バフンウニ(Hemicentrotus pulcherrimus)中に存在するラット血小板凝集惹起物質及び血小板凝集抑制物質

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Technical Report

Rat Platelet-Aggregation Inducer and Inhibitor in Sea Urchin (*Hemicentrotus pulcherrimus*) Extract

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Abstract : A rat platelet-aggregation inducer was extracted with methanol from gonads of sea urchin (*Hemicentrotus pulcherrimus*). The active substance in the extract was concentrated using a Sep-Pak C_{18} cartridge, Sephadex G-10 gel filtration and DEAE-Toyopearl 650M anion-exchange chromatographies. The active substance was identified as adenosine diphosphate (ADP) by UV spectroscopy and by HPLC (on an Asahipak GS-320 column), using authentic ADP as a reference. On the other hand, a rat platelet-aggregation inhibitor was found in the methanol extract from whole bodies (including gonads, alimentary canals, coelome liquids and shells) of *H. pulcherrimus*. This substance was concentrated using Sephadex G-10 gel filtration and DEAE-Toyopearl 650M anion-exchange chromatographies. The aqueous solution containing this substance had a maximum UV absorbance near 320nm.

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Recently, the pharmacological effects of natural products from marine organisms have received great interest (Kamiya, 1990 ; Sasaki and Endo, 1990 ; Fusetani, 1990 ; Kakisawa, 1992 ; Murakami and Yamaguchi, 1992). Of these pharmacological effects, the biological activities related to platelet aggregation are considered to be crucial as far as health is concerned. This is because platelet aggregation sometimes leads to thrombosis, which can induce heart attacks and strokes. New substances having the ability to induce and inhibit platelet aggregation have been found in animals and plants (Venable *et al.*, 1993 ; Grevelink *et al.*, 1993; Kumagai *et al.*, 1994; Sumi *et al.*, 1996). In this study, substances were analyzed from sea urchin *Hemicentrotus pulcherrimus* extract which have the ability to induce and inhibit rat platelet aggregation.

Materials and Methods

Materials Sea urchin samples (*Hemicentrotus pulcherrimus*) were sampled from the coast of Fukushima Prefecture in Japan. Gonads were separated and washed with 3% NaCl.

Extraction and Fractionation of Platelet Aggregation-Related Substances in Sea Urchin Gonads (140g) or whole bodies (1,200g, including gonads, alimentary canals, coelome liquids and shells) of sea urchin were homogenized with five volumes of methanol and filtrated with No. 2 filter paper (Advantec Toyo, Tokyo, Japan). The methanol solutions were concentrated with a rotary evaporator, and lipids in the extract were removed by diethyl ether extraction. The water soluble fraction of the methanol extract was adjusted to a 90% ethanol solution, and the precipitate was separated by centrifugation at 3,400 rpm. After dissolving it in distilled water, the precipitate fraction was applied to a Sep-Pak Plus C_{18} cartridge (Waters, Milford, MA USA), and eluted with water and methanol. The fraction eluted with water was applied on Sephadex G-10 gel filtration chromatography (2.5 x 40 cm) and developed with 5 mM potassium phosphate buffer (pH 6.86). The active fractions were combined and layered onto a DEAE-Toyopearl 650M anion-exchange chromatography (2.5 x 10 cm). The column was developed with 5 mM potassium phosphate buffer (pH 6.86) and 5 mM potassium phosphate buffer - 1 M NaCl (pH 6.86). Finally, the active substance was analyzed by high performance liquid chromatography (HPLC) with an Asahipak GS-320 column (7.6 x 500mm) using a developing solution of 0.1 M KH_2PO_4 (pH 3.8) and monitored at a wavelength of 257 nm.

Platelet-aggregation inhibitor was separated from active fractions of Sephadex G-10 gel filtration, and this was followed by DEAE-Toyopearl 650M anion-exchange chromatography (2.5 x 10 cm) with a linear-gradient elution of 0 - 1.0 M NaCl in 5 mM potassium phosphate buffer (pH 6.86).

Assay of Platelet Aggregation Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared from the whole blood of a male wistar rat, according to the method of Kanazawa *et al.* (1994). The PRP contained 5 x 10^5 cells/$\mu\ell$ of platelet. Platelet aggregation

and its inhibition were measured by the method of Born (1962) using an aggregometer (Hema Tracer 601, MC Medical, Tokyo, Japan) equipped with a NEC 9821 computer. For the measurement of platelet aggregation, 10$\mu\ell$ of sample or authentic ADP were added to 100 $\mu\ell$ of PRP, and the change of transmittance was monitored by an aggregometer, as shown in **Fig. 1.** In this study, the maximum aggregation rate was used as an index of platelet aggregation activity. On the other hand, the activity of platelet-aggregation inhibition was calculated by the following equation : 100 - (maximum aggregation rate of aggregation by 100μM ADP after addition of sample) / (maximum aggregation rate of aggregation by 100μ M ADP)x 100

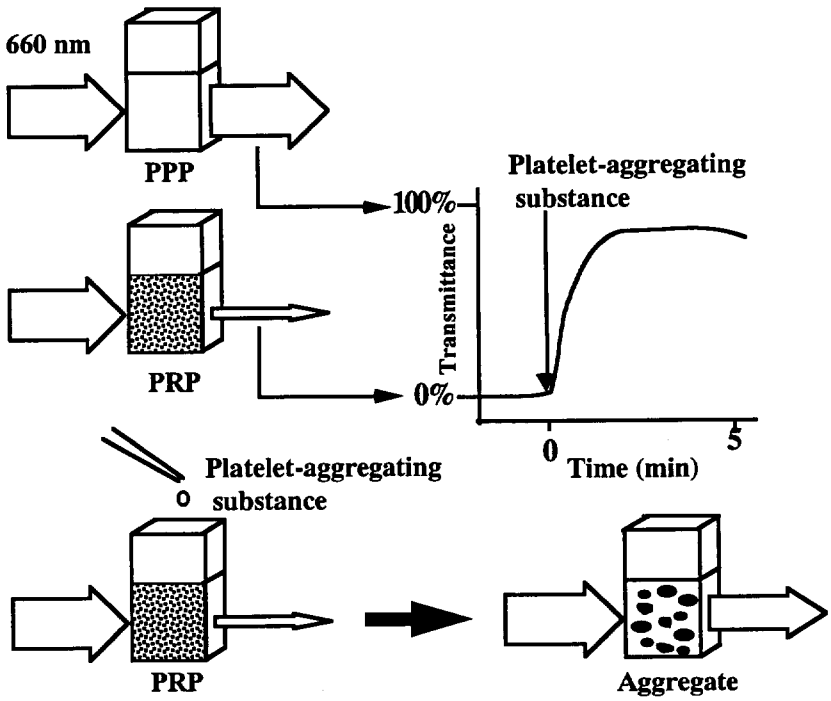

Fig. 1. Measurement of platelet aggregation.

Results and Discussion

The fractionation procedures of the platelet-aggregation inducer from sea urchin gonads is shown in **Fig. 2.** From preliminary experiments, we found that the methanol extract of sea urchin gonads has rat platelet-aggregation activity.

The results of a gel filtration is shown in **Fig. 3.** The fractions (Fr.) No. 16 to 25 had rat platelet-aggregation activity. The maximum aggregation rate of Fr. No. 20 was 47%. The active fractions (Fr. No. 18 to 25) were charged to a DEAE-Toyopearl anion-exchange chro-

matography and the fraction which was eluted with 5 mM potassium phosphate buffer - 1 M NaCl (pH 6.86) had the activity. The fraction had maximum absorption near 257 nm, similar to ADP. The HPLC results are shown in **Fig. 4.** The retention time of the major peak of the active fraction from a DEAE-Toyopearl 650M anion-exchange chromatography coincided with that of authentic ADP. From these results, one of the platelet-aggregation inducer of sea urchin gonads was identified as ADP, which is known to be a platelet activator (Weiss, 1986). In this study, the methanol extract of whole bodies of sea urchin was charged to a

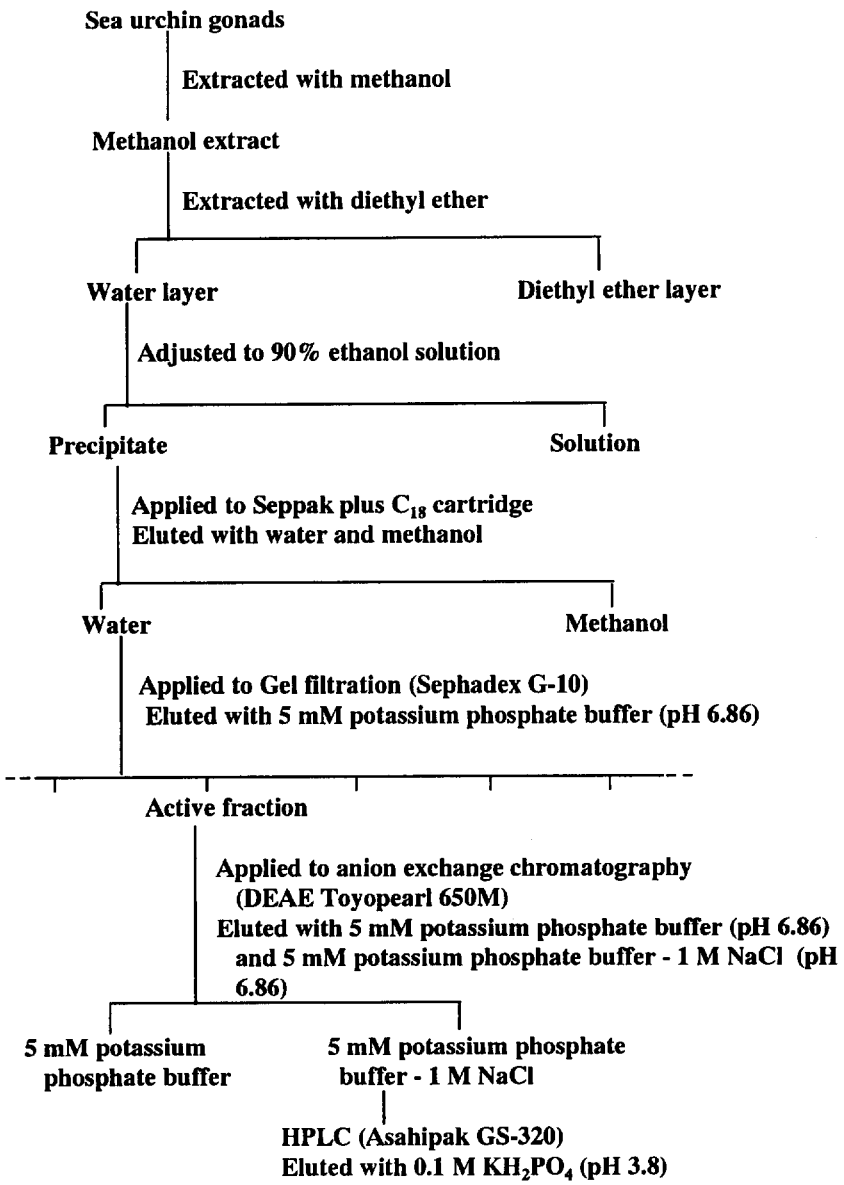

Fig. 2. Fractionation scheme for the platelet-aggregation inducer from sea urchin gonads.

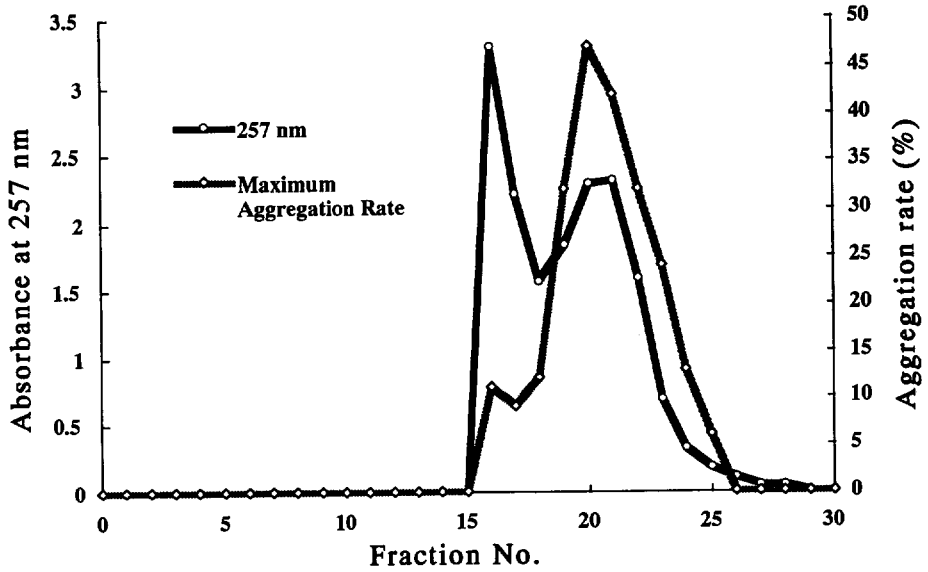

Fig. 3. Sephadex G-10 gel filtration of the methanol extract of sea urchin gonads. The water-eluted fraction of Sep-Pak C_{18} cartridge of the methanol extract of sea urchin gonads was applied to a Sephadex G-10 column (2.5 x 40cm), and eluted with 5mM potassium phosphate buffer. 4mℓ fractions were collected at a flow rate of 2.5 mℓ/min.

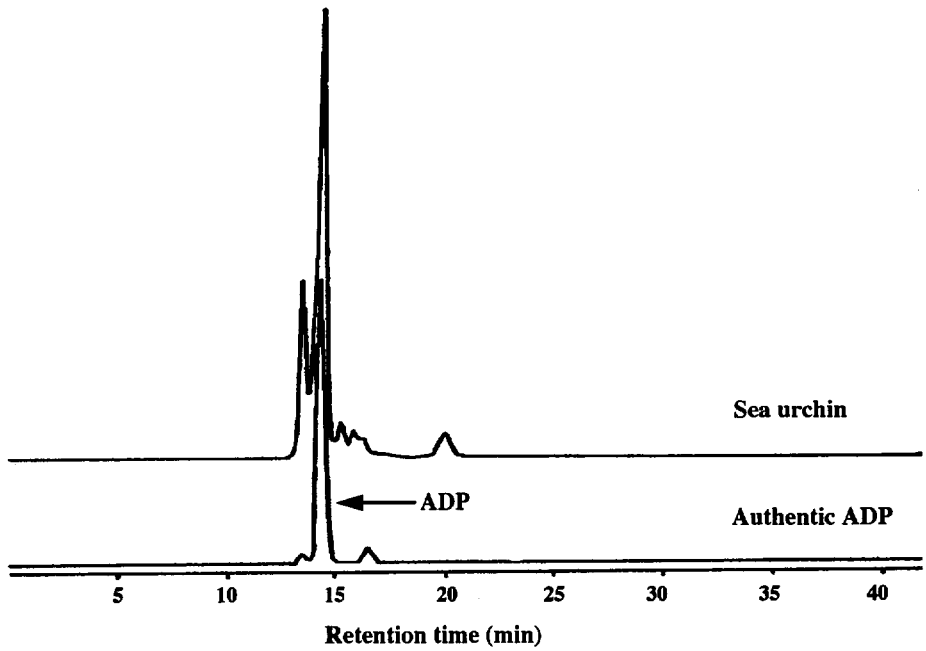

Fig. 4. HPLC chromatograms of the platelet-aggregation inducer from sea urchin gonads and authentic ADP. The fractions having platelet-aggregation activity were obtained using DEAE-Toyopearl 650M anion exchange chromatography and were subjected to HPLC on a Asahipak GS-320 column (7.6 x 500 mm). The column was eluted at a flow rate of 1mℓ/min with 0.1 M KH_2PO_4 (pH 3.8).

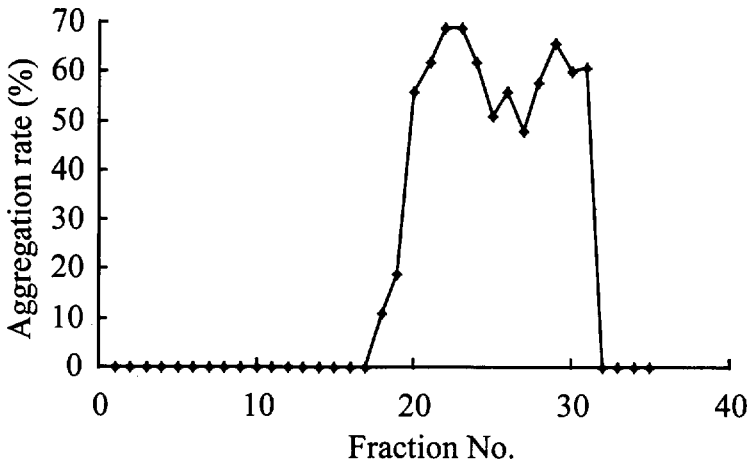

Fig. 5. Sephadex G-10 gel filtration of the methanol extract of whole bodies of sea urchin. The procedure for gel filtration was the same as that used Fig. 3.

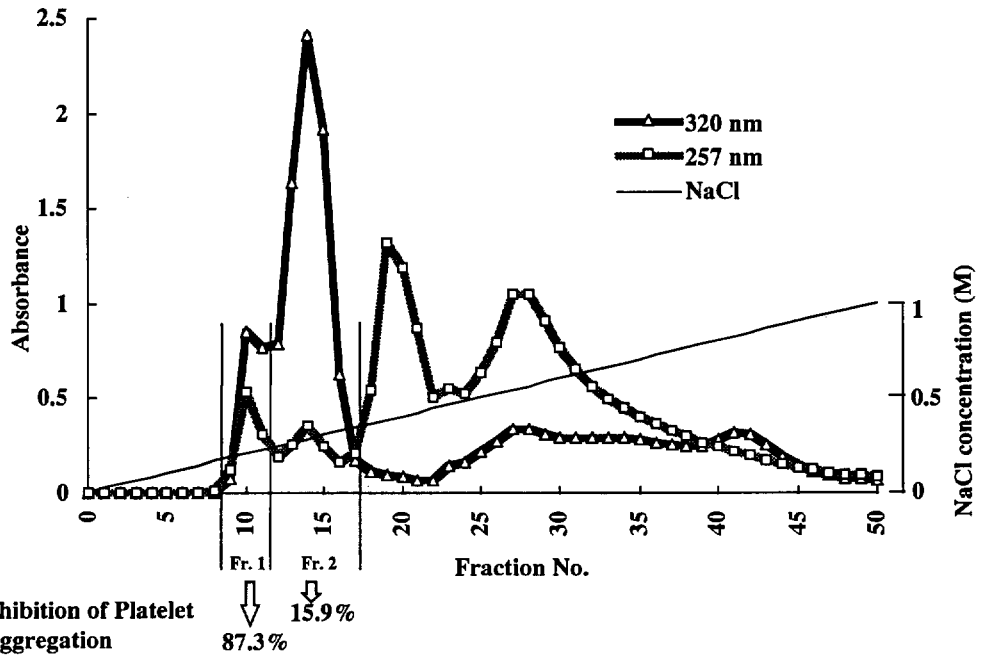

Fig. 6. DEAE-Toyopearl column chromatography of the methanol extract from whole bodies of sea urchin. The fractions having the platelet-aggregation inhibition activity were obtained by gel filtration (on Sephadex G-10) of a methanol extract of whole-body sea urchin. After gel filtration, the active fractions were combined and layered onto a DEAE-Toyopearl 650M anion-exchange chromatography (2.5 x 10cm) with a linear gradient elution of 0 - 1.0 M NaCl in 5 mM potassium phosphate buffer (pH 6.86). During this chromatographic separation, 4 ml fractions were collected at a flow rate of 2.5mℓ/min.

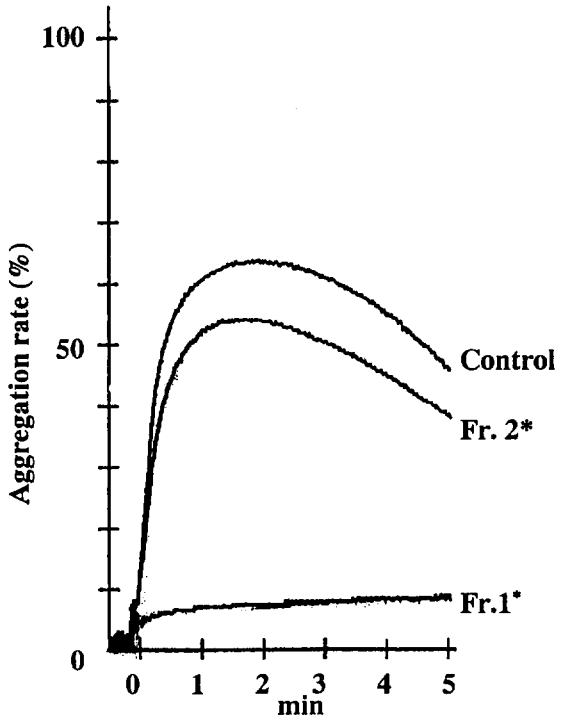

Fig. 7. The aggregation response in rat PRP treated with 10 $\mu\ell$ of ADP (100 μM). *Ten $\mu\ell$ of Fr. 1 or Fr. 2 of DEAE-Toyopearl column chromatography of methanol extracts of the whole body of sea urchin (**Fig. 6**) was added to PRP before the addition of ADP. The concentration of the fractions were adjusted to 30 mg/mℓ.

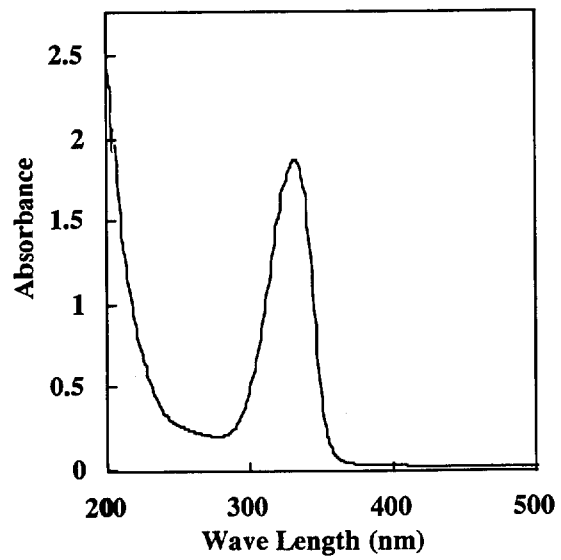

Fig. 8. Absorption spectrum of the active fraction (Fr. 2 of **Fig. 6**) having platelet-aggregation inhibition.

Sephadex G-10 gel filtration chromatography (**Fig. 5**). The fractions from 25 to 30 of **Fig.5** also had platelet-aggregation activities. It was suggested that other platelet-aggregation inducers exist in the whole bodies of sea urchin.

On the other hand, platelet-aggregation inhibitor was detected in the extracts from whole bodies of sea urchin during fractionation of the platelet-aggregation inducer on a DEAE-Toyopearl 650M anion-exchange chromatography (**Figs. 6** and **7.**). Fr. 1 and Fr. 2 of DEAE-Toyopearl anion-exchange chromatography (**Fig. 6**) inhibited the platelet aggregation induced by ADP. The inhibition rate of the fractions were 87.3% (Fr. 1) and 15.9% (Fr. 2). The fractions showed a maximum absorption near 320 nm (**Fig. 8**). This substance was not detected in the sea urchin gonad extracts - there was no activity or absorption at 320 nm. This substance is expected to have preventive effect on thrombosis. Further purification of this substance is now being carried out.

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バフンウニ(*Hemicentrotus pulcherrimus*)中に存在するラット血小板凝集惹起物質及び血小板凝集抑制物質

金庭正樹，村田裕子，横山雅仁，篠原和毅

摘　　要

バフンウニ（*Hemicentrotus pulcherrimus*）生殖巣のメタノール抽出物はラット血小板の凝集活性を有していたので，この抽出物中の血小板凝集惹起物質をゲルろ過，陰イオン交換クロマトグラフィーなどにより濃縮した。さらに紫外吸光分析，HPLC分析によってこの血小板凝集惹起物質はアデノシン二リン酸であることが判明した。また，バフンウニの個体全体からのメタノール抽出物中に320 nmに紫外吸収を有するラット血小板の凝集抑制画分が存在することが明らかになった。