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Isolation of an Inhibitor of Platelet Aggregation from a Fungus, *Ganoderma lucidum*

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The water-soluble fraction of *Ganoderma lucidum* was found to suppress platelet aggregation. It was further shown by gel chromatography, high performance liquid chromatography and nuclear magnetic resonance spectroscopy that the inhibitory substance present in this fraction was adenosine.

Keywords——fungus; *Ganoderma lucidum*; platelet aggregation; adenosine

Chinese medicines have long been used clinically. Their chemical bases, however, are not necessarily undestood in most cases. Some of them have attracted much attention as effective chemotherapeutic agents against cancer.[1-3] A fungus, *Ganoderma lucidum* (靈芝), has been clinically tested for the treatment of various diseases,[4] but despite many positive findings, it has never been reported to suppress thrombus formation. In this work, we have demonstrated for the first time that the water-soluble fraction of this fungus suppresses platelet aggregation.

The aggregation response induced by 0.21 unit/ml thrombin in the presence of the *G. lucidum* extract is shown in Fig. 1. A clear incubation time-dependent inhibition is apparent. A significant inhibitory effect was observed until the concentration of the extract was diluted approximately one-hundred times. These results indicate that *G. lucidum* contains a strong inhibitor(s) of platelet aggregation.

Our initial attempt to isolate the inhibitory substance(s) was made by gel filtration on a Sephadex G-25 column (1.3 × 47.5 cm) equilibrated with distilled water. The elution profile is shown in Fig. 2. The ultraviolet (UV) absorption and the inhibition of aggregation of each fraction are shown. Most of the materials with UV absorption were eluted within the void volume, but they had no inhibitory effect on platelet aggregation. A small UV absorption peak was observed at 1.6 times the gel volume. This peak (fraction numbers 65—74) contained the platelet aggregation-inhibitory substance(s) of *G. lucidum*. From this result, it is clear that the inhibitor(s) in the extract is weakly adsorbed by Sephadex G-25. It was reported that a tightly cross-linked dextran gel such as Sephadex G-25 has affinity for aromatic and pseudo-aromatic substances,[5] and Sephadex gel is routinely used for separation of ribo- and deoxyribonucleosides utilizing this feature.[6] Since the absorption spectrum of the active fraction from the gel chromatography showed a maximum at *ca.* 260 nm, the inhibitor might be a nucleoside-related compound.

Further purification was performed by Bio-Gel P-2 (polyacrylamide gel) chromatography, because nucleic acid components and related compounds may be separated by absorption chromatography on polyacrylamide gel.[7] The active fractions from Sephadex G-25 chromatography were concentrated by freeze-drying and the product was applied to a column of Bio-Gel P-2 (1.7 × 26 cm) equilibrated with distilled water. Each fraction of the eluate was checked for absorption at 260 nm (Fig. 3). The extents of inhibition of platelet

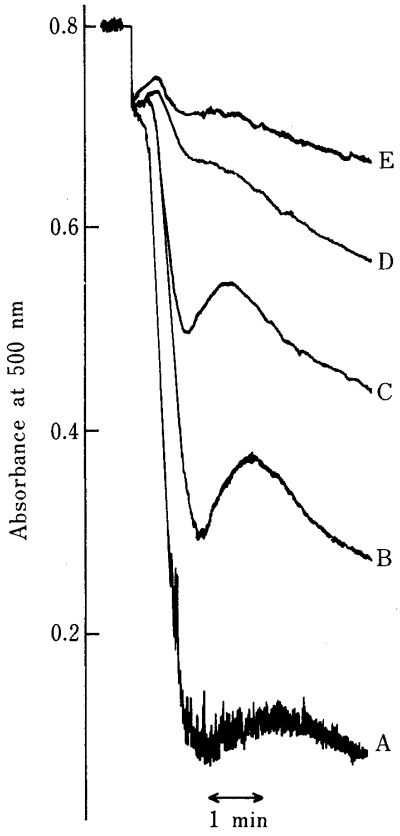

Fig. 1. Inhibitory Effect of *G. lucidum* Extract on Platelet Aggregation

A 0.45 ml aliquot of washed platelets in Tris–ACD (8×10^8/ml) was incubated with 50 μl of the water-soluble fraction of *G. lucidum* at 37 °C for 1 (A), 2 (B), 3 (C), 4 (D) or 6 min (E), then 50 μl of $CaCl_2$ (200 mM) and 50 μl of thrombin (2.5 units/ml) were added, and the aggregation was measured.

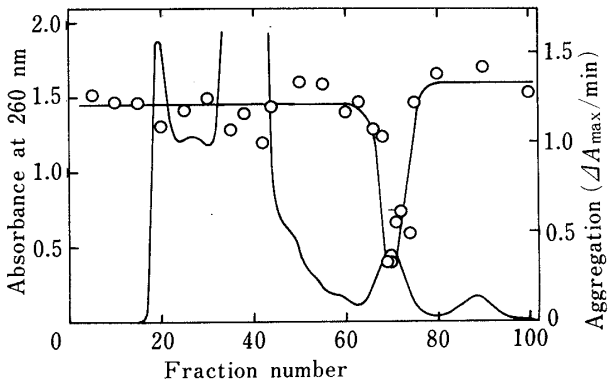

Fig. 2. Gel Filtration Chromatography of *G. lucidum* on a Sephadex G-25 Column

The water-soluble fraction of *G. lucidum* (2 ml) was applied to the column (1.3 × 47.5 cm). Elution was performed at 4 °C with distilled water at a flow rate of 7 ml/h, and fractions of 1.5 ml each were collected. The UV absorption was measured at 260 nm (——). The inhibition of platelet aggregation was determined as follows: 100 μl of a fraction was added to 0.40 ml of washed platelets in Tris–ACD (8×10^8/ml), and the mixture was incubated at 37 °C for 10 min, then 50 μl of $CaCl_2$ (200 mM) and 50 μl of thrombin (3 units/ml) were added. The aggregation was determined as the maximum decrease of turbidity at 500 nm per min (—○—).

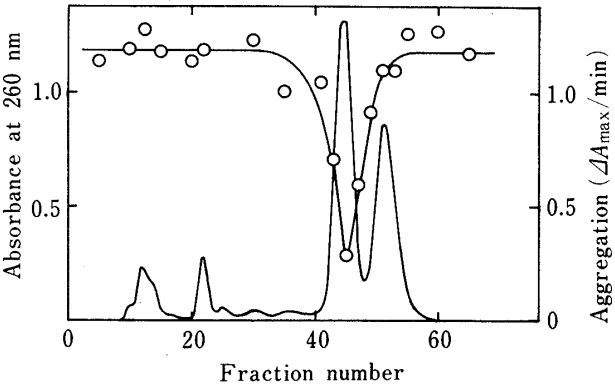

Fig. 3. Bio-Gel P-2 Gel Filtration Chromatography of the Active Fraction from Sephadex G-25 Chromatography

Concentrated active fraction from the experiment shown in Fig. 2 (fractions 65 through 74) was applied to a column of Bio-Gel P-2 (1.7 × 26 cm). Elution was performed at 4 °C with distilled water, at a flow rate of 5 ml/h and fractions of 2.2 ml each were collected. The inhibition of platelet aggregation was measured by the same method as described in Fig. 2.

aggregation are also shown in Fig. 3. The chromatography yielded two major peaks and several minor ones. The inhibitory activity was associated with only one peak, from fractions 42 through 47.

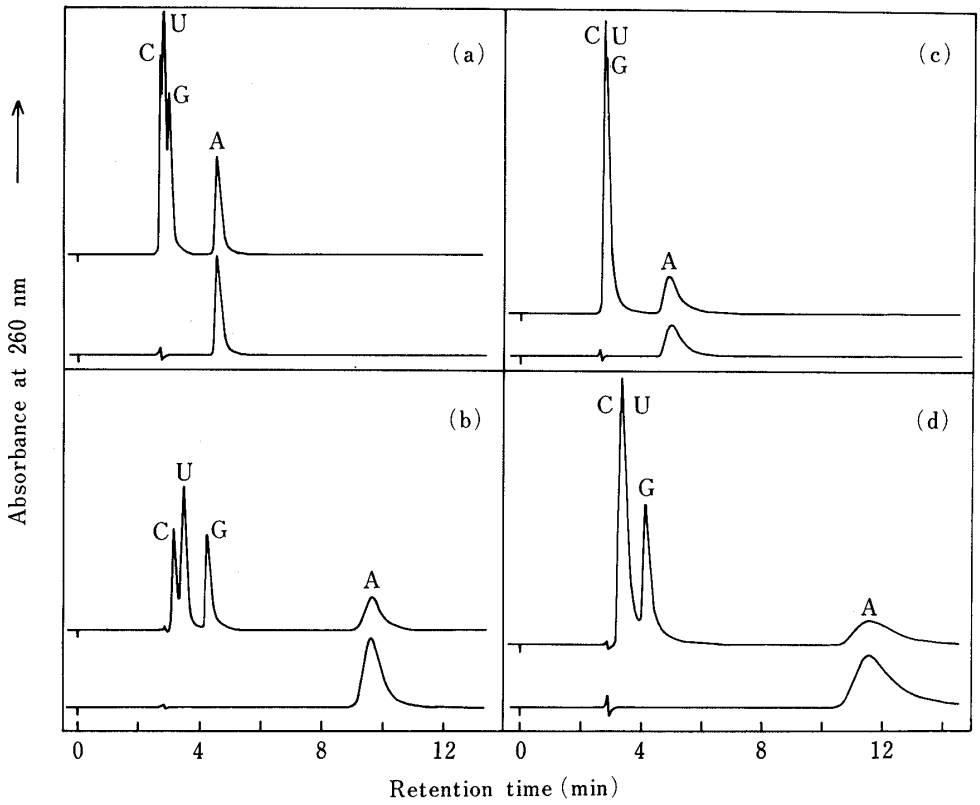

Fig. 4. Reversed-Phase HPLC of the Active Fraction from Bio-Gel P-2 Chromatography

Column: Zorbax-ODS (0.46 × 25 cm). Flow rate: 1.0 ml/min. Solvent: 10 mM potassium phosphate, pH 5.1, containing (a) 20% methanol or (b) 15% methanol, or 10 mM potassium phosphate, pH 8.5, containing (c) 20% methanol or (d) 15% methanol. The upper line represents the elution profile of standards (A, adenosine; C, cytidine; G, guanosine; U, uridine) and the lower one represents that of the active fraction (fractions 42—47) from Bio-Gel P-2 gel filtration in each figure.

We confirmed the purity of this fraction by high performance liquid chromatography (HPLC) as shown in Fig. 4. Only a single peak was detected with several different solvent systems. Furthermore, the retention time of this compound always coincided with that of adenosine. Other nucleosides, nucleotides and bases were separated from adenosine under these conditions. We also measured the nuclear magnetic resonance (NMR) spectrum of this compound. The spectrum coincided completely with that of adenosine (data not shown).

Several investigators have reported that adenosine inhibits platelet and thrombocyte aggregation.[8-11] Preincubation of platelets with adenosine is apparently required for the inhibition to be observed.[8] Adenosine seems to have a stronger effect on platelet aggregation induced by thrombin than on that induced by adenosine diphosphate (ADP). Adenosine clearly inhibits thrombin-induced platelet aggregation at concentrations of the order of μM.[10] These characteristics are in good agreement with what we have observed with the inhibitor obtained from *G. lucidum*. Adenosine was considered to act competitively with ADP,[12] but it has also been suggested that it inhibits the esterolytic activity of thrombin[13] or that it interferes with the energy flow from adenosine triphosphate[14] through its own phosphorylation. The mechanism of this inhibition has yet to be elucidated in detail.

We have thus demonstrated that adenosine is an inhibitor of platelet aggregation found in *G. lucidum*. The content of adenosine in this fungus was at least 40 mg/100 g of dried preparation.

Experimental

G. lucidum was a kind gift from Dr. Ikekawa of the National Cancer Center Hospital, Japan. Bovine thrombin and bovine serum albumin (BSA) (fraction V) were purchased from Mochida Pharmaceutical Co. and Sigma Chemical Co., respectively. All other reagents were of analytical grade.

A water-soluble fraction of *G. lucidum* was prepared as follows; 5 g of the fungus cut into small pieces was added to 300 ml of distilled water, and the mixture was heated at 70—80 °C for 8 h under constant stirring, then filtered through layers of gauze. The filtrate was freeze-dried and redissolved in 30 ml of distilled water.

Washed platelets were prepared from bovine blood by the method previously described,[15] and finally suspended in Tris–acid-citrate–dextrose (Tris–ACD) buffer containing 1.0% BSA to give a density of 8×10^9/ml. Aggregation of platelets was measured turbidimetrically with a Shimadzu MPS-5000 recording spectrophotometer after the addition of 50 μl of 200 mM $CaCl_2$ and 50 μl of thrombin (2—3 units/ml in Tris–ACD) to 0.5 ml of washed platelets (density, $7—8 \times 10^8$/ml) in Tris–ACD.

HPLC was conducted with a Shimadzu LC-3A using a Zorbax-ODS column (0.46×25 cm). The solvent was 10 mM potassium phosphate, pH 5.1 or 8.5, containing 15 or 20% methanol, and the column temperature and flow rate were 50 °C and 1.0 ml/min, respectively. The NMR spectrum was measured with a JEOL GX 400 spectrometer operating at 400 MHz.

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