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Purification and Characterization of Serine Protease Inhibitors from *Dolichos lablab* Seeds; Prevention Effects on Pseudomonal Elastase-Induced Septic Hypotension

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Three kinds of serine protease inhibitors, members of the Bowman-Birk trypsin inhibitor, were purified from *Dolichos lablab* seeds and named *Dolichos* protease inhibitor 1, 2 and 3 (DI-1, DI-2 and DI-3), respectively. Each inhibitor showed a single band with gel mobility at around 15.9, 12.1 and 14.6 kDa on 20% SDS-PAGE under reducing conditions. To characterize inhibitory specificity, the inhibition constant (Ki) for these inhibitors was measured against several known serine proteases. All three *Dolichos* protease inhibitors (DI-1, DI-2 and DI-3) inhibited the activity of trypsin and plasmin, but had no effect on thrombin and kallikrein (either for human plasma kallikrein or for porcine pancreas kallikrein). DI-1 inhibited chymotrypsin most effectively ($Ki = 3.6\times10^{-9}$ M), while DI-2 displayed inhibitory activity for porcine pancreatic elastase ($Ki = 6.2\times10^{-8}$ M). Pre-treatment of the 33 mg/kg of DI-mixture (active fractions from C_{18} open column chromatography that included DI-1, DI-2 and DI-3) inhibited the induction of pseudomonal elastase-induced septic hypotension and prevented an increase in bradykinin generation in pseudomonal elastase-treated guinea pig plasma. Also, the increase of kallikrein activity, by injection of pseudomonal elastase, was inhibited by the pretreatment of the DI-mixture in a guinea pig. Since the DI-mixture had no inhibitory effect on kallikrein activity when Z-Phe-Arg-MCA was used as a substrate *in vitro*, its inhibitory activity in the pseudomonal elastase-induced septic hypotension model might not be due to a direct inhibition of plasma kallikrein in the activation cascade of the Hageman factor and prekallikrein system. These results suggest that the *Dolichos* DI-mixture might be used as an inhibitor in pathogenic bacterial protease-induced septic shock.

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Introduction

Protease inhibitors are proteins that inhibit proteolytic enzymes and are found in animals, plants and microorganisms (Laskowski and Kato, 1980). Most plant protease inhibitors are inhibitors of serine proteases of microbial and animal origin, such as chymotrypsin, trypsin, elastase and substilisin (Ryan, 1990). In 1946, Kunitz (Kunitz, 1947a; Kunitz, 1947b) isolated the Kunitz-type soybean trypsin inhibitor (SBTI) from soybean, which comprises 181 amino acid residues with two-disulfide bridges (Koide and Ikenaka, 1973). The Bowman-Birk type soybean inhibitor (BBI) was also isolated and characterized by Birk (Birk, 1985). This inhibitor contains 71 amino acid residues, including seven-disulfide bridges (Ikenaka *et al.*, 1974).

It is known that pathogenic bacterial proteases induce the activation of one or more steps in the bradykinin-generating cascade: namely, bacterial protease activates precursors of the Hageman factor or prekallikrein to an active form protease, or cleaves high molecular weight kininogen to bradykinin, a potent vasodilator (Kaminishi *et al.*, 1990; Maruo *et al.*, 1993; Travis *et al.*, 1995; Maeda and Yamamoto, 1996). Bradykinin is known to relax a smooth vascular muscle that results in hypotension, and to enhance vascular permeability in tissues and organs. It is the principal endogenous substrate to cause pain *in vivo* (Colman, 1984). It was reported that intravenous administration of pseudomonal elastase (PE) to guinea pigs resulted in hypotensive shock due to activation of the Hageman factor dependent pathway (Khan *et al.*, 1993b). Furthermore, α_2-macroglobulin, a major plasma protease inhibitor that inhibits both kallikrein and bacterial protease, decreased in the blood of a PE-injected animal (Khan *et al.*, 1995).

Sepsis is the major cause of morbidity and mortality in hospitalized patients. Patients with the bacteria infection suffer septic shock (Roberts *et al.*, 1991). Several factors, such as nitric oxide (NO) (Goode *et al.*, 1995), cytokines (Ochoa *et al.*, 1992) and bradykinin (Katori *et al.*, 1989), are involved in the development of septic shock. For instance, NO-mediated endothelium-dependent vascular relaxation is elicited by trypsin via a second protease-activated receptor (PAR-2). This relaxation is inhibited by a soybean trypsin inhibitor (Hwa *et al.*, 1996). Since protease is the main participant in developing the septic syndrome, protease inhibitors would be an useful therapeutic means to improve the pathological condition. Aprotinin, a pancreatic protease inhibitor, has been widely used in treating septic shock, pancreatitis and other clinical states involving hyperfibrinolysis (Redl *et al.*, 1986). The Kunitz-type SBTI has been studied in order to estimate its physiological role and to improve its bioavailability (Shin *et al.*, 1996a; Shin *et al.*, 1996b).

Dolichos lablab bean seeds have been used as an oriental medicine for intestinal disorder diseases (Bensky and Gamble, 1993). Most intestinal disorder diseases are caused by bacterial infection that spreads exotoxin and endotoxin. This application assumed that *D. lablab* seeds contain some protease inhibitors, which inhibit the protease(s) inducing intestinal disorder diseases. In this study, three kinds of *Dolichos* protease inhibitors (DI-1, DI-2 and DI-3) were purified and characterized. Inhibition constants (Ki) were measured against several known serine proteases, and physiological functions of the purified *Dolichos* protease inhibitors were investigated using a pseudomonal elastase-induced guinea pig septic shock model. Not only the increase of bradykinin content and kallikrein activity in plasma, but also the decrease of blood pressure, was inhibited by pre-treatment with the DI-mixture in pseudomonal elastase injected guinea pigs.

Materials and Methods

Chemicals Crystallized pseudomonal elastase (PE) was obtained from Nagase Biochemicals (Fukuchiyama, Japan). Bovine albumin, soybean trypsin inhibitors (BBI and Kunitz-type), bovine pancreatic trypsin, porcine pancreatic elastase, bovine plasma plasmin, human plasma kallikrein, porcine pancreatic kallikrein and N-α-benzoyl-*D*, *L*-arginine-ρ-nitroanilide hydrochloride (BAPNA) were obtained from Sigma Chemical Co. (St. Louis, USA). We used chymotrypsin from Worthington (New Jersey, USA), Z-Phe-Arg-MCA, Boc-Gln-Ala-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, Boc-Val-Pro-Arg-MCA, Suc-Ala-Pro-Ala-MCA, and Boc-Val-Leu-Lys-MCA from Peptide Institute (Osaka, Japan), sodium pentobarbital from USP-Abbott Laboratories (Chicago, USA), heparin from ChoongWae Pharmaceutical Co. (Seoul, Korea), enzyme immunoassay kits for kinins (MARKIT-M Bradykinin) from Dainippon Pharmaceutical Co., LTD (Osaka, Japan), Sephadex G-50 from Pharmacia LKB Biotechnology (Uppsala, Sweden), ODS-A20 (C_{18}) from YMC-Gel (Osaka, Japan), ODS-120T column from Toyosoda (Tokyo, Japan), Chemosorb 5-ODS-H column from Chemco Scientific Co., Ltd. (Osaka, Japan). All other reagents were of analytical grade of the highest quality commercially available.

Purification of *Dolichos* protease inhibitors from *D. lablab* seeds

Preparation of the crude extract Seeds of *D. lablab* (500 g) were grounded and defatted with 2 liters of hexane with overnight stirrings at room temperature. Hexane was filtered through a 0.8 μm filter. The defatted powder was extracted using 3 liters of 10 mM sodium phosphate buffer containing 25 mM NaCl and 1 mM thiourea (pH 6.0) stirred for 3 h at 37°C. After the removal of seed debris by filtration through several folds of gauze, hydrochloric acid was added to the extract to adjust pH to 4.1. The precipitate was centrifuged at 32,000×*g* at 4°C for 20 min and the supernatant was heated at 100°C for 10 min to precipitate heat-labile proteins. After centrifugation, the supernatant was concentrated using an Amicon ultrafiltration cell (3,000 cut membrane).

Sephadex G-50 column chromatography The concentrate resulting from the ultrafiltration was gel-filtered through Sephadex G-50 column (3.5×120 cm), equilibrated with 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl, and eluted with the same buffer at flow rate 0.2 ml/min. Active fractions were selected by an inhibition assay of trypsin-catalyzed hydrolysis of BAPNA according to the described method (Erlanger *et al.*, 1961) as follows: The assay mixture contained 200 μl of 0.05 M Tris-HCl, pH 8.2, containing 0.05 M $CaCl_2$, 100 μl of 40 μg/ml trypsin and either 50 μl of the sample or the equal volume of buffer. After 10 min of incubation at 37°C, 600 μl of BAPNA (400 mg/ml) was added and incubated for another 10 min at 37°C. The reaction was stopped with 100 μl of 30% (v/v) acetic acid. The release of ρ-nitroaniline was monitored spectrophotometrically at 410 nm.

C_{18} open column chromatography Fractions showing inhibitory activity against trypsin from the Sephadex G-50 chromatography were loaded on a C_{18} open column (1.0×20.5 cm) equilibrated with 0.05% trifluoroacetic acid (TFA) in H_2O and washed with 0.05% TFA in 5% acetonitrile. It was then eluted with a linear gradient between 200 ml of 0.05% TFA in 5% acetonitrile and 200 ml of 0.05% TFA in 60% acetonitrile at a flow rate of 1 ml/min.

Reverse-phase C_{18}-HPLC In the final step of purification, a reverse phase C_{18} was used on HPLC (Gilson model 805). The column was equilibrated with 0.05% TFA in H_2O, and then eluted with a linear gradient of acetonitrile containing 0.05% TFA from 0 to 40% at a flow rate of 1 ml/min.

Rechromatography on reverse-phase C_{18}-HPLC Each peak containing trypsin inhibitory activity eluted from C_{18}-HPLC was rechromatographied on a Chemosorb 5-ODS-H HPLC column (21×150 cm) that was equilibrated with 0.06% TFA in H_2O. It was then eluted with a linear gradient of 80% acetonitrile containing 0.052% TFA from 0 to 60% in the equilibrating

solvent at a flow rate of 0.2 ml/min.

Determination of partial amino acid sequences of the purified *Dolichos* protease inhibitors

The purified *Dolichos* protease inhibitors (DI-1, DI-2 and DI-3) were reduced and S-pyridylated according to previously published methods (Jang *et al.*, 1998; Cho *et al.*, 1999). The S-pyridylated DIs were digested with trypsin, and the resulting peptides were separated by HPLC on a C_{18}-reverse phase HPLC column (Gilson). To determine the amino-terminal sequences of purified DIs, the lyophilized DIs were directly subjected to an automated amino acid sequence analysis.

Measurement of the inhibition constant (Ki) and specificity against known protease

Protease activities of trypsin, chymotrypsin, thrombin, elastase and plasmin were assayed fluorometrically using synthetic 4-methyl-7-coumaryl amide (MCA) substrates. 10 µl of the protease solution (15 µg/ml), and 50 µl of a properly diluted sample solution, were added to 500 µl Tris-HCl (pH 8.2) containing 50 mM NaCl and incubated at 37°C for 5 min. Then 2 µl of the MCA substrate (10 mM stock solution), dissolved in dimethylformamide (DMF), were added to the reaction mixture and incubated at 37°C for 5 min. The reaction was stopped by the addition of 17% acetic acid. The amount of 7-amino-4-methyl coumarin (AMC) was measured fluorometrically using a fluorescence spectrophotometer (Kontron SFM 25) with excitation at 380 nm and emission at 460 nm. The Ki value of each inhibitor for each protease was calculated by using the Dixon plot method (Dixon, 1953). The concentration of 50% inhibition (IC_{50}) was estimated by measuring protease activities in different inhibitor concentrations. To estimate the inhibitory effects of the DIs and of the DI-mixture against kallikrein *in vitro*, 10 µl of kallikrein solution (0.01 U/10 µl distilled water of porcine pancreas, or 0.1 U/10 µl distilled water human plasma) and 50 µl of the properly diluted sample solution were added to 500 µl of the assay buffer (10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 0.1% bovine serum albumin) and incubated at 37°C for 5 min. Then 5 µl of Z-Phe-Arg-MCA (10 mM) dissolved in DMF were added to the reaction mixture and incubated at 37°C for 10 min. The reaction was stopped by the addition of 17% acetic acid. The amount of AMC released from Z-Phe-Arg-MCA by kallikrein was measured by fluorescence spectrophotometer (at 380 nm and 460 nm). One inhibition unit (IU) was defined as inhibition activity that reduced kallikrein activity to 50% (0.1 U for human plasma kallikrein or 0.01 U for porcine pancreas kallikrein).

Electrophoresis

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) with 3.75% stacking gel and 20% separating gel. Proteins were stained with Coomassie brilliant blue R-250. Samples for SDS-PAGE were prepared by heating at 75°C for 20 min in a medium (pH 6.8) containing 1.6% SDS, 4.2% 2-mercaptoethanol, 0.17 M Tris, 0.047 M HCl, 11.2% glycerol and 0.04% bromophenolblue.

Measurement of mean arterial blood pressure (MAP) and bradykinin generation in guinea pig plasma

Guinea pigs (male Albino-Hartley strain guinea pig, weighing 250 to 350 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital at 30 mg/kg before the experimental procedures started. Measurements of mean arterial blood pressure (MAP) and bradykinin generation in guinea pig plasma were carried out by Kahn's method (Khan *et al.*, 1993a; Khan *et al.*, 1993b). Briefly, the right external jugular vein was cannulated for the measurement of blood pressure through a pressure transducer (Nihon Kohden, Tokyo, Japan) attached to a polygraph. After completion of the experimental setup, pseudomonal elastase (PE, 0.3 mg/kg/ml saline) was injected as a single bolus into the jugular vein. For control studies, only a saline solution (1 ml/kg) was injected. For inhibition of septic shock hypotension, purified DI or DI-mixture (33 mg/kg/ml saline) was infused into the vein. PE was injected 3 min after the end of the infusion of the DI or DI-mixture. To estimate bradykinin generation and kallikrein activity in plasma, 1 ml of blood was withdrawn through the jugular vein into a syringe containing a small amount of 200 mM EDTA 3 min before the injection of either the DI or DI-mixture, then 3 min after injection of PE, and after recovering the blood pressure from septic-shock hypotension. Then, 100 µl of 200 mg/ml trichloroacetic acid (TCA) was added to the collected blood to precipitate the protein. The TCA-soluble fraction was recovered by centrifugation for 5 min at 6,300×*g* (it was used to measure bradykinin and kallikrein activity in plasma). The method used to measure the concentration of bradykinin was an enzyme-linked immunoassay using a bradykinin measuring kit according to the manufacturer's instructions. For the measurement of plasma kallikrein activity, guinea pig plasma was taken under conditions as described above, and then the plasma solution was prepared by centrifugation. 100 µl of plasma solution was added to 500 µl of an assay buffer (10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 0.1% bovine serum albumin). Then 5 µl of Z-Phe-Arg-MCA (10 mM stock solution) dissolved in DMF was added to the reaction mixture and incubated at 37°C for 20 min. The reaction was stopped by the addition of 17% acetic acid. The amount of AMC released from Z-Phe-Arg-MCA by kallikrein was measured fluorometrically using a fluorescence spectrophotometer (at 380 nm and 460 nm).

Results

Purification of *Dolichos* protease inhibitors from *D. lablab* seeds

The crude extract from *D. lablab* was heated at 100°C for 10 min, and the precipitate was then removed by centrifugation. The specific activity was increased 11 times by heat treatment and the recovery of inhibitory activity for trypsin was not reduced as shown in Table 1. The supernatant from the heat-treatment was concentrated by ultrafiltration and then fractionated by the Sephadex G-50 column. The second major peak exhibited inhibitory activity against trypsin (Fig. 1A). The fractions showing trypsin inhibitory activity were pooled and subjected to C_{18}-reverse phase open column. The initial sharp phase contained DI-2 and DI-3, while the declined lag phase contained DI-1 (Fig. 1B). Fractions containing DI-1,

Table 1. Summary of purification of *Dolichos* protease inhibitors from *Dolichos lablab* seeds.

Purification	Protein (mg)	Total activity (IU[a])	Specific Activity (IU/mg)	Yield (%)	Purification fold
Seeds (500g) Crude extract	39900	7528.2	0.19	100.0	1
Heat treatment	3540	7363.2	2.08	97.8	10.9
Sephadex G-50	426	3540.0	8.31	47.0	43.7
C_{18} open column	257	2357.8	9.17	19.8	48.2
C_{18} HPLC DI-1	54.7	502.0	9.17	6.67	43.7
DI-2	21.0	140.6	6.71	1.87	35.3
DI-3	9.0	85.6	9.52	1.14	50.1

[a]One inhibition unit (IU) was defined as the amount of inhibitor, which reduced 0.17 mM trypsin activity to 50%.

DI-2 and DI-3 from C_{18}-open column chromatography were pooled and designated as the *Dolichos* inhibitor (DI) mixture. The DI-mixture was subjected to C_{18}-reverse phase HPLC after removing acetonitrile by a speed vacuum drier. To characterize the three peaks showing protease inhibitory activity, we obtained a large amount of DIs by repeated injections of the DI-mixture to the C_{18} HPLC column. Each peak was then named DI-2, DI-3 and DI-1, respectively, as shown in Fig. 1C, according to the order of the amount. DI-1, DI-2 and DI-3 showed a single band on SDS-PAGE and had gel mobility at 16.0, 12.0, and 14.6 kDa under reducing conditions, respectively (Fig. 1D). The results of the DI purification are summarized in Table 1.

Partial amino acid sequences of the purified *Dolichos* protease inhibitors

As three kinds of protease inhibitors had been purified to homogeneity, we determined their partial amino-acid sequences. As shown in Fig. 2A, DI-1 and DI-3 have the high sequence homology with the previously described BBI type of *Macrotyloma axillare* seed (Joubert *et al.*, 1979), Azuki bean (Ishikawa *et al.*, 1985) and Bowman-Birk Inhibitor (BBI type 1) of soybean (Odani and Ikenaka, 1972). DI-2 showed the homology with the BBI (type 3) of soybean (Odani and Ikenaka, 1977; Odani and Ikenaka, 1978) as shown in Fig. 2B. Although we did not determine the whole amino acid sequences of the purified DIs, the determined partial amino acid sequences of the DIs showed high homology with the BBIs of plants. Particularly, cysteine residues are perfectly conserved with those of BBIs. These results suggest that the purified *Dolichos* protease inhibitors belong to the family of BBI type serine protease inhibitors.

Protease inhibitory specificity of DIs compared against known protease

To find the inhibitory specificity of DIs, compared with known serine protease *in vitro*, we surveyed the inhibitory specificity of DIs against several known serine proteases. For this, the inhibition constant (Ki, Table 2) and the inhibition concentration (IC_{50}, Table 3) were estimated. The purified DI-1, DI-2 and DI-3 had similar inhibitory effects on trypsin, but they inhibited plasmin a little less effectively. DI-1 had the highest effect on chymotrypsin; DI-2 inhibited porcine pancreatic elastase. DIs did not affect thrombin or plasma kallikrein. For a comparison, enzyme specificity of BBI and SBTI (Kunitz) was examined. BBI inhibited trypsin (IC_{50}= 0.259 mg for 0.02 unit trypsin) and chymotrypsin (IC_{50}= 1.313 mg for 0.01 unit chymotrypsin). SBTI had an inhibitory effect on trypsin (IC_{50}= 0.155 mg) and plasma kallikrein (IC_{50} = 0.064 mg for 0.02 unit plasma kallikrein). Neither BBI nor SBTI had an inhibitory effect on porcine pancreatic elastase. To elucidate the synergic effect among DIs, IC_{50} was estimated for the DI-mixture and each DI. In the DI-mixture, the inhibitory activity for trypsin was increased almost 2.5 times compared with DI-1; however, activity for chymotrypsin was potentiated to the same degree for DI-1 alone. Finally, we estimated the Ki values for DI-1, DI-2 and DI-3 by using MCA-synthetic substrates as shown in Table 3. These results suggest that the DIs and the DI-mixture show potent inhibitory effects against trypsin and chymotrypsin.

Effects of *Dolichos* inhibitors on pseudomonal elastase (PE) induced hypotension

To investigate whether DIs could prevent bacterial protease-induced septic hypotension, a PE-induced guinea pig septic shock model (Khan *et al.*, 1993b) was employed in this study. As shown in Fig. 3A, an intravenous injection of PE at the dose of 0.3 mg/kg, induced hypotension in the guinea pigs (n = 10, from 64±6.4 mmHg to 14.7±9.4 mmHg). This hypotension reached its maximum 3 min after the injection and recovered within at least 30 min after the PE-injection. When each DI inhibitor (DI-1, DI-2 or DI-3) at 33 mg/kg was used as pretreatment 3 min before the PE-injection, PE-induced hypotension was not inhibited (Fig. 3B, C and D). However, pre-treatment with the DI-mixture at 33 mg/kg completely prevented PE-induced hypotension (n = 5, from 63 ±6.4 mmHg to 56.8±6.7 mmHg, Fig. 3E). Since the DI-mixture was assumed to contain some other materials besides DIs, each 11 mg/kg of purified DI-1, DI-2 and DI-3 were assembled, and then injected before the PE-injection. The assembled sample also inhibited PE-induced hypotension (Fig. 3F). This result eliminates the suspicion that the

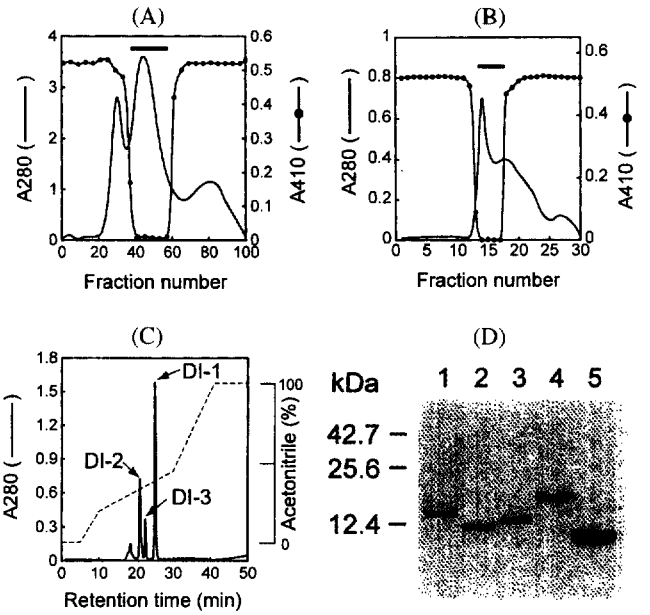

Fig. 1. Elution pattern of DIs from Sephadex G-50 column (A), C_{18} open column (B), C_{18} HPLC column (C) and SDS-PAGE pattern of purified DIs. (A) 8 ml of heat-treated crude extract containing about 1 g of protein was applied to the column (3.5×120 cm) equilibrated with a buffer A (10 mM phosphate, pH 7.2, containing 0.1 M NaCl). Elution was performed with the buffer at a flow rate of 0.2 ml/min. Fractions showing inhibitory activity against trypsin were indicated by a solid bar and pooled. —, protein (absorbance at 280 nm); -●-, protease activity (absorbance at 410 nm). (B) Pooled fractions from Sephadex G-50 were applied to a C_{18}-open column equilibrated with 0.05% TFA in H_2O and eluted with a linear gradient of 200 ml of 0.05% TFA in H_2O and 200 ml of 0.05% TFA in 60% acetonitrile at a flow rate of 1 ml/min after washing with 0.05% TFA in 5% acetonitrile. Fractions indicated by a solid line were pooled. —, protein (absorbance at 280 nm); -●-, protease activity (absorbance at 410 nm). (C) Active fractions from the C_{18}-open column chromatography were applied to a C_{18}-HPLC column equilibrated with 0.05% TFA in H_2O and eluted with a linear gradient of 0.05% TFA in H_2O and 0.05% TFA in acetonitrile. Each peak was assayed for inhibitory activity toward trypsin and designated as DI-1, DI-2, and DI-3 according to the order of amount of peaks. —, protein (absorbance at 280 nm); ------, acetonitrile concentration. (D) Samples were heated at 70°C with 2x SDS loading buffer containing 1.6% SDS and 4.2% 2-ercaptoethanol for 20 min before being loaded on a 20% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue 250A. The used size marker; albumin, 42.7 kDa; a-chymotrypsin, 25.6 kDa; cytochrome C, 12.4 kDa. Lane 1, the purified DI-1; lane 2, DI-2; lane 3, DI-3; lane 4, SBTI (Kunitz); lane 5, SBTI (BBI).

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(A)
DI-1          SG-HHQXT-- ---------- ------SIPP QCR-----LD SCHSA--SCL
DI-3          SG-HHQSTDE PSESSK---- ------SIPP QCR-----LD SCHSACK---
MAI DE-3      --DHHHSTDE PSESSKPCCD ECACTKSIPP QCRCTDVRLN SCHSACSSCV
AB-1          SGHHEHSTDZ PSZSSKPCCB HCACTKSIPP QCRCTDLRLD SCHSACKSCI
BBI           --------DD --ESSKPCCD QCACTKSNPP QCRCSDMRLN SCHSACKSCI

DI-1          CTFSINATCV CADTNDF--- ---------- ---
DI-3          ---SIPAQCV CADTNDFCYE PCK------- ---
MAI DE-3      CTFSIPAQCV CADTNDFCYE PCK------- ---
AB-1          CTLSIPAQCV CBBIBDFCYE PCKSSHSDDD NNN
BBI-1         CALSYPAQCF CVDITDFCYE PCKPSEDDK- -EN

(B)
DI-2          -------HHD DSSDEPSESL --ACCDLCIC TASIPPQCSC TDIRLNSCHS
BBI-3         MCILSFLKSD QSSSYDDDEY SKPCCDLCMC TRSMPPQCSC EDIRLNSCHS

DI-2          ACK------S VPGLCRCLDT NDFCYQP--- ---
BBI-3         DCKSCMCTRS QPGQCRCLDT NDFCYKPCKS RDD
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Fig. 2. Amino acid sequence comparison with *Dolichos* protease inhibitors and known serine protease inhibitors. (A) Partially determined amino acid sequences of DI-1 and DI-3 were compared with those of MAI DE-3 [*Macrotyloma axillare* seed (Joubert *et al.*, 1979)], AB-1 [Azuki bean (Ishikawa *et al.*, 1985)], and BBI [Bowman-Birk Inhibitor, type 1, of soybean (Odani and Ikenaka, 1972)]. (B) Partial amino acid sequences of DI-2 were compared with that of BBI (type 3) of soybean (Odani and Ikenaka, 1977; Odani and Ikenaka, 1978)

prevention of hypotension is due to the effect of other factors besides DIs in the DI-mixture. The decrease of blood pressure induced by PE in our animal model was probably due to the formation of bradykinin from a high molecular weight kininogen. Therefore, the DI-mixture might inhibit one protease engaging in the activation cascade of the Hageman factor and prekallikrein.

Effects of the DI-mixture on bradykinin generation and kallikrein activity in guinea pig plasma

When we measured the bradykinin content in the control plasma of guinea pigs, it was 122.3±18.8 pg/ml (n = 3, Fig. 4). However, bradykinin was markedly increased in plasma when the blood was collected during PE-induced hypotension 3 min after the PE-injection (n = 4, 1182.5±665.6 pg/ml plasma). The plasma bradykinin content dropped almost to the control levels when plasma was collected 30 min after the PE-injection (n = 3, 261.0±41.1 pg/ml plasma). The increase in bradykinin generation in PE-injected guinea pig plasma was significantly inhibited by pre-treatment with 33 mg/kg of the DI-mixture (n = 4, 224.25±45.33 pg/ml). To estimate the amount of plasma kallikrein in PE-injected guinea pigs, plasma was collected 3 min after the injection. As shown in Fig. 5, the kallikrein activity of PE-injected guinea pigs was significantly increased compared to the control plasma (n = 3, from 80.8±33.15 pg/ml to 238.7±34.9 pg/ml). The increase in kallikrein activity was also inhibited by pre-treatment with the DI-mixture at 33 mg/kg (n = 3, 105.0±22.2 pg/ml, Fig. 5). During *in vitro* experiment, we previously showed that the DI-mixture did not inhibit the activity of kallikrein, as shown in Table 2. However, pre-treatment with the DI-mixture in a PE-induced hypotension model inhibited the generation of plasma kallikrein. This result suggests that the DI-mixture may inhibit either the activation of plasma prekallikrein, or the activation of the Hageman factor in a kallikrein-kinin cascade.

Discussion

In this study, in order to obtain the potent protease inhibitors

Table 2. Dissociation constant (Ki) of the purified DIs (DI-1, DI-2 and DI-3) with various known proteases.

Proteases	Ki (M)			Substrates
	Inhibitors			
	DI-1	DI-2	DI-3	
Trypsin	2.5×10^{-8}	3.1×10^{-8}	2.4×10^{-8}	Boc-Gln-Ala-Arg-MCA
Chymotrypsin	3.6×10^{-9}	7.1×10^{-7}	2.1×10^{-7}	Suc-Leu-Leu-Val-Tyr-MCA
Thrombin	-[a]	-	-	Boc-Val-Pro-Arg-MCA
Elastase	-	6.2×10^{-8}	-	Suc-Ala-Pro-Ala-MCA
Plasmin	5.2×10^{-8}	1.7×10^{-8}	5.7×10^{-8}	Boc-Val-Leu-Lys-MCA
Kallikrein[b]	-	-	-	Z-Phe-Arg-MCA

[a]not determined because of the absence of inhibitory activity.
[b]both for human plasma kallikrein and for porcine pancreas kallikrein.

Table 3. Inhibitory activity of DIs and DI-mixture against several protease

Proteases[b]	IC_{50} (mg/ml)[a]			
	DI-1	DI-2	DI-3	DI-mixture
Trypsin	0.168	0.117	0.165	0.062
Chymotrypsin	0.027	1.323	0.937	0.027
Elastase	-	0.066	-	-
Thrombin	-	-	-	-
Plasmin	0.197	0.556	0.200	0.240
Kallikrein	-	-	-	-

[a]Inhibitory concentration (IC_{50}) was defined as the amount of inhibitor that inhibited protease activity to 50%.
[b]The amounts of proteases used to liberate 10 mM of AMC

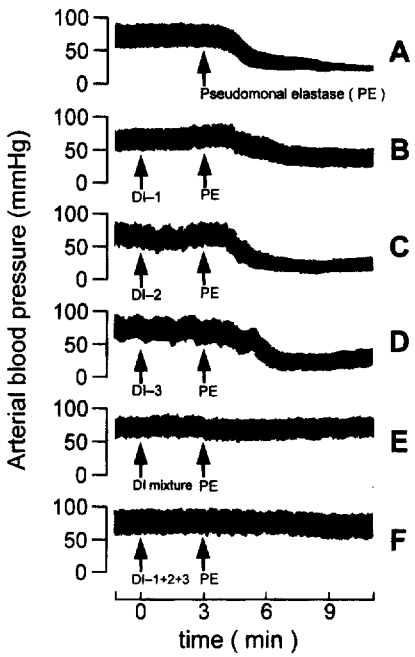

Fig. 3. Tracings showing the preventive effect of TM on hypotension induced by i. v. injection of pseudomonal elastase. The horizontal axis and the vertical axis represent the time period (min) and the mean systemic arterial blood pressure (MAP, mmHg), respectively. A, pseudomonal elastase (PE, 0.3 mg/kg); B, DI-1 (33 mg/kg) was injected 3 min before the PE-injection; C, DI-2 (33 mg/kg); D, DI-3 (33 mg/kg); E, DI-mixture (33 mg/kg); F, purified DI-1, DI-2 and DI-3 (11 mg/kg each) were assembled, and then injected before the PE-injection. See text for details.

from a plant source, we isolated and characterized three kinds of protease inhibitors (DI-1, DI-2 and DI-3) from *D. lablab* seeds. While preparing the crude extract, thiourea was included in the extraction buffer to prevent melanization. Without thiourea, the crude extract induced pigments. Since the DIs were heat-stable, heat-treatment was an efficient method for removing heat-labile proteins without affecting the activity and the recovery of the DIs (Table 1). The DIs were purified to homogeneity by chromatography on Sephadex G-50 and C_{18}-reverse-phase open column, followed by reverse-phase C_{18}-HPLC. Purification procedures employed in this study are useful for obtaining large amounts of purified DIs for the purpose of examining their biological functions.

The potent protease inhibitors will be useful as therapeutic materials for sepsis (Fritz, 1979; Takakura *et al.*, 1989). It was suggested that a potent kallikrein inhibitor, which can inhibit the activation of prekallikrein or kininogen, may be very important and useful for the improvement of septicemia states such as hypotension, shock, multiple organ failure (MOF), and disseminated intravascular coagulation (DIC). Aprotinin has been widely used in treating septic shock, pancreatitis, cardiopulmonary bypass surgery and other clinical states involving hyperfibrinolysis (Redl *et al.*, 1986; Harle and Rahman, 1989; Blauhut *et al.*, 1991). Recently, in an effort to increase the bioavailability of the Kunitz-type soybean trypsin inhibitor (SBTI), SBTI conjugated with gelatin was used to show the inhibitory effects against PE-induced hypotension. This derivative also inhibited the generation of kallikrein in PE-injected animal models (Shin *et al.*, 1996a, 1996b). In this study, the DI-mixture showed potent inhibitory effects in a PE-induced hypotensive shock model. However, the native

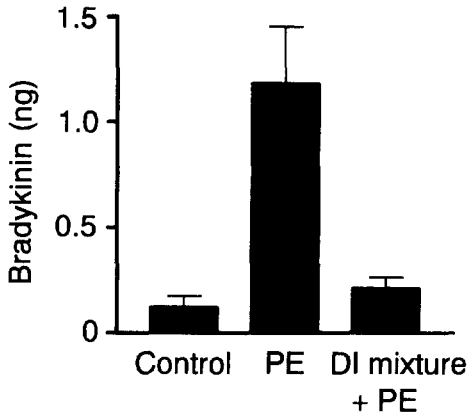

Fig. 4. The effect of the DI-mixture on the generation of bradykinin in guinea pig plasma. Control (n = 4), blood collected 3 min before PE; PE (n = 4), blood collected after PE-injection; DI-mixture+PE (n = 4), DI-mixture (33 mg/kg in 500 ml of saline) was injected 3 min before the PE infection. Bradykinin content was measured as described in "Material and Methods". Values are means±SEM (n = 4).

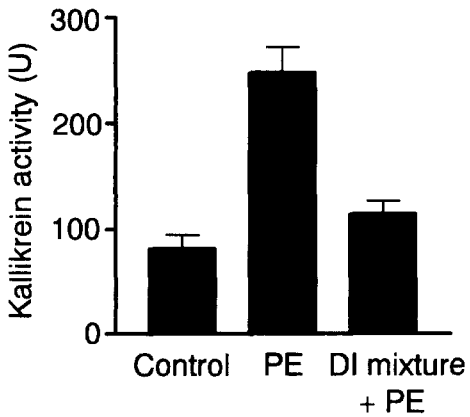

Fig. 5. The effect of the DI-mixture on kallikrein activity in guinea pig plasma. Kallikrein activity was measured with Z-Phe-Arg-MCA substrate in plasma collected before injection with PE or DI-mixture+PE; control (n = 3), blood collected 3 min before PE-injection (0.3 mg/kg); PE (n = 3), blood collected 3 min after the PE-injection; DI-mixture and PE (n = 3), DI-mixture (33 mg/kg in 500 ml of saline) was injected 3 min before the PE-injection. One inhibition unit (IU) was defined as inhibition activity, which reduced 0.1 U human plasma kallikrein activity to 50%. Values means±SEM (n = 3).

DI-mixture may exhibit too short of a half-life *in vivo* because of its molecular mass and that it is too small to be retained in circulating blood; hence it is necessary to enhance its retention time in blood. It is expected that a polymer-modified DI-mixture having a molecular mass more than the threshold of renal clearance, will be eliminated slower from the systemic circulation; therefore, it will more effectively prevent PE-induced hypotensive shock.

In addition to the DI-mixture's effects on hypotension, this study also investigated the effects on bradykinin generation on kallikrein activity, which induces activation kininogen to bradykinin. Increases in bradykinin generation and kallikrein activity by PE-injection were prevented by a pre-treatment with the DI-mixture (33 mg/kg, Fig. 3E). Because the SBTI is known to be a typical serine protease inhibitor with potent inhibitory activity against plasma kallikrein (Ikenaka *et al.*, 1974), the prevention of bradykinin generation by a pre-treatment of SBTI in PE-injected guinea pigs is assumed to be due to its inhibitory effect on plasma kallikrein. The DI-mixture did not inhibit plasma kallikrein activity during *in vitro* experiments using a Z-Phe-Arg-MCA substrate, even though the DI-mixture inhibited PE-induced hypotension and the accompanying increase in kallikrein activity and bradykinin generation. This result may suggest that the inhibitory effects of the DI-mixture against a PE-induced increase of kallikrein activity and bradykinin generation may be via a different way compared with the SBTI's direct inhibition of plasma kallikrein in kallikrein-kinin system. Further studies are necessary in order to purify the DIs-binding proteins to homogeneity and determine their primary structures. From these studies, it may be possible to postulate the molecular mechanism of DIs to inhibit the generation of bradykinin and kallikrein *in vivo*.

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