The effects on blood coagulation of dipicolinic acid (DPA, 2,6-pyridinedicarboxylic acid), an antibacterial substance known to be produced by Bacillus subtilis natto and contained in natto, a traditional Japanese fermented soybean food, were studied. It was found that addition of DPA with a final concentration of $5 \times 10^{-4}$ M results in substantial inhibition of platelet aggregation. DPA inhibition was found to be far stronger than that resulting from addition of aspirin. Furthermore, the clotting reaction of thrombin-fibrinogen was also found to be inhibited by DPA. It was confirmed by examination of thromboelastogram patterns that the coagulation of whole rat blood was completely inhibited by addition of $5 \times 10^{-4}$ M DPA. From the point of view of the blood coagulation system, these results show that DPA contained in natto may be effective in the prevention of thrombosis.

Keywords: Dipicolinic acid, Blood coagulation, Fibrinolysis, Platelet aggregation, Natto

Materials and methods

**Materials** Dipicolinic acid (DPA, 2,6-pyridinedicarboxylic acid), purchased from Nacalai Tesque Inc., and aspirin, purchased from Sigma Co., Ltd., were each dissolved in water and neutralized with NaOH before use. Bovine fibrinogen and adenosine diphosphate (ADP) were purchased from Sigma Co., Ltd., and bovine thrombin was purchased from Mochida Pharmaceutical Co., Ltd. Synthetic peptide substrate H-D-Phe-Pip-Arg-pNA (S-2238) was purchased from Chromogenix.

**Platelet aggregation** Blood was drawn from a Wistar male rat (weight: about 340 g) and a New Zealand White male rabbit (weight: about 3 kg), both etherized, using citrate at 1:10 by volume. Fractionation of platelet-rich plasma (PRP) was carried out by centrifugal separation performed at 100 × g for ten minutes, and fractionation of platelet-poor plasma (PPP) was carried out by centrifugal separation of the remainder at 2000 × g for ten minutes. Fifty microliters of PRP, 100 μl of Tyrode’s buffer solution and 50 μl of DPA solution were added to the cuvette of an aggregometer (PAT-4A: Mebanix). After pre-incubation at 37°C for five minutes, 22 μl of ADP solution (final concentration: 30 μM) was added as an aggregation trigger substance, and changes in turbidity over five minutes were measured by the aggregometer.

**Clotting time** Using 0.17 M borate-saline buffer (BSB), 0.5 ml of fibrinogen solution, prepared at 0.8%, and 0.5 ml DPA solution were placed in a test tube ($\phi 13 \times 100$ mm), and after pre-incubation at 37°C for ten minutes, 0.1 ml of 20 U/ml thrombin solution was added and the elapsed
time until complete formation of fibrin was measured at 37°C.

Amidolytic activity Ten microliters of 10 U/ml thrombin solution, 30 μl of PBS and 50 μl of DPA solution were mixed on a 96-well micro test plate, and after pre-incubation at 37°C for ten minutes, 10 μl of 5 × 10^{-3} M synthetic peptide substrate solution (S-2238) was added and changes in absorbance at 405 nm were measured using a Bio-Kinetics Reader (EL+80, Bio-Tec Instruments Inc.).

Thromboelastography Whole rat blood (0.3 ml) and DPA solution (0.01 ml) were added to a cell, and coagulation and fibrinolysis patterns were monitored for two hours at 37°C using a Clot-Tracer (TE-700: Erma Inc.) (Otsuka et al., 1980).

Results and Discussion Platelet aggregation patterns were observed for rat blood with ADP as the aggregation trigger substance, and it was found that the addition of DPA caused a reduction in aggregation patterns. Figure 1 shows the aggregation inhibition rate of DPA at various concentration levels; the rate shows a dose-dependent rise at DPA concentrations of 1.0-5.0 × 10^{-3} M. The calculated IC_{50} was a low concentration of 1.68 × 10^{-3} M, which shows that aggregation inhibition was stronger than that observed for aspirin (IC_{50}: 4.0 × 10^{-2} M). Although not shown in the figure, the results were almost identical in the case of rabbit blood platelets (IC_{50}: 2.2 × 10^{-3} M).

Figure 2 shows the results of the tests studying the effects of DPA on the coagulation reaction in which fibrinogen, a water-soluble protein, is converted by thrombin into insoluble fibrin. Whereas the clotting time was three minutes when one unit of thrombin was used as a control, this was extended by three times by addition of DPA at 1.37 × 10^{-2} M. Furthermore, the clotting time was extended by 8.2 times by addition of DPA at 2.75 × 10^{-2} M, and 16.4 times by addition of DPA at 5.5 × 10^{-2} M, which shows that DPA is effective for inhibition of fibrin formation. As shown in Figure 2, the inhibition of the coagulation reaction by DPA was stronger than that of aspirin. (The clotting time measured for aspirin solution at a concentration of 1.0 × 10^{-2} M was five minutes.)

However, a study of the effects using H-D-Phe-Pip-Arg-pNA (S-2238), a specific substrate of thrombin, revealed that, as shown in Fig. 3, no inhibition at or exceeding 50% was observed for one unit of thrombin with addition of DPA at 1.25 × 10^{-3} M-2.0 × 10^{-2} M. In order to examine the effects of DPA on the fibrinolysis system, DPA was added to urokinase (UK), but no inhibition was observed.

Anti-platelet Aggregation and Anti-blood Coagulation Activities of DPA

Fig. 1. Anti-platelet aggregation activity of DPA.
Analysis was performed as described in the Materials and Methods section. The final concentration of ADP in the reaction mixture was 30 μM.

Fig. 2. Effect of DPA on fibrin clot formation.
Clotting times were recorded when fibrin formation was completed.

Fig. 3. Effect of DPA on thrombin-amidolytic activity. A H-D-Phe-Pip-Arg-pNA (S-2238) concentration of 5 × 10^{-3} M was used for the substrate.

Fig. 4. Thromboelastography patterns in rat plasma after addition of DPA.
A: control; B: 2.5 mM DPA; C: 5.0 mM DPA.
A study of the effects of DPA on whole rat blood was conducted based on thromboelastogram patterns (Fig. 4). With no DPA added, the reaction time (r value) was 9.1–10.1 min, the clot formation time (k value) was 2.6–4.2 min, and the maximum amplitude (Ma value) was 57.8–60.3 mm (n = 3); a reduction in each value was observed as a result of the addition of DPA at \(2.0 \times 10^{-3} \) M, with the r value at 11.6–14.3 min, the k value at 3.5–7.4 min, and the Ma value at 46.0–54.8 mm. It was found that further raising the concentration of DPA to \(5.0 \times 10^{-3} \) M, resulted in an r value of 15.5 min or more, a k value of 0 min and an Ma value of 0–12.5 mm, which indicates strong blood coagulation inhibition.

It was reported as a result of preliminary tests that, although it was previously thought that a substantial amount of DPA exists among Bacillus spores, recent logical point of view, these amounts cannot be ignored. It was found that DPA is very active in anti-platelet aggregation inhibition activity is derived from DPA itself.

From a calculation based on overall consumption of natto clarified that, in the current series of tests (Figs. 1–4), it was established for the first time that platelet aggregation inhibition activity is derived from DPA itself. It was found that DPA is very active in anti-platelet aggregation; its inhibition reaction is stronger than that of aspirin under the same conditions. It is estimated from a calculation based on overall consumption of natto that the average intake of DPA by Japanese people is around 0.4–4.0 mg/day (Nagayama, 1990); from a physiological point of view, these amounts cannot be ignored. Although it was previously thought that a substantial amount of DPA exists among Bacillus spores, recent analyses of the fermented foodstuff natto clarified that the DPA content is in fact quite substantial, and that some DPA is also present outside the spores in the water-soluble fractions (Sumi and Ohsugi, 1990; Sumi et al., 2000b).

Because the platelet aggregation reaction acts as a trigger for blood coagulation, it is believed that inhibiting subsequent platelet aggregation is of considerable value. In view of the effects of DPA on the blood coagulation system, it is hoped that active ingestion of DPA may prove helpful in the prevention and treatment of thrombosis.

References