Existence of Thermally Stable Thrombin Inhibitors in Soybean Paste, *Doenjang*

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Received November 22, 1998

Antithrombotic function of *doenjang* was investigated using thoroughly desalted aqueous extract. In the presence of the extract, thrombin suffered loss of its fibrin-clotting activity, whose extent increased in a dose-dependent manner. The active substance involved in thrombin inhibition showed a high thermal stability. Results of Sephadex G-25 permeation chromatography indicated that there were at least two soluble thrombin inhibitors in *doenjang* with different molecular sizes.

Key words : Doenjang *extract, thrombin inhibitors.*

Thrombosis, a pathological phenomenon resulting from an excessive formation of thrombus in vessels and the heart, is closely related to the biochemical function of thrombin that acts as an enzyme to convert fibrinogen into fibrin threads that enmesh platelets, blood cells and plasma to form clots.[1,2] The inhibition of thrombin activity by certain compounds, either synthetic or natural, would therefore provide an effective means of thrombosis control. Naturally, much effort has long been made to find potent thrombin inhibitors aiming at developing antithrombotic drugs. Heparin, widely used as anticoagulant of blood, is probably the best known among thrombin inhibitors.[3] Another natural inhibitor of thrombin is hirudin, a single chain polypeptide isolated from the salivary gland of *Hirudo medicinalis*.[4] Several small synthetic compounds also appear to inhibit thrombin.[5,6]

It has been implicated that typical soybean-fermented foods in the oriental diet, such as *natto* in Japan and *doenjang* and *chungkook-jang* in Korea, contain certain thrombolytic and/or antithrombotic agents. Potent fibrinolytic enzymes were isolated from *natto*[7] as well as from *Bacillus* sp. strain CK 11-4 screened from *chungkook-jang*,[8] and possible use of these enzymes for oral fibrinolytic therapy was speculated. The peptide fractions of *doenjang* were reported to suppress ADP-induced platelet aggregation.[9] In the present communication, we report that in *doenjang* there exist at least two bioactive compounds with different molecular sizes that act as inhibitors of thrombin and have a very high thermal stability.

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Materials and Methods

Materials. *Deonjang* (fermented soybean paste) was purchased from local market. Human thrombin and bovine fibrinogen were obtained from Sigma Chemical Co (St. Louis, MO).

Preparation of *Deonjang* extract. *Doenjang* (1.5 kg) was suspended in distilled water (1.7 *l*), stirred for 4 h at room temperature, and then filtrated through three layers of cheese cloth. After centrifugation at 10,000 g for 20 min, the supernatant was further filtrated using Whatman No. 42 filter paper and desalted for 2.5 h using an electrodialyzer (model; TS3B-2-5, Tokuyama Corp., Japan). The extract was lyophilized for storage until use.

Quantification of protein and sugar. Protein was measured by the Bradford method using bovine serum albumin as the standard.[10] Total sugar content was determined by the phenol-sulfuric acid method with glucose as the standard as in Dubios *et al.*[11]

Determination of antithrombin activity. Human plasma thrombin and bovine plasma fibrinogen were dissolved in 50 mM Tris buffer (pH 7.5), respectively. Dried *doenjang* extract, dissolved in 20 μ*l* of the same buffer at various concentrations, was mixed with 200 μ*l* of 0.125% (w/v) fibrinogen solution and incubated for 3 min at 37°C. Thrombin (0.5 unit, 100 μ*l*) was added to the mixture, then the clot formation time was measured as described

by Irving *et al.*[12] using a blood coagulation analyzer (model; Coag-Stat™, Kyoto Daichi, Japan).

Measurement of thermal stability. The extract in 50 mM Tris buffer (pH 7.5) at the concentration of 50 mg/ml was incubated at 65°C as well as at boiling temperature for up to 120 min. After cooling, antithrombic activity of the incubated extract was assayed as above.

Gel permeation chromatography. The dried extract (1 g) was dissolved in 150 mM NaCl (1 ml) and centrifuged at 10,000 g for 20 min. The supernatant was subjected to gel permeation chromatography using a Sephadex G-25 column (1.2×106 cm) equilibrated with 150 mM NaCl. The column was developed at 30 ml/h. The absorbance at 280 nm and the activity of thrombin inhibitor were measured for each fraction.

Results and Discussion

Since many enzymes are readily inactivated by high concentrations of salt, it would be essential to desalt such highly salted crude extract of *doenjang* prior to undertaking the screening of an enzyme inhibitor presumably present in the sample. Molecular size-exclusion techniques such as conventional dialysis and filtration may, in theory, be employed for desalting. In a work with active compounds whose molecular weights are unknown and supposedly low, however, it is likely time-consuming to use those conventional techniques. Furthermore, it might be difficult to find a dialysis membrane or a molecular sieve with a suitable molecular weight cut-off. In this respect, electrodialysis could be an answer to such problems from a practical viewpoint. Results summarized in Table 1 demonstrate that effective and rapid desalting of *doenjang* extract was achieved by electrodialysis; most of salts were removed within 2.5 h, as assessed by electric conductivity, while peptides and sugars were mostly retained.

When thrombin was briefly incubated with desalted *doenjang* extract, a significant loss of activity for fibrin clot formation was observed. The extent of thrombin inhibition increased with increasing amount of the extract added (Fig. 1), indicating the presence in the extract of bioactive substance that acts as potent inhibitor of thrombin. The active substance appeared to have a very high thermal stability. Thrombin inhibitory activity of the extract was lowered only marginally, by ca. 5%, by prolonged treatment even at boiling temperature: virtually no change in the activity was seen in the extract when heat-treated for 2 h at 65°C (Fig. 2).

Preliminary results of purification experiments indicated that there were at least two thrombin inhibitors in the extract that were differentiated on the basis of gel permeation property. Sephadex G-25 chromatography performed with the extract revealed two clearly resolved activity peaks with the major one appearing at the void volume (Fig. 3). Because the fractionation range of molecular mass in a Sephadex G-25 column is 1~5 kDa for

Table 1. Electrodialysis of doenjang extract.

Quantities	Fractions*	Electrodialysis	
		before	after
Volume (L)	R	3.0	2.5
	P	3.0	3.5
Conductivity (mS/cm)	R	50.4	7.8
	P	2.2	49.6
Peptide (g)	R	0.73	0.71
	P	0	0.3
Total sugar (g)	R	45.4	36.0
	P	0	0.4
Total solid (%)	R	12.0	8.7
	P	0.1	4.0

*R and P indicate the retentate and the permeate, respectively.

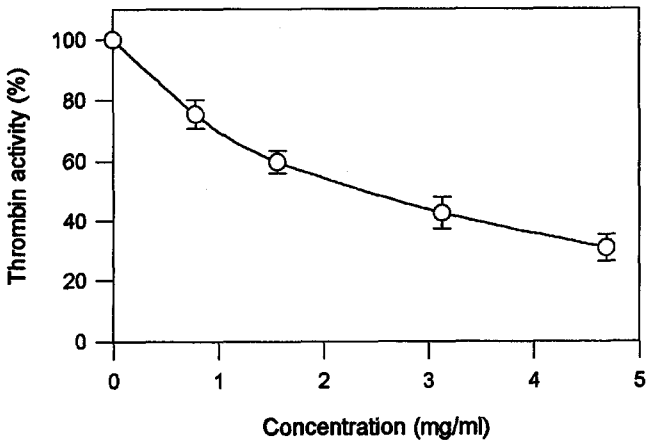

Fig. 1. Inhibition of thrombin as a function of the concentration of desalted *doenjang* extract in the mixture of the extract, fibrinogen and thrombin. The inhibition was assessed by an increase in fibrin clotting time. Data are presented as mean±SE (n=3).

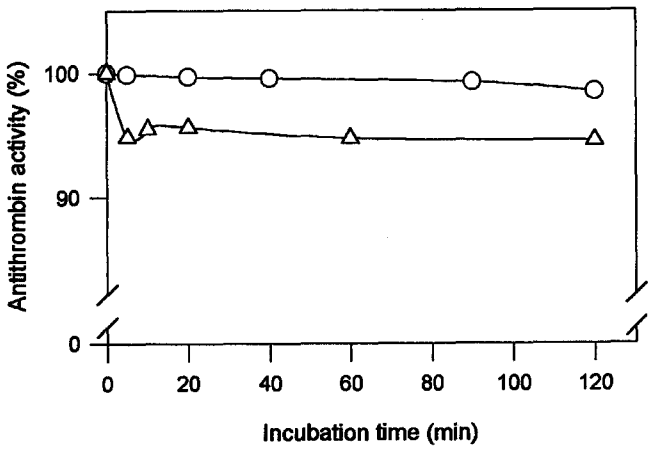

Fig. 2. High temperature effect on antithrombin activity of desalted *doenjang* extract. Incubation temperature of the extract was 65°C (○) and boiling temperature, 98°C (△). Results are averages of duplicated measurements.

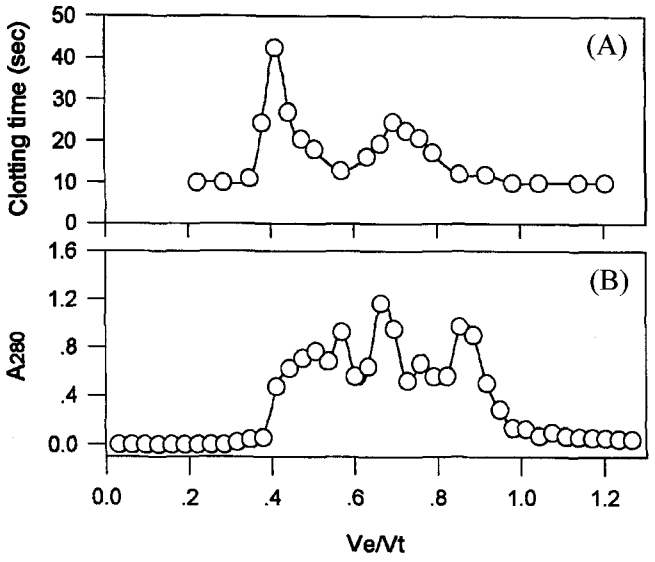

Fig. 3. Elution of thrombin inhibitors from a Sephadex G-25 column. The profiles were made based on antithrombin activity (A) and absorbance at 280 nm (B).

protein, it is assumed that the major component of antithrombin substance in *doenjang*, eluted at the void volume, has a molecular size corresponding to that of a protein with a molecular mass larger than 5 kDa, and the minor one apparently has a molecular size much smaller than this.

Because thrombin is a serine protease, as is the case with trypsin, and because soybean, the raw material for *doenjang*, contains number of trypsin inhibitors of both Kunitz and Bowman-Birk type,[13] these inhibitors might also act as antithrombin substances only if the inhibitors remain active in *doenjang*. In order to access this possibility, we checked whether or not *doenjang* extract can cause inhibition of trypsin as well as chymotrypsin, finding out that there were virtually no activities of trypsin inhibitors in the extract (data not shown). It may be conjectured that those trypsin inhibitors lost almost completely their activities during the processing of soybean for *doenjang* manufacture through thermal denaturation and also seemingly through enzymatic degradation.

To our knowledge, this investigation is the first attempt to search into the existence in soybean fermented foods of antithrombin agents rather than thrombolytic agents like fibrinolytic enzymes. The so-called 'physiologically functional' foods are presently gaining popularity and some foods unfamiliar in the ordinary diet are frequently claimed to be functional, based on somewhat vague conception. In the case of *doenjang*, however, this popular fermented food of Korea may deserve to be classified as a truly functional food, for it could be a potential dietary source of thrombosis-controlling substance without any adverse side-effects on human health.

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