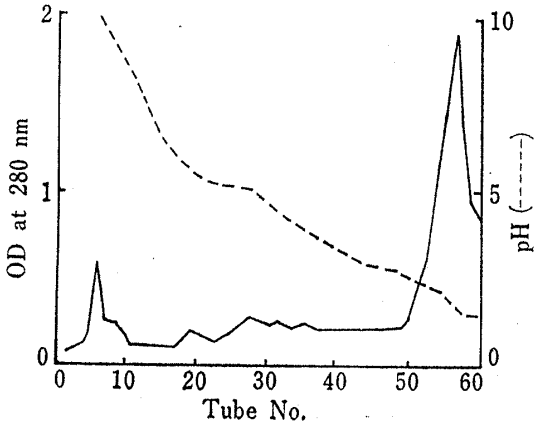

Fig. 4. Isoelectric Focusing of Fr. OM on an Ampholine Column

Biological Activities of Fr. OM

i) Effect of Fr. OM on Squirming and Capillary Permeability Tests——Intraperitoneal administration of Fr. OM did not induce squirming at a dose of 5 mg/kg and had no effect on peritoneal capillary permeability in mice at a dose of 10 mg/kg.

ii) Effect of Fr. OM on Body Temperature in Rats——Administration of Fr. OM (*i.p.* and *i.v.*) had no effect on the rectal temperature of rats through 4 h after the administration.

Discussion

In the previous paper, we have already reported that melanin obtained from ink bags of *Ommastrephes bartrami* Lesuel consisted of high molecular fraction Fr. SM I and low molecular fraction Fr. SM II. In the present experiment, we found that melanin of *Octopus vulgaris* Cuvier was composed of only high molecular fraction and was different from the squid melanin. However, this melanin fraction exhibited the same effect as squid melanin on gastric secretion inhibitory and had strong inhibitory activity on both ulcer formation in pylorus-ligated rats and aspirin-induced ulcer based on its inhibitory effect on gastric secretion.

In the squirming test and the capillary permeability test in mice Fr. OM had no irritative

action. Thus, we concluded that the biological activities of Fr. OM were not based on irritative factors.

On the other hand, we found several kinds of proteins which dissociated from Fr. OM in the presence of SDS.

These proteins binding to melanin pigment were assumed to enhance the solubility of the pigment. Fr. OM was considered to be slightly soluble in water after removal of protein by hydrolysis in hydrochloric acid.

Further investigations are required to clarify the mechanisms of gastric inhibitory activity of Fr. OM as well as those of squid melanin.

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1) This work was presented at the Annual Meeting of the Nippon Suisan Gakkai, Tokyo, Apr. 1981.
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