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Platelet aggregation is inhibited by phycolectins

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Abstract. Lectins from four marine algal species were examined for interaction with human platelets. The lectin designated hypnin A, from the red alga *Hypnea japonica*, inhibited adenosine diphosphate (ADP)- or collagen-induced human platelet aggregation in a dose-dependent manner. Complete inhibition was observed at concentrations of 100 and 5 μg/ml of the lectin with ADP (2 μM) and collagen (0.2 μg/ml)-induced platelet aggregation, respectively. At the inhibitory concentration of 0.5 to 100 μg/ml, the lectin did not induce aggregation of resting platelets. Lectins from the other three algal species also inhibited ADP-induced human platelet aggregation. These results indicate that the algal lectins are a new group of inhibitors and may be useful to study glycoconjugates on platelet membranes and to design novel platelet aggregation inhibitors.
Key words. Lectin; hemagglutinin; platelet; aggregation; inhibition.

Lectins are glycoproteins or proteins which bind to specific carbohydrate structures on cell surfaces, and some lectins induce interesting phenomena such as mitogenic stimulation of lymphocytes or inhibition of growth of tumor cells[1]. Moreover, several lectins have been shown to interact with human platelets. Phytohemagglutinin (PHA)[2], wheat germ agglutinin (WGA)[3] and snake venom lectins[4] induced platelet aggregation by binding to the platelet membrane, whereas lentil lectin[5] bound to platelets but did not induce aggregation. Tsunehisa et al. reported that lentil lectin and *Pisum sativum* lectin inhibited collagen-induced platelet aggregation but did not affect ADP-induced platelet aggregation[6].

We have characterized several lectins from marine algae. Algal lectins are commonly low molecular weight monomeric proteins or glycoproteins, with affinity for glycoproteins bearing *N*-glycans but not for monosaccharides, and do not require divalent cations for activity[7–9], although there are a few exceptions[8–10]. A typical lectin, designated hypnin A, from the red alga *Hypnea japonica*, has been characterized as a very low molecular weight monomeric peptide with binding specificity for complex *N*-glycans of glycoproteins[11]. It has also been shown that some algal lectins have mitogenic or antineoplastic activity[12, 13]. However, the interaction of algal lectins with platelets has not yet been investigated.

In this study, we examined the interaction of four algal lectins, including the *H. japonica* lectin, with human platelets. These lectins inhibited platelet aggregation induced by adenosine diphosphate (ADP), while one of them induced the aggregation of resting platelets.

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Materials and methods

Materials. The lectin designated hypnin A was purified from the red alga *H. japonica* by slightly modifying the method described previously[11]. Lectins from the green alga *Boodlea coacta*[14], and the red algae *Solieria robusta*[12] and *Carpopellis flabellata*[13], were purified according to previous papers. All lectin specimens purified here gave only a single band in SDS-polyacrylamide gel electrophoresis (data not shown), although lectin specimens from *H. japonica* and *B. coacta* included, respectively, three (hypnin A-1–3) and four isolectins (boonins A ~ D). ADP and collagen were purchased from Sigma Chemical and Hormon Chemie, respectively.

Preparation of human platelets. Human blood from healthy volunteers was collected, adding nine volumes of blood to one volume of 3% sodium citrate anticoagulant. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared from fresh citrate-anticoagulated blood by differential centrifugation according to the manual for the aggregometer used (Nikkou Bioscience Aggregometer, HEMA TRACER 601, Tokyo, Japan). The platelet count was adjusted to $3–7 \times 10^8$/ml with PPP.

Aggregation studies. Platelet aggregation was carried out in cuvettes using the platelet aggregation tracer (Nikkou Bioscience Aggregometer, HEMA TRACER 601) at 37 °C with stirring at 1000 rpm. Twenty microliters of lectin solution at various concentrations were added to 200 μl of PRP which had been pre-incubated for 1 min at 37 °C. After incubation for 2 min, 24 μl of ADP (20 μM) or collagen (2 μg/ml) was added to the mixture to give a final concentration of about 2 μM or

0.2 μg/ml. Aggregation was recorded as the increase in light transmission through the mixture. To examine whether the lectins themselves induce the aggregation of human platelets, 20 μl of each lectin solution was added to 200 μl of PRP at 37 °C for 10 min with stirring.

Hemagglutination assay. Hemagglutinating activity of lectins was determined using 2% of trypsin-treated rabbit erythrocyte suspension as described previously[11].

Protein concentration. Protein concentrations were determined by using a Micro BCA protein assay reagent Kit (Pierce), using bovine serum albumin as a standard.

Results and discussion

The hemagglutinating activities and sugar-binding properties of algal lectins used in this study are summarized in table 1. The minimum concentrations of the algal lectins required to agglutinate trypsin-treated rabbit erythrocytes were at or below nanograms per milliliter, indicating the very strong hemagglutinating activities of these lectins. Based on binding to glycoprotein, these lectins have been grouped in three types: complex, high mannose, or both *N*-glycan-specific lectins[7] (table 1). The algal lectins had no binding affinity for monosaccharides.

We screened the effects of marine algal lectins on human platelets. The results are shown in table 1. *Boodlea coacta* lectin, the only one with sugar-binding specificity restricted to high mannose *N*-glycan, induced platelet aggregation. All algal lectins examined inhibited ADP-induced platelet aggregation. The *B. coacta* lectin acted like WGA, which not only induced but also inhibited aggregation[6]. Hypnin A had strong inhibitory effects on ADP-induced platelet aggregation. Furthermore, we investigated the inhibitory effects of hypnin A on platelet aggregation induced by ADP or collagen. Hypnin A inhibited human platelet aggregation induced by ADP or collagen in a dose-dependent manner, as shown in figures 1 and 2. Complete inhibition was observed at concentrations of 100 and 5 μg/ml of the lectin with ADP- and collagen-induced aggregation, respectively. Hypnin A also inhibited ADP- and collagen-induced platelet aggregation with IC_{50} of 10 μg/ml and 1.0 μg/ml, respectively (fig. 2). Thus, the concentration of the lectin necessary to inhibit collagen-induced aggregation was lower than that requred to inhibit the ADP-induced aggregation. At the inhibitory concentration of 0.5 to 100 μg/ml the lectin did not aggregate resting platelets, although it agglutinated trypsin-treated rabbit erythrocytes with the minimum hemagglutination concentration of 10 ng/ml.

The interaction of some plant and animal lectins with human platelets has been examined as described in the introduction. However, there are a few lectins which inhibit platelet aggregation, indicating that the inhibitory marine algal lectins may be useful for investi-

Table 1. Inhibition of platelet aggregation by algal lectins, and hemagglutinating activities and sugar-binding specificities of their lectins.

Algal lectin	Minimum hemagglutination concentration (ng/ml)[a]	Sugar-binding specificity	Concentration (μg/ml)	Platelet aggregation[b]	Inhibition of ADP-induced platelet aggregation[c]
Hypnea japonica[11] (a mixture of hypnin A-1–3)	10	Complex *N*-glycans	10	–	++
Boodlea coacta[14] (a mixture of boonins A–D)	95	High mannose *N*-glycans	8	+	+
Solieria robusta[12] (Solnin B)	0.3	Complex and high mannose *N*-glycans	24	–	+
Carpopellis flabellata[13] (Carnin)	5.8×10^{-5}	Complex and high mannose *N*-glycans	4	–	+

[a]The minimum concentration of lectin solution required to agglutinate trypsin-treated rabbit erythrocytes.
[b]+, aggregation with more than 10% maximum right transmission; –, aggregation with less than 10% maximum right transmission.
[c]++, more than 80% inhibition; +, 20–80% inhibition; –, less than 20% inhibition.

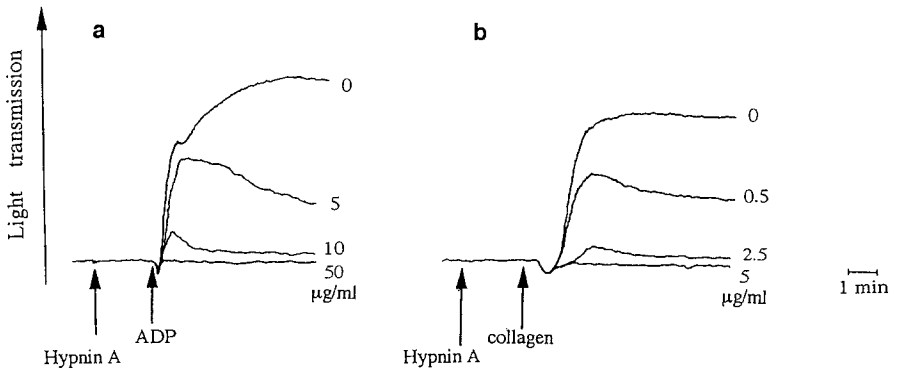

Figure 1. Inhibition of platelet aggregation by the *H. japonica* lectin hypnin A. The analysis was performed as described in Materials and methods. The final concentration of ADP (*a*) and collagen (*b*) was 2 μM and 0.2 μg/ml, respectively.

gating the surface of platelets. On the other hand, it is known that proteins or peptides containing the tripeptide sequence Arg-Gly-Asp (RGD), which is a recognition sequence in adhesive proteins, can inhibit platelet aggregation by preventing the binding of fibrinogen to the platelet-receptor glycoprotein GPIIb/IIIa[15–17]. Binding of fibrinogen to GPIIb/IIIa is critical for platelet aggregation[18–20]. These inhibitors include proteins from natural sources such as snake venom[21–25] and leeches[26,27]. There are also reports that a protein, designated disagregin, from the tick[28], and the 29 kDa fragment of fibronectin[29] have the ability to inhibit ADP-induced human platelet aggregation in spite of having neither the RGD sequence nor an amino acid sequence homologous to the inhibitory proteins from snake venom. The inhibitory algal lectin hypnin A included three isolectins, designated hypnin A-1, -2 and -3, which can be separated from each other by reversed-phase high-performance liquid chromatography (HPLC) and have almost the same hemagglutinating activity, sugar-binding properties, molecular mass and sequence of the first 18 N-terminal amino acid residues (unpubl. obs.), indicating that the three isolectins have closely similar chemical structures. The primary structures, except for hypnin A-3, have recently been determined to be a single polypeptide composed of 90 amino acid residues (unpubl. obs.). Hypnin A-1 and -2 do not contain either the RGD sequence or any amino acid sequences similar to the inhibitory proteins so far reported. The structural difference of hypnin A-1 and -2, compared with the known inhibitors of platelet aggregation, require that the mechanism by which the lectin inhibits platelet aggregation be clarified. It is interesting that hypnin A-1 and -2 contained a tripeptide sequence of Asn-Gly-Asp (NGD) which is analogous to the RGD sequence. Another analogous tripeptide sequence, Lys-Gly-Asp (KGD), has also been detected in an inhibitory protein, barourin, from snake venom[30]. These findings suggest that the arginine residue of the RGD sequence could be replaced by another amino acid for inhibition of the platelet aggregation.

Hypnin A did not affect the change in platelet shape when ADP or collagen was added (fig. 1). Thus, hypnin A might block the binding of fibrinogen to its receptor and inhibit platelet aggregation. However, it remains unclear whether the NGD sequence can be recognized by GPIIb/IIIa, or hypnin A can bind to the carbohydrate structures of GPIIb/IIIa and inhibit the binding of fibrinogen. It will be of interest to identify the binding site of hypnin A and of the receptor on platelet membranes. Thereafter, the inhibitory algal lectins demonstrated in this study could be used to investigate glycoconjugates on platelet membranes and to design novel inhibitors.

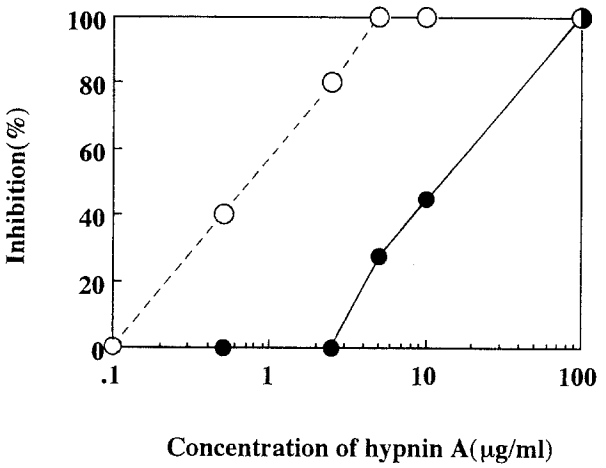

Figure 2. Dose–response curves of platelet aggregation inhibition by hypnin A. Platelet aggregation was induced by ADP (●—●) and collagen (○—○). The results are the average of the two separate experiments.

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