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석사학위 논문

Isolation and Characterization of a Fibrin(ogen)olytic Protease from *Lepidasthenia izukai*

조선대학교 대학원

생명과학과

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이즈카긴비늘갯지렁이로부터 피브린분해 단백질분해효소의 분리 및 생화학적 특성분석

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지도교수 이 정 섭

이 논문을 이학 석사학위 신청 논문으로 제출함.

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윤상구의 석사학위논문을 인준함.

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ABSTRACT

Isolation and Characterization of a Fibrin(ogen)olytic protease from *Lepidasthenia izukai*

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This study was performed to purify and characterize biochemically a serine protease from a marine polychaete *Lepidasthenia izukai*. To purify the enzyme, a five-step procedure consisting of ammonium sulfate fractionation (20~80% in saturation concentration), Hiprep 16/10 Q FF, two-sequential Mono Q 4.6/100 PE column anion exchange, and finally Superdex 75 10/300 GL size exclusion chromatographies were employed in order. The resulting purified enzyme was named LIZ (stands for *Lepidasthenia izukai*). The purified LIZ enzyme had a specific activity of 1,628 units/mg and the yield was 0.9% from approximately 50 g of the *L. izukai* worm cell lysate. The estimated molecular weight of the purified enzyme was found to be approximately 28 kDa, as determined by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The optimal pH for the activity of LIZ was approximately 7.5 and the enzyme activity was relatively stable under the temperature range of 20~60℃. In addition, the proteolytic activity of LIZ was clearly inhibited by serine protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP), but not by metalloprotease inhibitors, including 1,10-phenanthroline (1,10-PT) and

ethylenediaminetetraacetic acid (EDTA), suggesting that the enzyme is a typical serine protease. Among the chromogenic substrates tested, S-2288 (H-D-Ile-Pro-Arg-*p*NA), a typical substrate for tissue plasminogen activator (t-PA), was the most suitable one for the purified enzyme, implying that the enzyme can cleave the carboxyl side of Arg in the synthetic peptide substrate. The purified enzyme also efficiently cleaved various blood coagulation-related proteins, including fibrinogen, prothrombin, and plasminogen. The purified LIZ enzyme showed a typical fibrin(ogen)olytic activity *in vitro*, as it could digest all the Aα, Bβ, and γ chains of fibrinogen within 20 min and cleave cross-linked fibrin polymer and fibrin as well. In addition, the enzyme could exhibit a proteolytic activity in cleaving the fibrin clots formed in human blood plasma and also on the fibrin plate. The fibrin plate assay also showed that 1 μg each of LIZ and plasmin formed 1.8 cm, and 1.2 cm of halo zones in diameters, respectively, on the fibrin plate, indicating that the adjusted plasmin unit of LIZ is equivalent to 0.004 plasmin units in digesting the fibrin clots. All these results suggest that the purified LIZ enzyme is active fibrin(ogen)olytic serine protease that can dissolve blood thrombi.

Ⅰ. INTRODUCTION

Marine polychaete, *Lepidasthenia izukai* (*L. izukai*) is included in the family Polynoidae. The body of this specific worm has no hair on the edge of the backbone. The back legs are cylindrical. The foot is large and triangular. The worm lives in a soft or mixed bottom, sandy, or attached organism. It is distributed in Korea (Wolpo, Ulleung island, Haeundae, Geojeum, Gogeum island, and Gunsan *et al.*), Japan, Yellow Sea, East China Sea, and there are many individuals (Fig. 1) (Kim, 2015). This organism was first scientifically discovered by Imajima and Hartman in 1964 (Imajima and Hartman, 1964).

According to a recent report by the World Health Organization (WHO, 2016), cardiovascular disease (CVD) is the leading cause of death worldwide. CVDs cause 17 million deaths every year, accounting for 31% of total deaths 85% of which are due to by heart attack and stroke (WHO, 2016). Intravascular thrombosis, the formation of a clot of blood in a blood vessel, is one of the main causes of a variety of CVDs (WHO, 2016). The major protein component of blood clots, fibrin, is formed from fibrinogen via thrombin-mediated proteolysis (Goldhaber and Bounameaux, 2001). Moreover, the fibrin clots can be hydrolyzed by plasmin to remove thrombosis from blood vessels. In some situations, there is an imbalance due to some disorders and the clots are not hydrolyzed, thus thrombosis occurs (Tough, 2005). Over the years, thrombolytic therapies like injecting or orally administrating thrombolytic agents to lyse thrombi in blood vessels have been extensively investigated (Peng *et al.,* 2005).

Hemostasis is the physiologic system, which supports the blood in the fluid state. A normal hemostatic system suppresses the formation of a blood clot in the bloodstream, but reacts in the vascular injury to prevent blood loss. The blood homeostasis is maintained by a balance between the formation and degradation of fibrin (Fig. 2) (Park *et al.*, 2014).

Fig. 1. Photograph of *L. izukai.* The body length and width are about 120 mm and 9 mm, respectively. The worm has scales on the right and left and is exposed at the center. The body is round and flat, and there is a small round pattern of mottling at the green (Kim, 2015).

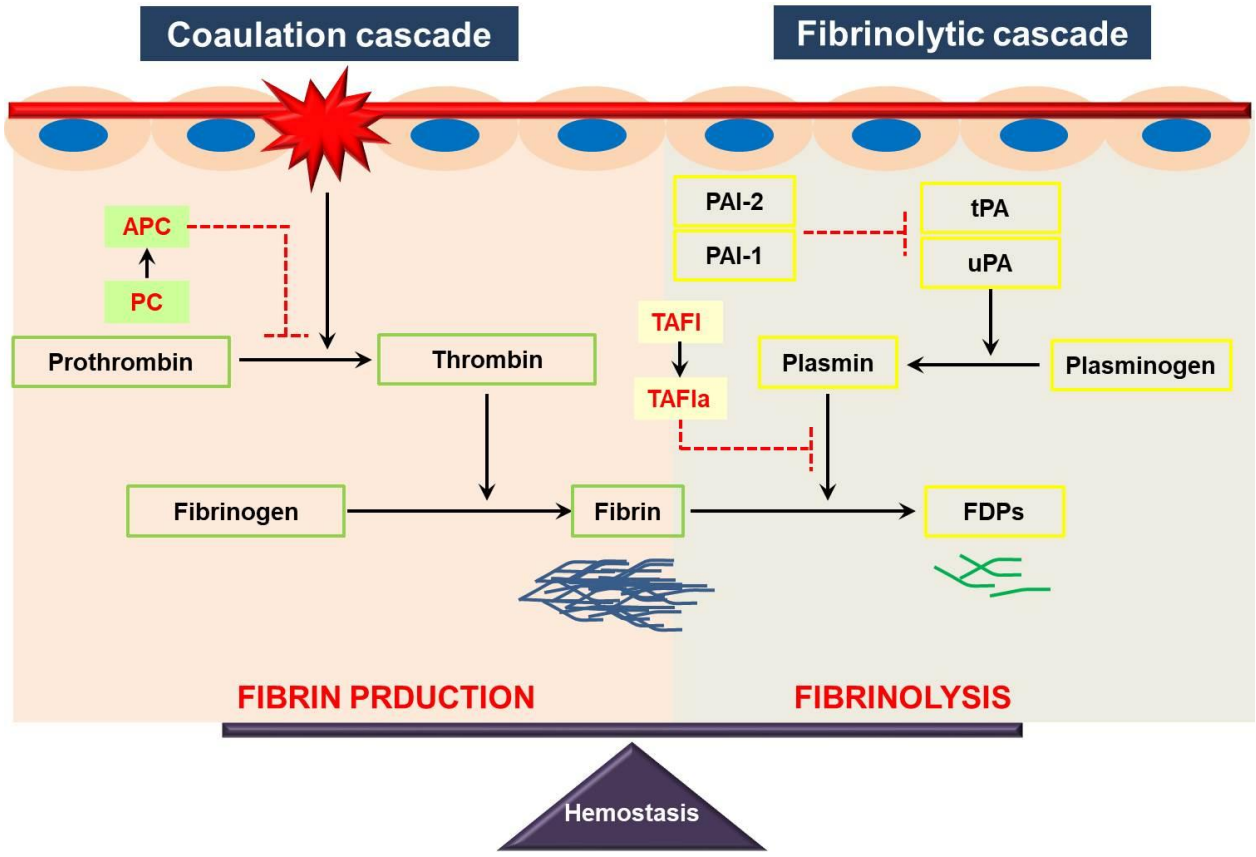

Fig. 2. The balance between coagulation and fibrinolysis. TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa, thrombin activatable fibrinolysis inhibitor activated; PC, protein C; APC, activated protein C; FDP's, fibrin degradation products.

The primary function of the coagulation system is to stop bleeding on an injury until repair occurs. Through the activation of a cascade of plasma proteins, blood clots are formed at the site of injury or blood flow disturbance through fibrin deposition and platelet congregation (Dahlback *et al.,* 2005; Sun, 2005). In the event of injury to the blood vessel wall, tissue factor (TF) normally sequestered in the subendothelial layer is exposed. TF binds and activates circulating factor VII (FVII). The complex of TF and activated FVII (FVIIa) then activates factor X (FX) and factor IX (FIX). The activated FXa forms the prothrombinase complex with activated factor V (FV). The prothrombinase complex (FVa, FXa) then cleaves prothrombin to thrombin, which then cleaves fibrinogen to form fibrin. FIX activation also amplifies the clotting reaction through interaction with activated factor VIII, which accelerates FX activation (Sun, 2005). This is achieved through anticoagulation and fibrin(ogen)olytic systems. Together, coagulation, anticoagulation, and fibrinolysis maintain a delicate physiological balance (Sun, 2005).

The main function of the anticoagulation system is to prevent or slow the propagation of clots. The major anticoagulants include antithrombin (AT), tissue factor pathway inhibitor (TFPI), and activated protein C (APC). AT is produced by the liver and inhibits several coagulation factors such as thrombin, FVIIa, FIXa, and FXa (Quinsey *et al.,* 2004; Roemisch *et al.,* 2006; Sun, 2005). TFPI is a serine protease that inhibits FXa. In the presence of FXa, TFPI also inhibits the TFFVIIa complex (Broze *et al.*, 1995; Broze *et al.*, 1998; Price *et al.*, 2004; Sun, 2005). When thrombin is produced, it can bind to thrombomodulin present on the vascular endothelial surfaces. Protein C is an inactive plasma serine protease. The thrombin/thrombomodulin complex can then cleave protein C into APC. APC generation is enhanced by the endothelial cell protein C receptor (EPCR) on the endothelial surface. APC, with cofactor protein S, can cleave and inactivate FVa and FVIIIa to negatively regulate coagulation (Aird *et al.*, 2004; Dahlback *et al.,* 1997; Esmon *et al.*, 1999; Sun, 2005).

The fibrin(ogen)olytic system functions to break down existing fibrin clots. The major protease of fibrin(ogen)olytic system is plasmin. Plasminogen is the inactive form of plasmin and circulates in the plasma. Plasminogen is activated by tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). This system can be inhibited by plasminogen activator inhibitor-1 (PAI-1), which inhibits both tPA and uPA, or by α2-antiplasmin, which inhibits plasmin (Collen *et al.*, 1999; Sun, 2005). The coagulation system is carefully regulated by pro-coagulant and anticoagulant proteins. However, the overwhelming infection can lead to dysregulation of this system, resulting in the pathological systemic thrombosis, known as disseminated intravascular coagulation, signifying the role of coagulation in pathology of infectious disease (Sun, 2005).

Accumulation of fibrin in the blood vessels can interfere with blood flow and lead to myocardial infarction and other serious CVDs. Unless the blockage is removed promptly, the tissue that is normally supplied with oxygen by the vessel die or become severely damaged. If the damaged region is large, the normal conduction of electrical signals through the ventricle may be disrupted, leading to an irregular heartbeat, cardiac arrest or death (Mihara et al., 1991).

Fibrin(ogen)olytic enzymes can be widely found in nature. They have been found in hemorrhagic toxins from snake venoms (Nikai et al., 1984), marine green alga (Matsubara et al., 2000), earthworm secretions (Mihara et al., 1991), food-grade microorganisms (Chang et al., 2005; Jeong et al., 2001), insects (Amarant et al., 1991; Hellmann and Hawkins, 1964; Matsushima et al., 1993), marine creatures (Mihara et al., 1991), and herbal medicines (Choi et al., 2001). They have been found in hemorrhagic toxins from snake venoms (Nikai et al., 1984), earthworm secretions (Mihara et al.,e 1991), food-grade microorganisms (Chang et al., 2005; Jeong et al., 2001), marine creatures (Mihara et al., 1991), and herbal medicines (Choi et al., 2001).

In particular, fibrin(ogen)olytic protease has been isolated from Spirodela polyrhiza, an ingredient of traditional oriental medicine has been used for lowering blood pressure and the detoxification of snake venom (Choi et al., 2001). Strong fibrin(ogen)olytic enzymes are also produced by *Bacillus* sp. Strains that are used in food fermentation, invertebrates like *Stichopus japonicus*, as well as the seaweed *Codiales codium* (Jeon et al., 1995).

This study describes the purification and characterization of the serine fibrin(ogen)olytic enzyme from a marine polychaete species, *L. izukai,* mainly focusing on the biochemical properties and fibrin(ogen)olytic activity.

ll. MATERIALS AND METHODS

ll-1. Materials

Tosyl-L-lysine chloromethyl ketone (TLCK), tosyl-phenylalanyl chloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride (PMSF), prothrombin, sodium dodecyl sulfate (SDS), 1,10-phenanthroline (1,10-PT), diisopropyl fluorophosphate (DFP), ammonium sulfate, azocasein, bovine serum albumin (BSA), thrombin, trizma base, β-mercaptoethanol, plasmin, fibrinogen, and other chemicals were purchased from Sigma (St. Louis, MO, USA). The homogenizer was purchased from PRO Scientific (Oxford, USA). Chromatographic columns, including HiPrep 16/10 Q FF, Mono Q 4.6/100 PE, Superdex 75 10/300 GL, and PD-10 were purchased from Amersham PharmaciaBiotech Co. (Uppsala, Sweden). The protein size marker was purchased from Bioprince (Chuncheon, Republic of Korea). Human plasma was prepared as follows: blood samples collected from healthy volunteers were put into a BD vacutainer tube containing 0.072 ml of 7.5% EDTA (Becton Dickinson, MD, USA) to prevent coagulation and centrifuged for 15 min at 990 x*g* to remove blood cells. The resulting plasma was stored at −70℃ until used. The synthetic chromogenic substrates H-D-Ile-Pro-Arg-*p*NA (S-2288), H-D-Pro-Phe-Arg-*p*NA (S-2302), H-D-Val-Leu-Lys-*p*NA (S-2251), H-D-Phe-Pip-Arg-*p*NA (S-2238), pyro-Glu-Pro-Arg-*p*NA (S-2366), H-D-Pro-Phe-Arg-*p*NA (S-2302), and N-α-Z-D-Arg-Gly-Arg-*p*NA (S-2765) were purchased from Chromogenix Co. (Milan, Italy). *L. izukai* individuals were collected from the coast of Wando island (34°20′27″N, 126°9′5″E, Republic of Korea), immediately washed, frozen, and stored at −70℃ until used. They were kindly supplied by Prof. S. M. Yoon's laboratory (Chosun University, Gwangju, Republic of Korea).

ll-2. Purification of a proteolytic enzyme from *L. izukai*

For the purification of the proteolytic enzyme, the frozen worms (approximately 50 g) were thawed in 100 ml of ice-cold 25 mM Tris-HCl buffer (pH 7.5), homogenized three times at two intervals of 5 min at the maximal speed using an electric homogenizer, and stirred for 6 h at 4℃ (Park *et al.*, 2014). Cell debrises were removed from the homogenate by centrifuging at 12,000 x*g* and 4℃ for 15 min and the supernatant was subjected to ammonium sulfate precipitation (Park *et al.*, 2014). Ammonium sulfate was added to the crude extracts at a saturation concentration of 20%, stirred for 3 h at 4℃, and then centrifuged for 40 min at 12,000 x*g*. The resulting protein precipitate was discarded. The soluble proteins that remained in the supernatant were further fractionated with 80% saturated ammonium sulfate, in which the sample was stirred for 6 h at 4℃ and the resulting protein precipitate was collected by centrifuging for 40 min at 12,000 x*g* and 4℃ (Park *et al.*, 2014). The proteins obtained were dissolved in 25 mM Tris-HCl buffer (pH 7.5) and desalted on a PD-10 column equilibrated with the 1.4-time buffer volume. The desalted proteins (approximately 450 mg) were loaded onto a HiPrep 16/10 Q FF column, equilibrated with 25 mM Tris-HCl buffer (pH 7.5) and the bound proteins were eluted by a linear NaCl gradient ranging from 0 to 0.5 M in the same buffer. The active fractions were pooled and then desalted on a PD-10 column equilibrated with 25 mM Tris-HCl buffer (pH 7.5) (Park *et al.*, 2014). The desalted proteins (19.2 mg in total) were loaded onto the first Mono Q 4.6/100 PE column, equilibrated with 25 mM Tris-HCl buffer (pH 7.5) and the bound proteins were eluted by a linear NaCl gradient from 0 to 0.5 M in the same buffer. Once again, the active fractions were pooled and then desalted on a PD-10 column equilibrated with 25 mM Tris-HCl buffer (pH 7.5). The desalted proteins (3.7 mg in total) were loaded onto the second Mono Q 4.6/100 PE column, equilibrated with 25 mM Tris-HCl buffer

(pH 7.5) and the bound proteins were eluted by a linear NaCl gradient from 0 to 0.4 M in the same buffer. Protein peak fractions were pooled (3.15 mg) concentrated and loaded separately onto a Superdex 75 10/300 GL gel filtration column, equilibrated with 25 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl (Park *et al.*, 2014). The active fractions were collected and stored at −20℃ as the purified enzyme. Protein concentrations were determined by the Bradford method (Bradford, 1976).

ll-3. Assay of protease activity on casein-agarose plate

A casein-agarose plate was made in 25 mM Tris-HCl buffer (pH 7.5) with 2% agarose, 2% sodium casein and poured into a petri dish. Each of the Seventy-microgram ammonium sulfate samples was placed in 5 mm wells on the plate, incubated at 37℃ for 21 h and the enzyme activity was determined by measuring the size of the clear zone.

ll-4. Determination of protein concentration

The determination of protein concentration was performed according to the Bradford procedure using BSA as the standard (Bradford, 1976). Bradford reagent was added to the reaction mixture and vortexed. The amount of protein was determined by measuring the absorbance at 595 nm after 2 min.

Ⅱ-5. Protease activity assay

Azocasein assay was routinely used for examining protease activity in the chromatographic steps (Chang *et al.*, 2005). In a typical azocasein assay, the

reaction mixture (200 μl) composed of 0.5% azocasein, 25 mM Tris-HCl (pH 7.5) and 0.5 μg of the enzyme to be assayed was incubated at 37℃ for 30 min (Park *et al.*, 2014). The reaction was stopped by the addition of 100 μl of 10% TCA and centrifuged at 10,000 x*g* for 10 min (Park *et al.*, 2014). From the resulting supernatant, 200 μl sample was withdrawn and the absorbance at 440 nm was measured. In this assay, one unit of enzyme activity was defined as the amount of protease digesting 1 μg of azocasein per min.

ll-6. Substrate specificities of LIZ protease

The chromogenic substrates used for assaying the amidolytic activity of protease were as follows: VPR for Thrombin; LGR for Factor-Xa; S-2238 for thrombin; S-2251 for plasmin; S-2288 for t-PA; S-2302 for FXIIa; S-2444 for u-PA; S-2765 for Factor-X. The reaction was carried out in a total volume of 100 μl with 0.5 μg of the purified enzyme. Approximately 10 μl of a 4 mM stock of each substrate was added to the reaction mixture and the reaction was continued for 1 h. Amidolytic of a chromogenic substrate, S-2288 was used as the control. LIZ enzyme was able to cleave various plasma protein substrates, including fibrinogen, plasminogen, and prothrombin. Each protein substrate (10 μg) was incubated with LIZ (0.5 μg) for 10 min at 37℃ and then electrophoresed on a 12% SDS-polyacrylamide gel.

ll-7. Optimal pH and temperature for LIZ enzyme activity

The buffer systems used in the chromogenic substrate for the pH requirement of enzyme were as follows: 25 mM sodium acetate (pH 4.0~5.0); 25 mM PIPES (piperazine-N,N-bis) (pH 5.5~6.0); 25 mM phosphate (pH 6.0~7.0); 25 mM Tris-HCl

(pH 7.5~11.0) (Park *et al.*, 2014). The effects of temperature on enzyme activity in the assay was examined for 1 h at various temperature conditions. The effects of various protease inhibitors and divalent cations on enzyme activity were examined using chromogenic as a substrate with or without the corresponding additives at 37℃ for 1 h (Park *et al.*, 2014).

ll-8. Effects of protease inhibitors on proteolytic activity of LIZ

Various protease inhibitors were tested with the purified enzyme under optimum reaction conditions in a total volume of 100 μl. Aliquots (0.5 μg) of the purified protease were pre-incubated with several inhibitors for 10 min at 37℃. The 10 μl of 10 mM protease inhibitors were added to the reaction mixture and the protease activity was assayed as described at 1 h. Residual activities in the presence of the inhibitors were compared with that of control without inhibitors (Park *et al.*, 2012).

ll-9. Fibrin(ogen)olytic activity assay

For the fibrin(ogen)olytic activity assay, 200 μl of reaction mixture consisting of fibrinogen (100 μg) and purified enzyme (3 μg) in 25 mM Tris-HCl buffer (pH 7.5) were placed in a microcentrifuge tube and incubated at 37℃ and 20 μl of reactants were withdrawn at the intervals of 0, 0.5, 1, 2, 3, 4, 5, 6, 10, and 20 min (Park *et al.*, 2014). The reaction was stopped by the addition of 1 μl of 10 mM PMSF and the resulting products were analyzed by SDS-PAGE as previously described (Chang *et al.*, 2005). The fibrin(ogen)olytic activity of the enzyme was measured on the fibrin plate and also by turbidity assay as previously described. Fibrin(ogen)olytic activity was determined by an artificial fibrin plate assay, synthesized by the method

described by Astrup and Mullertz (Astrup and Mullertz, 1952). Plasmin from human plasma (2 U/mg protein) was applied as a positive control. Fibrin plate was made by mixing 2 ml of 1% fibrinogen, 2 ml of 1% agarose, and 90 μl of thrombin (17.7 U/ml) in 25 mM Tris-HCl buffer (pH 7.5). In the wells (5 mm in diameter) made in the plate, 20 μl each of Tris-buffered saline (TBS; 50 mM Tris-HCl buffer pH 7.5, NaCl 150 mM), purified enzyme (1 μg), and plasmin (1 μg) were inoculated and incubated at 37℃ for 21 h to visualize halo zones (Park *et al.*, 2014). A turbidity assay was performed by measuring the decrease in fibrin polymer turbidity caused by the enzyme in a 96-well plate. Typically 90 μl of 1 mg/ml fibrinogen in 25 mM Tris-HCl buffer (pH 7.5) was added to 10 μl thrombin (17.7 U/ml) and incubated for 2 h at 37℃ to allow the forming of fibrin polymer (Park *et al.*, 2014). Thereafter, purified enzyme (1 and 2 μg) and plasmin (1.5 μg) were added and incubated for 1 h at 37℃. The decrease in absorbance at 350 nm was then recorded with a 96-well plate reader (Molecular Devices Corp., Orleans, CA, USA). Cross-linked fibrin was formed by incubating each 20 μl of reaction mixture consisting of 20 μg fibrinogen, 0.002 U FXIIIa, 0.02 U thrombin, and 1 mM $CaCl_2$ in 25 mM Tris-HCl buffer (pH 7.5) at 25℃ for 1 h. For the detection of cross-linked fibrin cleavage, 0.5 μg of the purified enzyme was added to the cross-linked fibrin sample and incubated at 37℃ for 30 min (Park *et al.*, 2014). The reaction was stopped by the addition of 6 μl of 6x SDS-PAGE sample buffer and heated at 100℃ for 3 min. The reaction products were electrophoresed onto 8% SDS-polyacrylamide gel and visualized by staining the gel with Coomassie brilliant blue (Park *et al.*, 2014).

ll-10. Turbidity assay in human plasma

The turbidity assay was performed by measuring the decrease in turbidity of fibrin polymer formed by the enzyme in a 96-well plate as described previously

(Park *et al.,* 2014). Typically 90 μl of 20% human plasma was added to 10 μl thrombin (17.7 U/ml) and incubated for 2 h at 25℃ to allow the formation of the fibrin polymer. Thereafter, purified enzyme (3 μg) and plasmin (3 μg) were added and incubated for 3 h at 37℃. The decrease in absorbance at 350 nm was then recorded with a 96-well plate reader (Molecular Devices Corp., Orleans, CA, USA).

ll-11. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (Laemmli, 1970). Protein samples were mixed with an equal volume of reducing 6x SDS-PAGE sample buffer, boiled at 100℃ for 3 min, and then loaded onto 8~12% SDS-polyacrylamide gel. After electrophoresis, protein bands were visualized by staining the gel with Coomassie brilliant blue.

III. RESULTS AND DISCUSSION

Ⅲ-1. Purification of LIZ enzyme

To purify the enzyme, five purification steps, including ammonium sulfate fractionation (20~80%), HiPrep 16/10 Q FF anion exchange column, sequential repeated Mono Q 4.6/100 PE column, and Superdex 75 10/300 GL chromatographies were used in the order (Fig. 3). Initially the worm crude extract (approximately 700 mg) was fractionated with 20~80% range of ammonium sulfate (Fig. 4). The ammonium sulfate-fractionated proteins showing protease activity on the casein plate were applied onto a HiPrep Q FF column (Fig. 5). The proteins bound on the column were eluted by a linear NaCl gradient of 0 to 0.5 M. The proteolytic activity of each fraction was assayed by using azocasein as a substrate. As shown in Fig. 5, sixteen fractions (30 to 45) eluted by 0.3 to 0.45 M range of NaCl showing protease activities were pooled as an active fraction. The proteins pooled were concentrated by ultrafiltration using a YM 10 membrane and then desalted on a PD-10 column. The desalted proteins were loaded onto the first Mono Q 4.6/100 PE column and the bound proteins were eluted by a linear NaCl gradient of 0 to 0.5 M (Fig. 6). As shown in the activity profile of Fig. 6, four fractions (19 to 22) eluted by the 0.19 to 0.22 M range of NaCl showed protease activity and the proteins contained were pooled as an active fraction. The proteins pooled were loaded onto the second Mono Q 4.6/100 PE column, and the bound proteins were eluted by a linear NaCl gradient of 0 to 0.4 M (Fig. 7). As shown in the activity profile in Fig. 7, five fractions (21 to 25) eluted by the 0.28 to 0.3 M range of NaCl exhibited protease activity. These fractions were pooled and further separated by a Superdex 75 10/300 GL column chromatography, in which the typical protease activity appeared at the fraction number 12 and the proteins contained were

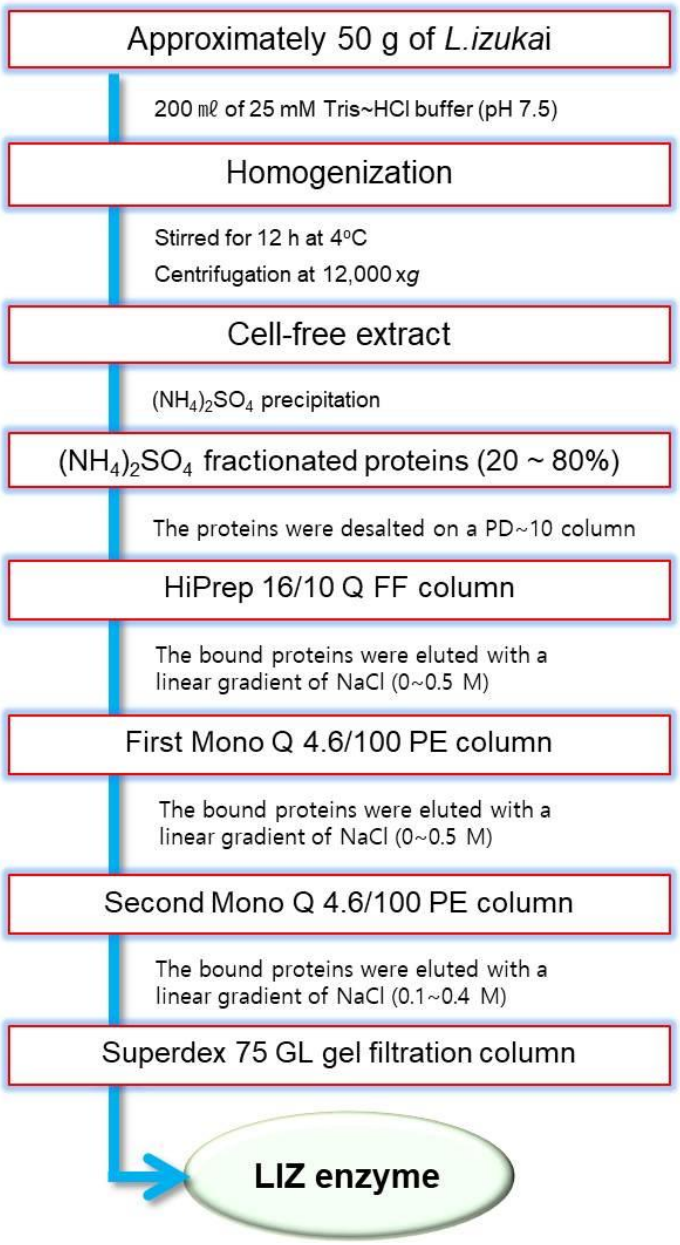

Fig. 3. Purification scheme for LIZ enzyme from *L. izukai*. To purify the enzyme, five purification steps, including ammonium sulfate fractionation (20~80%), HiPrep 16/10 Q FF anion exchange column, sequential repeated Mono Q 4.6/100 PE column, and Superdex 75 10/300 GL chromatographies were used in the order.

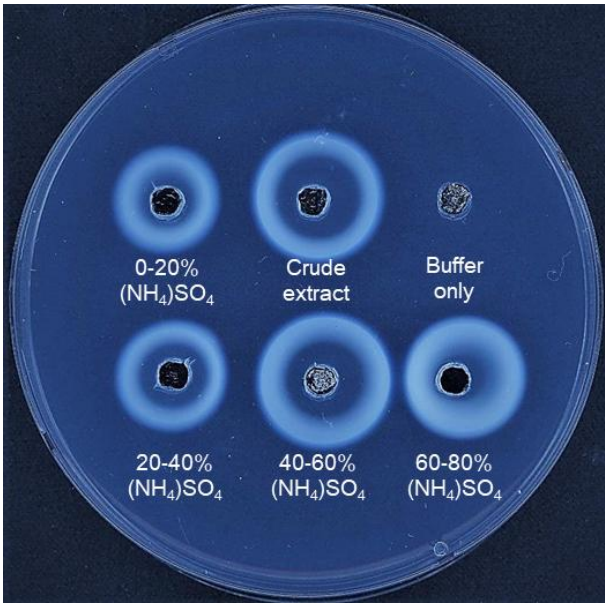

Fig. 4. Proteolytic activities of the ammonium sulfate-fractionated proteins on 2% casein plate. Seventy micrograms each of the fractionated proteins were inoculated in the hole and incubated for 21 h at 37℃ to visualize halo zones.

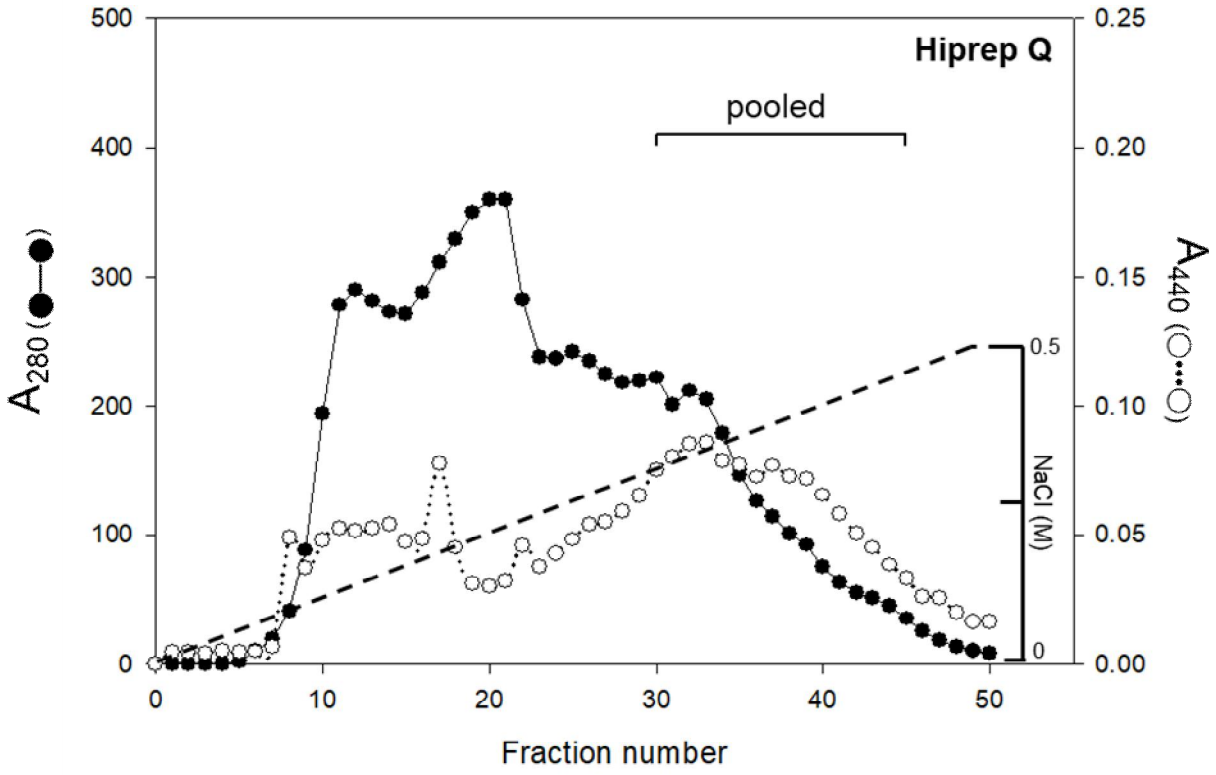

Fig. 5. Purification of LIZ enzyme from *L. izukai* using HiPrep Q column chromatography. Proteins fractionated with 20~80% ammonium sulfate were loaded onto an anion exchanger HiPrep Q column and the bound proteins were eluted by a linear NaCl gradient ranging from 0 to 0.5 M. Active fractions from fraction numbers 30 to 45 were pooled as indicated, and 50 μl aliquots of the active fractions were assayed for protease activity with azocasein as a substrate, in which the absorbance at 440 nm was measured (○). In the chromatographic step, protein concentrations were monitored by measuring the absorbance at 280 nm (●).

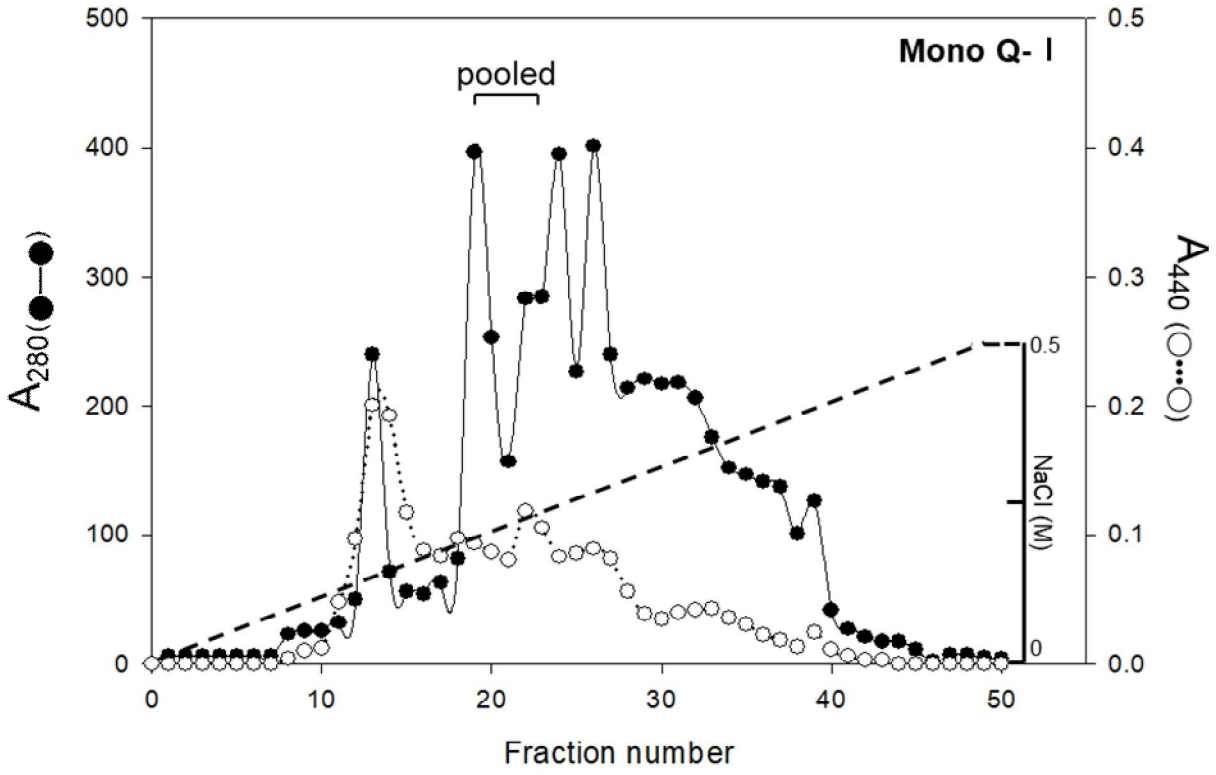

Fig. 6. Purification of LIZ enzyme from *L. izukai* using Mono Q－Ⅰ column chromatography. Proteins collected from the HiPrep Q column (Fig. 5) were applied onto an anion exchanger Mono Q column and the bound proteins were eluted by a linear NaCl gradient ranging from 0 to 0.5 M. Active fractions from fraction numbers 19 to 22 were pooled as indicated, and 50 μl aliquots of the active fractions were assayed for protease activity with azocasein as a substrate, in which the absorbance at 440 nm was measured (○). In the chromatographic step, protein concentrations were monitored by measuring the absorbance at 280 nm (●).

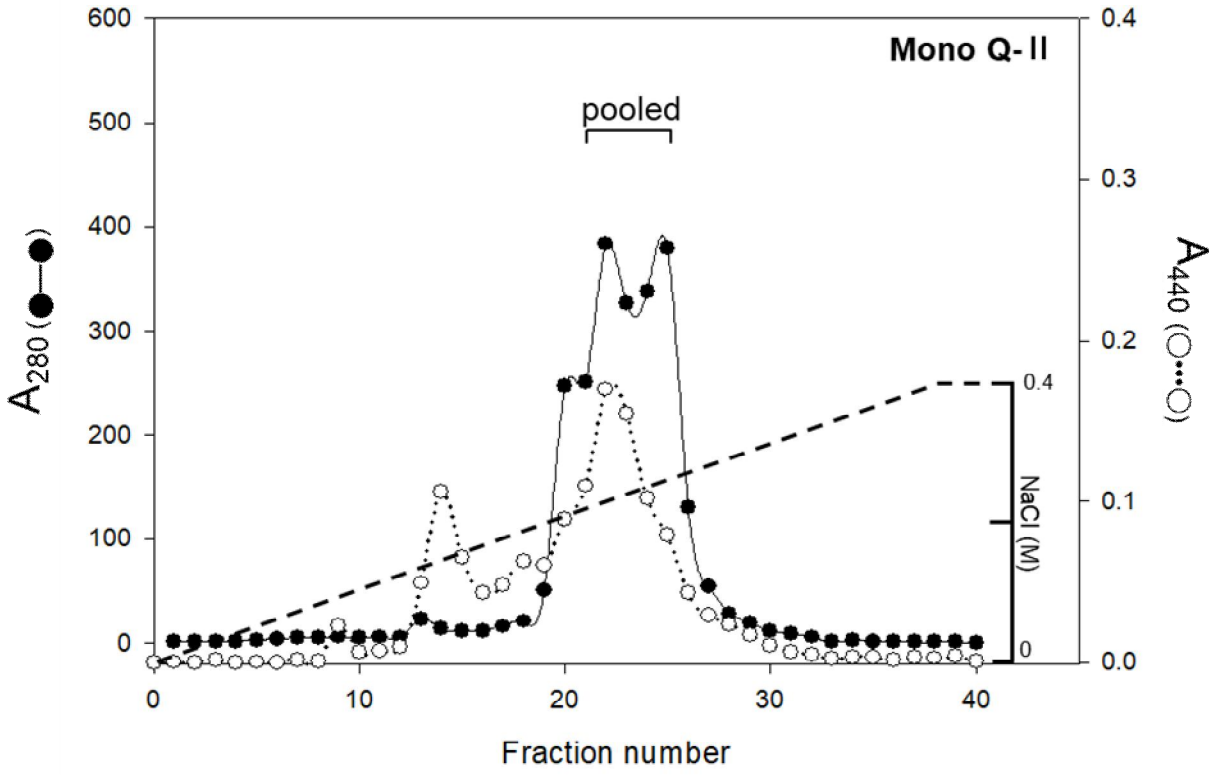

Fig. 7. Purification of LIZ enzyme from *L. izukai* using Mono Q−II column chromatography. Proteins collected from the Mono Q-I column chromatography (Fig. 6) were applied onto an anion exchanger Mono Q-II column and the proteins bound were eluted by a linear NaCl gradient ranging from 0 to 0.4 M. Active fractions from fraction numbers 21 to 25 were pooled as indicated, and 50 μl aliquots of the active fractions were assayed for protease activity with azocasein as a substrate, in which the absorbance at 440 nm was measured (○). In the chromatographic step, protein concentrations were monitored by measuring the absorbance at 280 nm (●).

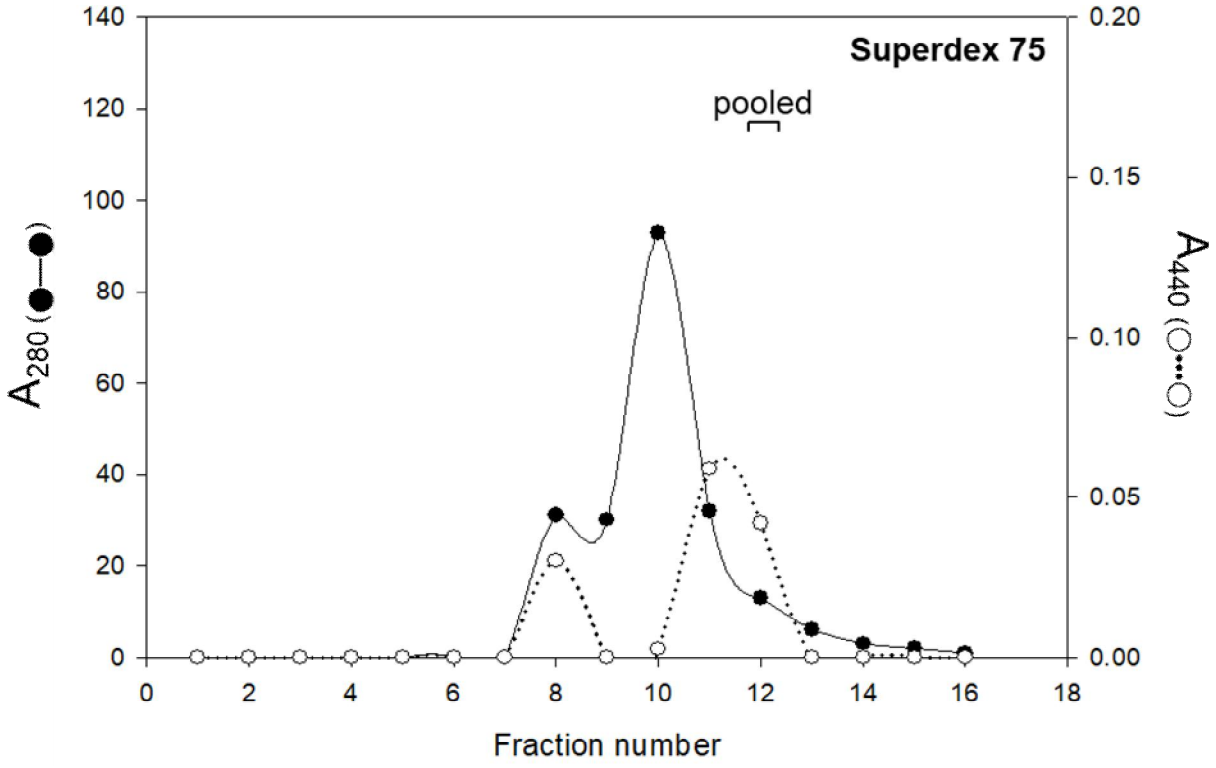

Fig. 8. Purification of LIZ enzyme from *L. izukai* using Superdex 75 column chromatography. Proteins collected from the Mono Q-II column chromatography (Fig. 7) were applied onto a Superdex 75 10/300 GL column and the proteins were eluted with Tris-HCl buffer (pH 7.5). LIZ enzyme appeared in fraction number 12. Protein concentrations were monitored by measuring the absorbance at 280 nm (●). The protease activity was assayed with azocasein as a substrate in which the absorbance at 440 nm was measured (○).

regarded as finally purified enzyme (Fig. 8). The purified enzyme was designated to as LIZ (stands for *Lepidasthenia izukai*). As shown in Fig. 9, the purified LIZ enzyme appeared as a single band on a 12% SDS-polyacrylamide gel after staining with Coomassie brilliant blue and its molecular weight was estimated to be 28 kDa. Table 1 summarizes the purification results. The specific activity of purified LIZ was estimated to be 1,628 U/mg protein and 0.8 mg of enzyme could be obtained from 700 mg of total cell extracts in a yield of 0.9% (Table 1).

III-2. Effects of various inhibitors and metal ions on LIZ enzyme activity

It is well known that the biochemical properties of an enzyme can be revealed by using various inhibitors and cofactors (Sigma and Mooser 1975; Adinarayana *et al.*, 2003). In this study, the effects of various protease inhibitors and metal ions on the proteolytic activities of LIZ were examined as summarized in Table 2. The proteolytic activity of LIZ enzyme was inhibited by serine protease inhibitors such as PMSF and DFP, but not by metalloprotease inhibitors, including ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline (1,10-PT). PMSF is known to sulphonate the essential serine residue in the active site of serein protease and has been reported to result in the complete loss of enzyme activity (Gold, 1964). Therefore, these results suggest that LIZ is a typical serine protease. Some metal ions such as Ca^{2+}, Mg^{2+}, and especially Zn^{2+} were considered as important cofactors for protease activity. Therefore, the effect of metal ions on LIZ enzyme activity was investigated (Table 2). Except for Zn^{2+}, the other metal ions had no inhibitory effect of LIZ.

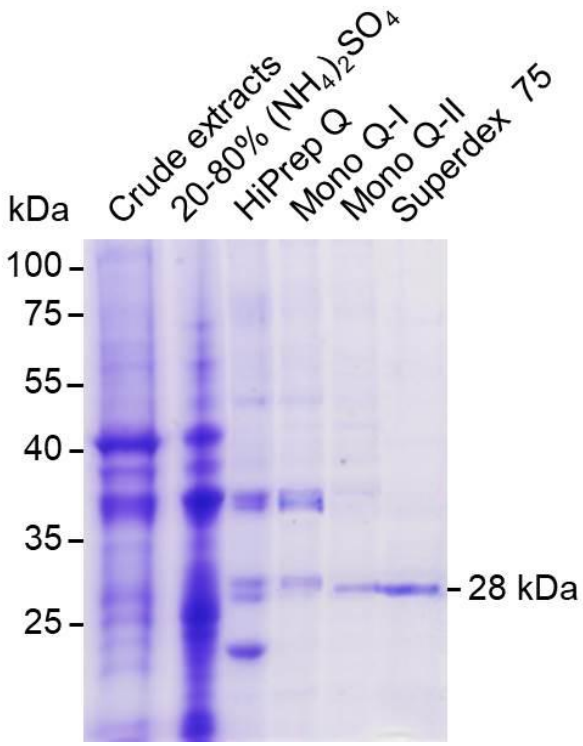

Fig. 9. SDS-PAGE of proteins obtained from various purification steps. The active fractions collected from each chromatographic step were subjected to 12% SDS-PAGE under reducing condition and then stained with Coomassie brilliant blue for visualization. Protein samples were prepared in the presence of 1 mM of PMSF to prevent autodegradation.

Table 1. Summary of the purification of LIZ enzyme from *L. izukai*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)[a]	Yield (%)[b]
Crude extract	700	145,038	207	100
20~80% $(NH_4)_2SO_4$	450	104,798	232	72.2
HiPrep Q FF 16/10	19.2	20,086	1,046.1	15.2
Mono Q-I 4.6/100 PE	3.7	5,307	1,434.3	4.0
Mono Q-II 4.6/100 PE	3.15	4,797	1,523.1	3.6
Superdex 75 10/300 GL	0.8	1,302	1,628	0.9

[a] One unit of an enzyme was defined as the amount of a protease digesting 1 μg of azocasein/min.

[b] The total activity of the crude extract was assigned the value of 100%.

Table 2. Effects of various inhibitors and metal ions on LIZ enzyme activity.

Additive	Concentration (mM)	Relative activity (%)[a]
Control	-	100 ± 3.4
TPCK	0.1	66 ± 5.3
TLCK	1	104 ± 11
PMSF	1	0 ± 0
Aprotinin	0.1	47 ± 0.2
EGTA	1	119 ± 8.2
EDTA	1	117 ± 2.4
DTT	1	107 ± 0.7
DFP	1	10 ± 1.2
1,10-PT	1	108 ± 0.6
Ca^{2+}	1	100 ± 2.3
Cu^{2+}	1	106 ± 6.6
Mg^{2+}	1	103 ± 7.9
Mn^{2+}	1	96 ± 1
Ni^{2+}	1	83 ± 1.5
Zn^{2+}	1	75 ± 6.5

[a] In the activity assay, S-2288 was used as a substrate for LIZ with the corresponding additive at 37℃ for 1 h. Data from the duplicate experiments were expressed as mean values ± S.D.

III-3. Optimal pH and temperature for LIZ enzyme activity

The proteolytic activity of LIZ was examined with a chromogenic substrate S-2288. As shown in Fig. 10, LIZ enzyme exhibited maximal activity at pH 7.5 when the synthetic substrate was used. This proteolytic activity of LIZ was similar to those of fibrin(ogen)olytic enzymes, TMFE from *Tenebrio molitor* (Huang *et al.*, 2012), a serine protease from *Petasites japonicus* (Kim *et al.,* 2015), and a protease from *Cordyceps militaris* (Xiaolan *et al.,* 2017). LIZ showed pH stability (7.0 to 8.0) like subtilisin DFE (Peng *et al.,* 2003). The LIZ activity was the highest in the pH range of 7.0 to 8.0 and sharply decreased above pH 8.5 (Fig. 10). The enzyme seemed to be relatively stable at 20℃ to 60℃, although the optimal temperature was found to be 60℃ (Fig. 11). These properties were similar to those of NK from *B. natto* (Fujita *et al.,* 1993), a fibrin(ogen)olytic peptidase from *Bacillus thuringiensis* IMB B-7324 (Matseliukh *et al.,* 2011), a serine fibrin(ogen)olytic protease from *Bacillus megaterium* KSK-07 (Kotb. E., 2015), and subtilisin from *Bacillus* (Peng *et al.,* 2003).

III-4. Substrate specificity of LIZ enzyme

LIZ enzyme was also able to cleave various blood coagulation-associated proteins, including fibrinogen, plasminogen, and prothrombin (Fig. 12). LIZ enzyme showed amidolytic activity towards various synthetic peptide substrates. Among the 9 chromogenic substrates tested, S-2238, S-2251, S-2288, S-2366, S-2302, S-2444, VPR, LGR, and S-2765 were hydrolyzed by the enzyme at different rates, suggesting that they have different specificities to the enzyme (Table 3). As shown in Table 3, the highest amidolytic activity of LIZ was found with S-2288. All these results suggest that LIZ may have a proteolytic activity that can cleave the carboxyl side of arginine in the peptide substrate. However, the cleavage site of LIZ on its protein substrate should be elucidated in further study.

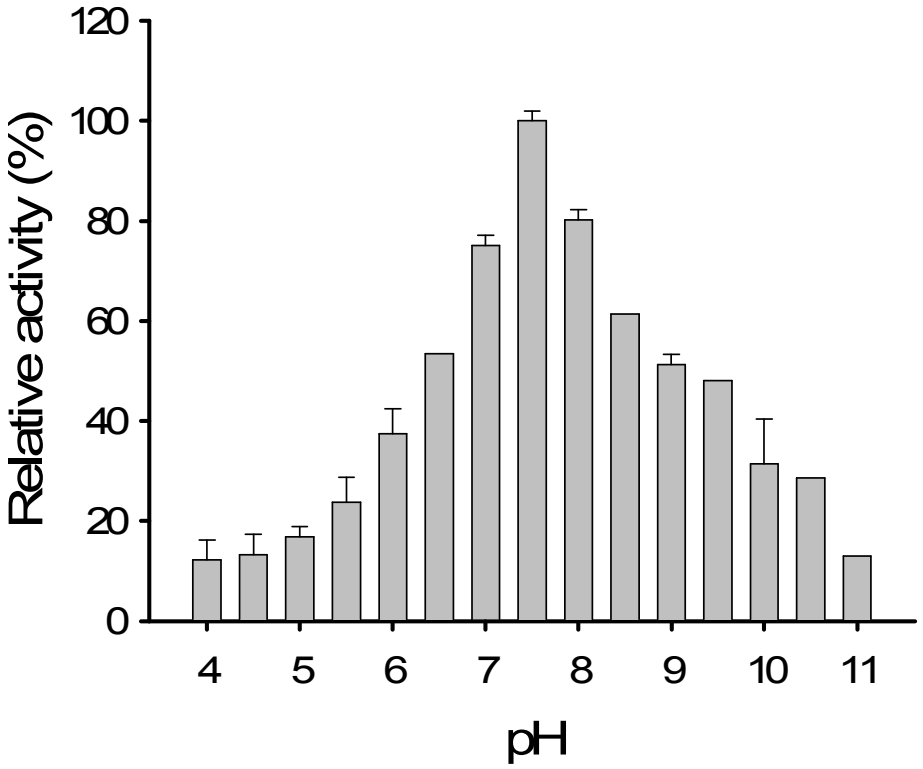

Fig. 10. Effect of pH on LIZ enzyme activity. LIZ enzyme (0.5 μg) was incubated at 37℃ for 1 h with S-2288 as a substrate under different pHs and the absorbance at 405 nm were measured.

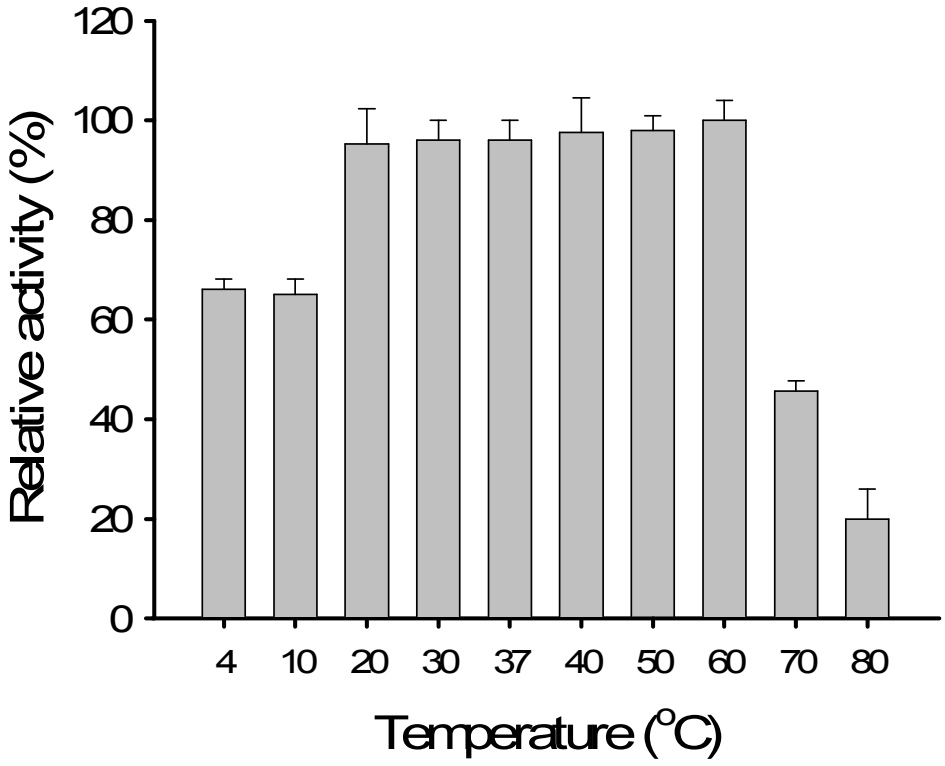

Fig. 11. Effect of temperature on LIZ enzyme activity. LIZ enzyme (0.5 μg) was incubated with S-2288 as a substrate at various temperatures for 1 h as indicated and the absorbance at 405 nm was measured.

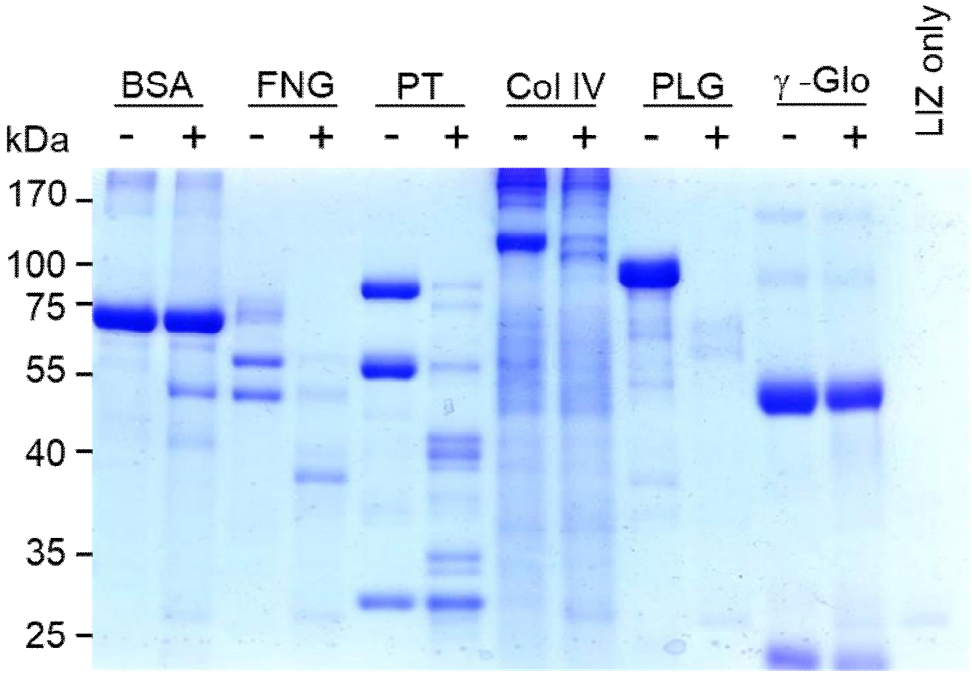

Fig. 12. Cleavage of various plasma protein substrates by LIZ enzyme. Each protein substrate (10 μg) was incubated with of LIZ enzyme (0.5 μg) for 10 min at 37℃ and electrophoresed on a 12% SDS-polyacrylamide gel. The symbols - and + indicate the omission or addition of LIZ respectively. BSA, bovine serum albumin; FNG, fibrinogen; PT, prothrombin; Col IV, type IV collagen; PLG, plasminogen; γ-Glo, γ-globulin.

Table 3. Amidolytic activity of LIZ enzyme against synthetic protease substrates.

Substrate	Relative activity (%)[a]	Typical substrate for	Formula
S-2288	100 ± 5	t-PA	H-D-Ile-Pro-Arg-*p*NA
S-2238	53 ± 12	Thrombin	H-D-Phe-Pip-Arg-*p*NA
S-2251	5 ± 1.6	Plasmin	H-D-Val-Leu-Lys-*p*NA
S-2366	4.4 ± 0	FXIa	pyro-Glu-Pro-Arg-*p*NA
S-2302	0 ± 0	FXIIa	H-D-Pro-Phe-Arg-*p*NA
S-2765	0 ± 0	FXa	Z-D-Arg-Gly-Arg-*p*NA
S-2444	0 ± 0	Urokinase	Pyro-Glu-Gly-Arg-*p*NA
VPR	51 ± 1.8	Thrombin	Boc-Val-Pro-Arg-*p*NA
LGR	0 ± 0	FXa	Boc-Leu-Gly-Arg-*p*NA

[a] The relative activity was expressed as a percentage of the activity appeared with S-2288 as a substrate. Data from the duplicate experiments were expressed as mean values ± S.D.

Ⅲ-5. Fibrin(ogen)olytic activity of LIZ enzyme

The LIZ enzyme also exhibited fibrinogen-degrading activity as shown by 12% SDS-PAGE (Fig. 13). The Aα chain of fibrinogen could be totally degraded by the enzyme within 30 sec at the mass ratio of 1:33 (enzyme versus fibrinogen) and the Bβ, and γ chains of the protein were also completely digested for 20 min (Fig. 13). It has been reported that the γ-chains of fibrinogen from a triple-stranded α-helical coiled-coil structure, which is often extremely resistant in cleaving by most of the fibrin(ogen)olytic enzymes (Martinez *et al.*, 2013). Even plasmin does not efficiently cleave the γ-chain, while it actively degrades the Aα and Bβ chains of fibrinogen (Siritapetawee *et al.*, 2012). Therefore, it has been postulated that LIZ enzyme has a relatively unique property in that it can cleave the γ-chain of fibrinogen, unlike other fibrinogenolytic enzymes. In this study, the proteolytic activity of LIZ to fibrin polymer was also examined with turbidity assay (Swenson and Markland 2005; Xiao *et al.*, 2007). As shown in Fig. 14, the relative turbidity of fibrin polymers was decreased by treatments with 1.5 μg of plasmin and LIZ enzyme in a time-dependent manner. These results suggest that the enzyme can actively cleave fibrin polymers that are spontaneously formed by thrombin-catalyzed conversion of fibrinogen to fibrin monomers (Fig. 14). The fibrinolytic activity of LIZ to cross-linked fibrin that was formed from fibrinogen by FXIIIa and thrombin in the presence of 1 mM Ca^{2+} was also observed by cleavage assay on an 8% SDS-polyacrylamide gel (Fig. 15). As shown in Fig. 15, the cross-linked α-α and γ-γ chains of fibrin that were catalyzed by FXIIIa were resistant to plasmin degradation because of their stable and insoluble structures (Mosesson, 1992; Mosesson, 2005). However, the α–α and γ–γ chains were susceptible to the cleavage by LIZ enzyme (Fig. 15). The susceptibility of cross-linked fibrin to cleavage by LIZ enzyme could also be observed on the fibrin plate (Fig. 16). When plasmin (1 μg) and

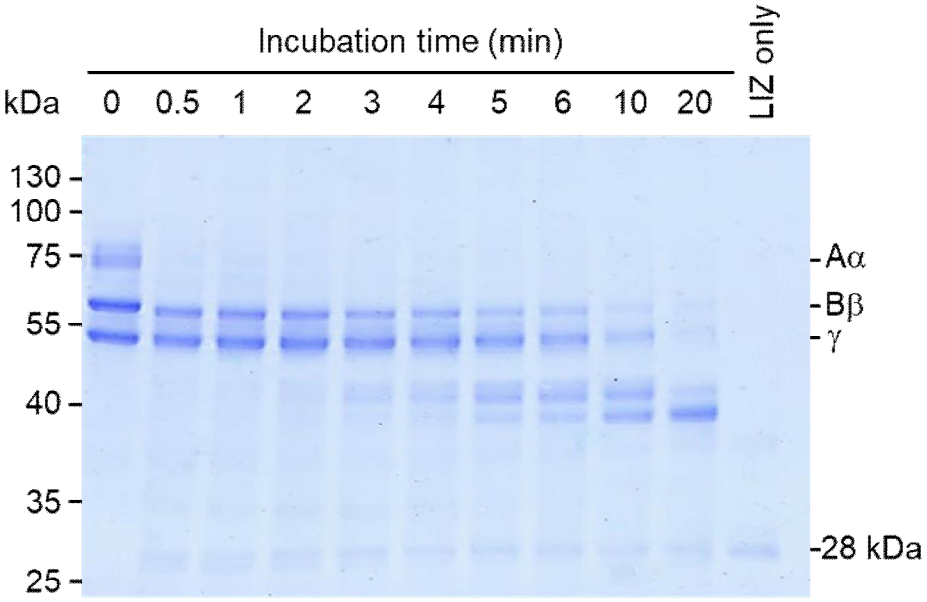

Fig. 13. Fibrin(ogen)olytic activity of LIZ enzyme. In this assay, fibrinogen (10 μg) was incubated with LIZ (0.3 μg) for various time periods at 37℃. The reaction products were electrophoresed on a 12% polyacrylamide gel and stained with Coomassie brilliant blue. The positions of Aα, Bβ, and γ chains of fibrinogen on the gel were shown on the right side of the panel.

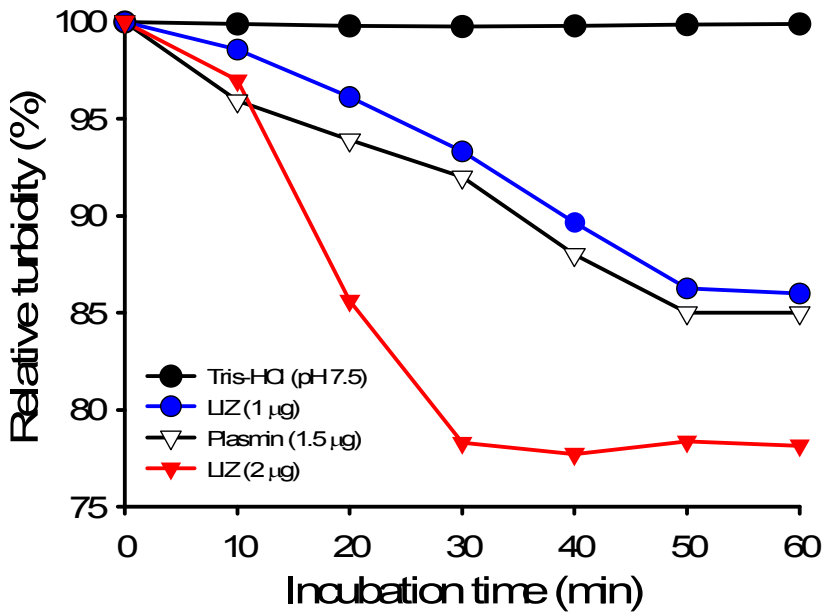

Fig. 14. Turbidity assay for fibrinolytic activity of LIZ. In this assay, 90 μl of fibrinogen (1 mg/ml) were treated with 10 μl of thrombin (17.7 U/ml) for 1 h at 37℃ and then 10 μl each of Tris-HCl (pH 7.5), plasmin (1.5 μg), and LIZ (1 and 2 μg) were treated for 1 h at 37℃, during which the decrease in absorbance at 350 nm was recorded with a 96-well plate reader. The cleavage of the fibrin polymer was expressed as a percentage of a decrease in turbidity, relative to that at the beginning of incubation at t=0. Symbols ●, Tris-HCl; ▽, plasmin (1.5 μg); ●, LIZ (1 μg); ▼, LIZ (2 μg).

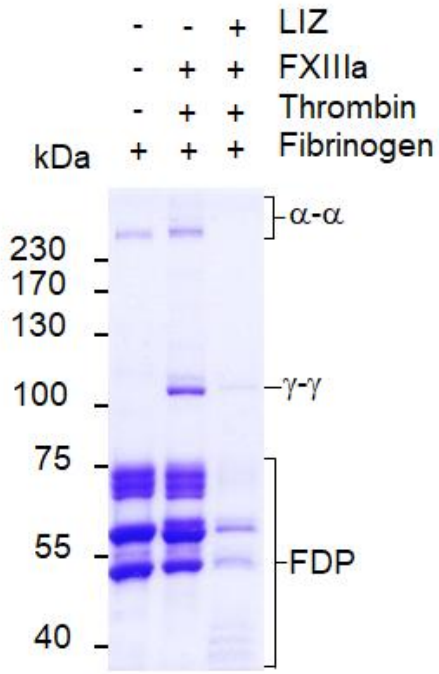

Fig. 15. Fibrinolytic activity of LIZ enzyme against to cross-linked fibrin. The cross-linked fibrin formed by incubating fibrinogen (20 μg) and thrombin (0.02 U) in the presence of FXIIIa (0.002 U) was incubated with LIZ enzyme (0.5 μg) for 30 min at 37℃. The reaction products were electrophoresed on an 8% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Symbols + and − represent addition and omission of the corresponding factor, respectively. The positions of α-α and γ-γ chains of cross-linked fibrin are shown on the right side of the gel. FDP, fibrin degradation products.

LIZ enzyme (1 μg) were applied into the wells of the fibrin plate, followed by incubation for 21 h at 37℃, halo zones clearly appeared (Fig. 16). The fibrin assay showed that the radii of halo zones formed by treatments with plasmin and LIZ enzyme were 1.8, and 1.2 cm, respectively. These results suggest that the adjusted plasmin (0.002 U) unit of LIZ can be calculated to be 0.004, indicating that LIZ enzyme exhibited stronger fibrinolytic activity than plasmin.

Ⅲ-6. Efficacy of LIZ in cleaving fibrin clots under blood plasma milieu

In human blood plasma, a large amount of proteins are contained (Anderson and Anderson, 1977). These include albumin (~55%), globulin (~38%), fibrinogen (~7%), (pro)enzymes, and protease inhibitors (less than 1% each) (Adkins et al., 2002; Anderson and Anderson, 1977; Park et al., 2014). Plasma also contains a variety of inorganic materials such as Na^{2+}, Cl^{-}, K^{+}, and Mg^{2+}. In this study, the fibrinolytic activities of LIZ enzyme and plasmin as a positive control within human blood plasma were examined (Fig. 17). As shown in Fig. 17, plasmin (3 μg) and LIZ (3 μg) decreased the turbidity of plasma clot to 12.3% and 17.5%, respectively, at 3 h (Fig. 17). These results suggest that LIZ could the fibrin clots under the plasma milieu as with plasmin. Taken together, all these results suggest that the purified LIZ enzyme is active fibrin(ogen)olytic serine protease that can dissolve blood thrombi.

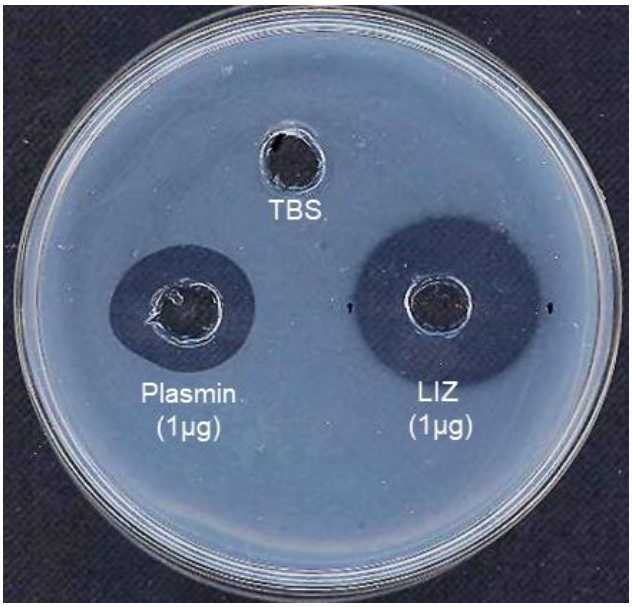

Fig. 16. Fibrinolytic activity of LIZ enzyme on fibrin plate. Tris-buffered saline (TBS; 50 mM Tris-HCl buffer pH 7.5, NaCl 150 mM), plasmin (1 μg), and LIZ enzyme (1 μg) were inoculated in the holes made in the fibrin plate and incubated for 21 h at 37℃.

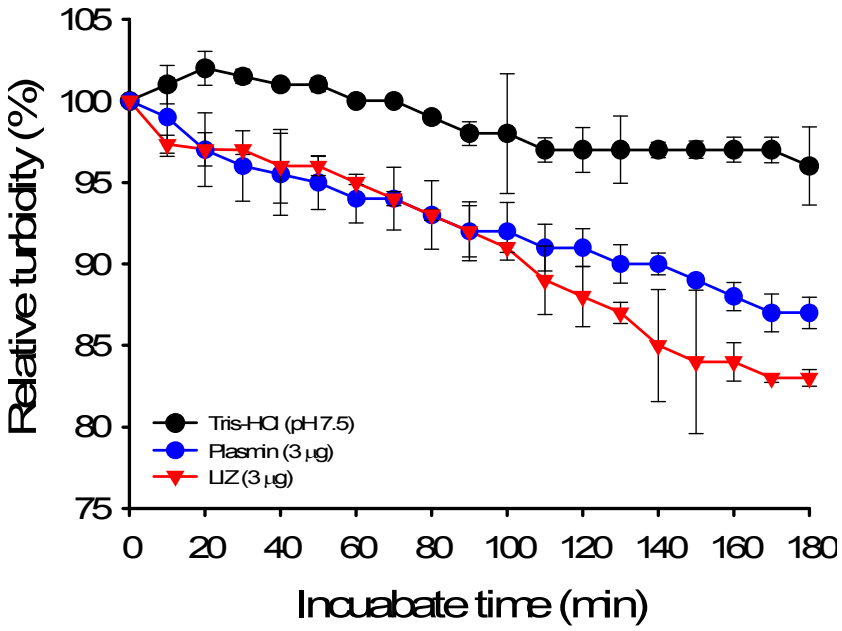

Fig. 17. Fibrinolytic activity of LIZ enzyme in human blood plasma. In this assay, 90 μl of 20% human plasma were incubated with 10 μl of thrombin (17.7 U/ml) for 2 h at 25℃ and then 10 μl each of 25 mM Tris-HCl (pH 7.5), plasmin (0.01 U), and LIZ (3 μg) were added for 3 h at 37℃, during which the decrease in absorbance at 350 nm was recorded with a 96-well plate reader. Data from duplicate experiments were expressed as mean values ± S.D. Symbols ●, Tris-HCl; ●, Plasmin; ▼, LIZ.

IV. 초록

이즈카긴비늘갯지렁이로부터 피브린분해 단백질분해효소의 분리 및 생화학적 특성분석

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본 연구는 국내 서·남해안에 서식하는 다모강 환형동물문의 하나인 이즈카긴비늘갯지렁이(*Lepidasthenia izukai*)로부터 피브리노겐 및 피브린을 분해하는 단백질분해효소를 순수 분리하여 그 생화학적 특성을 규명하기 위하여 수행되었다. 이즈카긴비늘갯지렁이를 균질기로 갈아 원심분리하여 상층액을 모은 뒤, 20~80% ammonium sulfate로 분획하여 농축하였고, HiPrep 16/10 Q FF와 Mono Q 4.6/100 PE 등을 이용한 두 종류의 음이온 크로마토그래피와 Superdex 75 10/300 GL 크기-배제 크로마토그래피를 사용하여 해당 단백질분해효소를 정제하였다. 순수분리한 단백질분해효소를 LIZ(*Lepidasthenia izukai*)라 명명하고, 효소활성 등을 포함한 생화학적 특성을 규명하였다. SDS-polyacrylamide gel electrophoresis(SDS-PAGE)로 확인한 결과, LIZ 효소의 분자량은 약 28 kDa이었으며, PMSF(phenylmethylsulfonyl fluoride)가 없는 경우, 자가분해가 일어남을 알 수 있었다. LIZ 효소의 최적 pH는 약 7.5이었으며, 효소 활성은 20~60℃에서 일정하게 유지되었다. 또한 LIZ의 활성은 PMSF 및 diisopropyl fluorophosphate(DFP)와 같은 전형적인 세린계열 단백질분해효소 저해제들에 의해서 억제되었지만, ethylenediaminetetraacetic acid(EDTA) 및 1,10-phenanthroline(1,10-PT)과 같은 금속성단백질분해효소 저해제들에 의해서는 억제되지 않았다. 이러한 결과는 LIZ는 전형적인 세린계열 단백질분해효소임을

시사한다. LIZ 효소는 프로트롬빈, 피브리노겐, 플라스미노겐과 같은 다양한 혈장 단백질들을 10분 내에 빠르게 절단할 수 있었다. 발색성 펩타이드 기질을 이용한 기질 절단자리 분석을 통하여 LIZ는 전형적인 t-PA 기질인 S-2288(H-D-Ile-Pro-Arg-*p*NA)을 가장 잘 절단함을 확인하였다. LIZ 효소는 피브리노겐의 Aα 사슬을 30초 내에, Bβ 사슬을 10분 내에, γ 사슬은 20분 내에 모두 절단하였고, 교차연결 피브린의 α-α와 γ-γ 사슬도 0.5 μg의 적은 양으로도 잘 분해한다는 사실도 확인하였다. 또한 이 효소는 혈장 내의 피브린 섬유도 분해할 수 있는 효소활성을 지니고 있었다. 피브린 평판법을 통해 3 μg의 LIZ는 0.01 U의 플라스민과 동일한 피브린 분해능을 가지고 있음도 확인하였다. 이상의 결과는 LIZ는 피브린의 Aα 또는 Bβ 사슬만을 가수분해할 수 있는 여타의 혈전분해효소들과는 달리, 혈전을 매우 효과적으로 분해할 수 있는 가능성을 지닌 효소임을 시사하는 것이다.

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