[Chem. Pharm. Bull. 33(12)5568–5571(1985)]

A New Platelet Aggregation Factor from *Gynostemma pentaphyllum* MAKINO

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(Received May 8, 1985)

The hot water extract of *Gynostemma pentaphyllum* MAKINO (Japanese name: Amachyazuru) contained platelet aggregation factor which differs from other agonists that are known to activate platelets. The aggregation was completely inhibited by prostaglandin I_2, but was not affected by aspirin. This heat-stable factor was inactivated by treatment with β-glucosidase, but not with proteases, which suggests that it is a kind of glycoside or polysaccharide.

Keywords——*Gynostemma pentaphyllum*; platelet; aggregation; glycoside; agonist

Recently, it has been elucidated that *Gynostemma pentaphyllum* MAKINO (Japanese name: Amachyazuru; abbreviated as G.p. throughout this paper) contains saponins which are pharmaceutically valuable.[1] Since the plant is distributed over large areas in Japan and is easily found, G.p. is of interest as a potential crude drug. In this paper, the effect of G.p. extract on bovine platelets was investigated, and it was found that the hot water extract from dried leaves aggregated platelets.

Figure 1 (a) shows the platelet aggregation pattern obtained with G.p. extract. The pattern resembles that induced by adenosine diphosphate (ADP) (b), in which the aggregation is accompanied with disaggregation when the concentration is low.

The aggregation factor in the extract required calcium ion for its activity, and the dependency upon calcium concentration was quite similar to that of other agonists (data not shown).

Actin polymerization and shape change, which are known to occur prior to aggregation,[2] were measured and were found to occur in the same dose-dependent manner (Fig. 2).

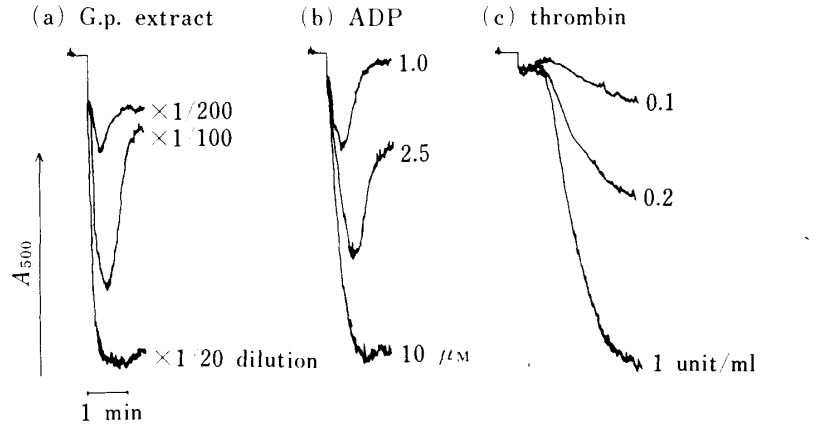

Fig. 1. Aggregation Patterns with Various Stimulants

Fifty μl of $CaCl_2$ (100 mM) and 50 μl of thrombin (c) or the G.p. extract (a) were added to 0.5 ml of washed platelets in Tris–ACD (8×10^8 cells/ml). In the case of ADP (b), 50 μl of $CaCl_2$ (100 mM) and ADP were added to 0.5 ml of platelet-rich plasma (5×10^8 cells/ml).

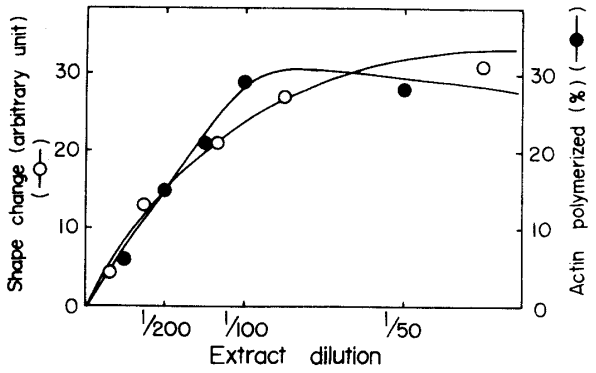

Fig. 2. Shape Change and Actin Polymerization

Twenty-five μl of the G.p. extract was added to 2 ml of washed platelets in Tris–ACD (5×10^8 cells/ml) to give the final dilutions indicated on the abscissa, and the decrease of light scattering (arbitrary unit) was measured as an index of platelet shape change. Actin polymerization was measured by the method previously described.[2]

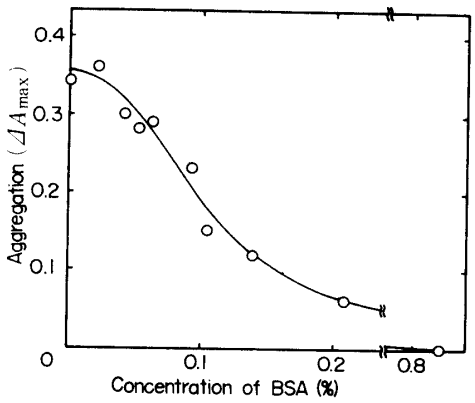

Fig. 3. Inactivation by BSA

BSA (in a volume of 50 μl) was added to 0.5 ml of washed platelets and incubated for 1 min before addition of the extract and $CaCl_2$.

Scanning electron microscopy revealed that the shape of platelets changed from discoid to spheroidal form with some pseudopods, as is usually observed with other agonists (data not shown). These results indicate that this factor brings about genuine platelet aggregation through activation, not mere clumping or precipitation of platelets.

However, the aggregation induced by this factor occurred only in washed platelet suspension, and could not be observed in platelet-rich plasma. To find the reason for this phenomenon, bovine serum albumin (BSA) was added to washed platelet suspension. The aggregation was quickly inhibited in a dose-dependent manner (Fig. 3). The binding of the factor to BSA was confirmed by the use of a BSA–Sepharose column (data not shown). The aggregation factor in the extract was proved to be trapped by BSA, and therefore we concluded that it was inactivated in plasma, in which the BSA content is 3.5—5.5%.[3] This inactivation of the factor is in contrast to the behavior of known agonists, which do aggregate platelets in plasma.

Table I shows the effects of various aggregation inhibitors. Adenosine triphosphate (ATP), hirudin and imipramine are specific inhibitors of ADP-, thrombin- and 5-hydroxytryptamine(5-HT)-induced aggregations, respectively. The aggregation induced by this factor was unaffected by any of these inhibitors, and it was confirmed that this factor is different from ADP, thrombin, or 5-HT, and that its aggregating activity is not due to the secretion or activation of such agonists. Aspirin also had no effect on the activity of this factor, which indicates that activation by the factor does not require thromboxane synthesis.[4] This activation seems to depend on intracellular calcium mobilization, since prostaglandin I_2(PGI_2), which is known to increase the level of cyclic-adenosine monophosphate (c-AMP) in platelets and may prevent calcium mobilization,[5] completely inhibited the aggregation induced by the factor. On the other hand, a lectin (wheat germ agglutinin)-induced platelet aggregation[6] was not affected by PGI_2 (data not shown). This result suggests that this factor is not a lectin.

We tried to establish some of the characteristics of this factor. This aggregation factor was very stable to heat, and was not inactivated by treatment at 80 °C for 1 h over a wide pH range (pH 2—8). In a preliminary experiment using paper chromatography, the active fraction in the extract was shown to contain reducing sugars but not proteins. Thus, the

Table I. Effects of Various Aggregation Inhibitors

Inhibitor	Agonist			
	ADP	Thrombin	5-HT	G.p. extract
ATP	+	−	−	−
Hirudin	−	+	−	−
Imipramine	−	−	+	−
Aspirin	−	−	−	−
PGI_2	+	+	+	+

The symbols + and − indicate the presence and absence of inhibition, respectively.

Table II. Effects of Protease and Glycosidase Treatments on Aggregating Activity

Enzyme	Remaining activity (%)
Trypsin	98
Subtilisin BPN′	105
α-Amylase	89
β-Galactosidase	96
β-Glucosidase	0

extract was treated with some proteases and glycosidases to investigate whether these enzymes could decompose the factor (Table II). Subtilisin BPN′, which has very low specificity,[7] did not inactivate the factor, and only β-glucosidase could inactivate the factor in a time-dependent manner. It seems that this factor is not a peptide or protein, and it may be a kind of glycoside or polysaccharide. However, it is not certain that the factor requires β-glucoside bonds for activity, because the β-glucosidase used here was not highly purified and might contain other glycosidase activity.

Since this factor was caught by BSA, it is unlikely to cause thrombosis *in vivo* if G.p. extract is ingested. However, this factor may affect cells or tissues other than platelets, because other agonists, such as 5-HT, ADP, thrombin, or collagen, all have various important biological functions *in vivo*. Further research into the medicinal utilization of G.p. seems desirable. We might also use this aggregation factor as a new probe for investigation of platelet activation. The mechanism of platelet activation is still unclear in detail, and many investigators have been exploring the problem using various agonists. Calcium ionophore and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), which do not exist in mammalian tissues, are frequently used for such studies.[8] This aggregation factor in G.p. might also be useful as a probe, if its chemical properties and biological functions are defined. We are currently working on the isolation and detailed characterization of this factor.

Experimental

Hirudin, imipramine, BSA (fraction V), β-galactosidase (*E. coli*), α-amylase (*Bacillus* species) and trypsin (porcine pancreas) were purchased from Sigma Chemical Co., and 5-HT, thrombin (bovine) and β-glucosidase (almond emulsin) were obtained from Tokyo Kasei Co., Mochida Pharmaceutical Co. and Calbiochem-Behring Corp., respectively. PGI_2 and subtilisin BPN′ were kind gifts from Toray Industries Inc. and Nagase Biochemicals Ltd., respectively.

Gynostemma pentaphyllum Makino were collected in the northern part of Kanagawa prefecture and dried under the sun. The leaves were extracted twice with distilled water at 80 °C for 30 min. The extract was concentrated by lyophilization and applied to a Sephadex G-50 column equilibrated with distilled water. The fraction which had platelet aggregating activity was collected, and used for experiments as the G.p. extract.

Washed platelets were prepared from bovine blood by the method previously described,[9] and were suspended in Tris–acid citrate dextrose (ACD) buffer (pH 7.35). Platelet aggregation was measured turbidimetrically with a Shimadzu MPS-5000 recording spectrophotometer. The maximum decrease of absorbance at 500 nm (ΔA_{max}) was utilized as an index of aggregation.

The effects of various aggregation inhibitors were investigated by the following method. Washed platelets were incubated with ATP (0.1 mM), hirudin (5 U/ml), imipramine (0.5 mM) or PGI_2 (0.1 mM) for 30 s before addition of 50 μl of $CaCl_2$ and the agonist. Aspirin was dissolved in ethanol and diluted with Tris–ACD, then added to washed platelet suspension to give a final concentration of 1 mM. The final ethanol concentration was lower than 1%, which did not affect platelet aggregation. After incubation for 5 min, the agonist and $CaCl_2$ were added.

Actin polymerization was measured by deoxyribonuclease (DNase) I inhibition assay, and platelet shape change

was observed by light scattering measurement and scanning electron microscopy as described previously.[2,10]

Protease and β-galactosidase treatments were performed in 50 mM Tris–HCl buffer (pH 7.5), while in other glycosidase treatment, 0.1 M acetate buffer (pH 5.0) was used (180 μl of buffer was added to 0.3 ml of G.p. extract). After preincubation for 5 min, 20 μl of enzyme solution (10 mg/ml) was added. Treatment was continued for 24 h at 25 °C, then the cuvettes were heated in boiling water to stop the reaction and the pH was readjusted to 7.35. Inactivation of enzymes was checked in the control sample. Aggregation was measured by the method described in the legend to Fig. 1.

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