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碩士學位論文

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뱀독으로부터 열 안정성 트롬빈-유사 단백질분해효소의 정제 및 특성분석

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ABSTRACT

Purification and characterization of a thermostable thrombin-like serine protease from a snake venom

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Snake venom proteases affect various physiological functions including platelet aggregation, blood coagulation, fibrinolysis, blood pressure, and nervous system. In this study, a thermostable thrombin-like serine protease (TLSP) was purified and characterized from a venom of *Gloydius halys ussuriensis* (Russian viper). Purification of the TLSP was performed using various chromatographic steps including anion exchange columns with Hiprep 16/10 Q FF and Source 15 Q 4.6/100 PE, a gel filtration with Superdex 75 10/300 GL in order, and then another anion exchange column with Mono Q^{TM} HR 5/5 as a final step. The purified TLSP appeared to be a single polypeptide on sodium dodecyl sulfate (SDS)-polyacrylamide gel. The amino terminal sequence of TLSP was found to be NH_2-VVGGDEHNIN-COOH,

showing a significant similarity ≥ 80% to those of thrombin-like serine proteases from other snake venoms. On SDS-polyacrylamide gel, the apparent molecular weights of TLSP was estimated to be 35 kDa and 26 kDa under reducing and non-reducing conditions, respectively. TLSP was stained with periodic acid-Schiff (PAS) and the size was reduced to about 33 kDa after treatment with N-glycosidase F under the reducing condition. These results suggest that TLSP is a glycosylated protease presumably having N-linked sugar chains. TLSP completely degraded the β-chain of human fibrinogen within 2 hr at 37℃, with much slower digestion of the α-chain. However, TLSP failed to induce formation of fibrin polymer through its fibrinogen cleavage. TLSP cleaved prothrombin, but the resulting products did not show any thrombin activity. In addition, TLSP did not cleave the fibrin polymer. TLSP showed an amidolytic activity against VPR-*p*NA (specific for thrombin), LGR-*p*NA (for factor Xa), and S-2288 (for t-PA and a broad serine proteases). Among these substrates, Boc-VPR-*p*NA was the most favorable one for TLSP. When Boc-VPR-*p*NA was used as a substrate, the enzyme kinetics of TLSP could be obtained as follows: K_M = 0.3 mM and K_{cat} = 8.69 s^{-1}. The enzyme activity of TLSP was inhibited by various serine protease inhibitors including aprotinin, PMSF, and DFP, not by metalloprotease inhibitors including EDTA, DTT and 1,10-PT. These results suggest that TLSP belongs to a member of the serine proteases. On the other hand, the enzyme activity of TLSP was inhibited by various divalent cations such as Ni^{2+}, Zn^{2+}, and Cu^{2+}. The optimal pH for TLSP enzyme activity was about pH 9.0. Surprisingly, the 59% enzyme activity remained, even at 100℃. Furthermore, TLSP showed no enzyme activity when it was treated with

N-glycosidase F, suggesting that a glycosylation on the enzyme may have greatly influence on enzyme activity. Taken together, the results obtained by this study suggest that TLSP is a novel glycosylated thermostable thrombin-like serine protease that cleaves the β-chain more preferentially than the α-chain of fibrinogen.

I. INTRODUCTION

Snakes use their venoms as offensive weapons in immobilizing, killing, and digesting the preys. Snake venoms evoke many signs and symptoms such as flaccid paralysis, systemic myolysis, coagulopathy and haemorrhage, renal damage and failure, and cardiotoxicity and local tissue injury at the bite site (Koh *et al.*, 2006).

Until now, more than two hundred species of venomous snakes have been found and classified by *Hidrophidae, Elapidae, Viperidae,* and *Crotalidae.* Nerve toxins are generally found in *Hidrophiae* and *Elapidae* venoms. Blood coagulation and hemostatics system are known to be contained in *Viperidae* and *Crotalidae* venoms (Ouyang *et al.*, 1992).

The snake venom contains many of enzymatic proteins, which act upon the different stages of the blood coagulation. The snake venom is particularly rich source of serine proteases and metalloproteases that are involved in platelet aggregation, blood coagulation, and fibrinolysis. (Matsui *et al.*, 2000). Specifically, the serine-type proteases include thrombin-like enzymes (Matsui *et al.*, 1998), bradykinin releasing enzymes (Nikai *et al.*, 1998; Serrano *et al.*, 1998), capillary permeability-increasing enzymes (Shimokawa *et al.*, 1993), factor X activators (Hofmann *et al.*, 1983), and non coagulant platelet aggregation inducers (Basheer *et al.*, 1995). Among these serine-type proteases, snake venom thrombin-like enzymes (so called SVTLEs) have been widely studied due to their features distinguished with thrombin (Castro *et al.*, 2004).

As shown in Fig. 1, intrinsic and extrinsic pathways lead blood coagulation, in which thrombin activated by factor Xa plays a central role. The thrombin

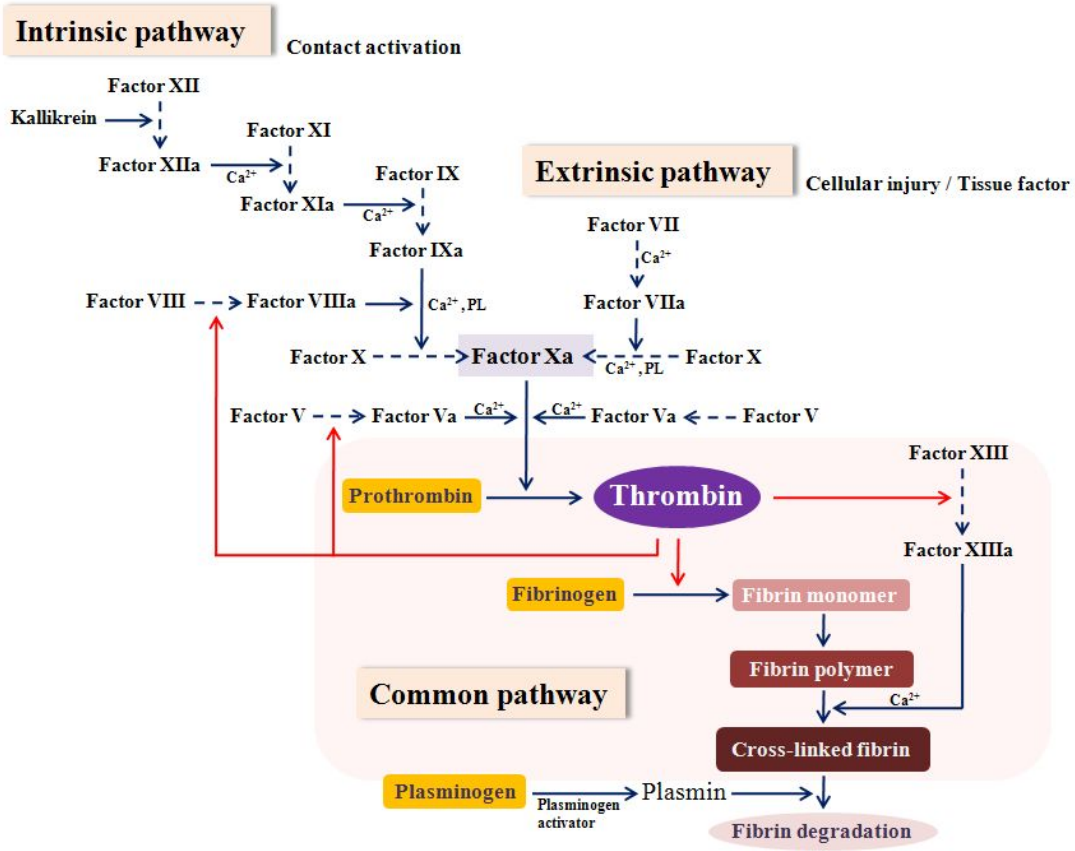

Figure 1. Blood coagulation pathways in part.

produced from prothrombin by factor Xa cleaves fibrinogen to fibrin. The resulting fibrin monomers spontaneously polymerize to make a fibrin clot in the presence of factor XIIIa *in vivo*. The fibrin clots can be dissolved by plasmin activated by plasminogen activators such as tissue-type plasminogen activator (t-PA) from plasminogen (Fig. 1).

SVTLEs are produced from several pit viper genera (*Akistrodon, Bothrops, Crotalus, Lachesis* and *Trimeresurus*), as well as some true vipers (*Bitis* and *Cerastes*). SVTLEs share a high degree of sequence homology among themselves (~67%), and show a low sequence identity with thrombin (26-30%), kallikrein (34-40%), and trypsin (31-44%) (Castro *et al.*, 2004). In addition, almost all SVTLEs are not inhibited by thrombin inhibitors like anti-thrombin III, heparin, and hirudin. However, most of SVTLEs affect coagulants related to thrombin, including fibrinogen, factor V, factor VIII and factor XIII.

SVTLEs preferentially release only one of the fibrinogen chains (α- or β -chain), rarely both with α- and β-chain (Aronson, 1976, Bell, 1997, Matsui *et al.*, 2000). Based on this feature, SVTLEs are classified by venombin A, B or AB. Venombin A group is characterized by cleaving preferentially fibrinopeptide A and include Ancrod from *T. flavoviridis* (Nolan *et al.*, 1976), Batroxobin from *Bothrops atrox,* and Crotalase from *Crotalus adamanteus* (Stocker *et al.*, 1976). Venombin B group act preferentially upon fibrinopeptide B, in which contortrixobin from *Agkistrodon contortrix contortrix* (Amiconi *et al.*, *et al.*, 2000) and halystase from *Agkistrodon halys blomhoffii* (Matsui *et al.*, 1998) are included. Abnormal cleavage of both α- and β -chains can lead the failure of the clots to be cross-linked and also can

induce a break down in fibrinolytic system. Venombin AB group evoke the clotting of fibrinogen by hydrolytic release of both fibrinopeptides A and B. Jararacussin from *B. jararacussu* (Bortoleto *et al.*, 2002), THLE from *Trimeresurus flavoviridis* (Komori *et al.*, 2002), and Kangshuanmei from *Agkistrodon halys brevicaudus stejnegeri* (Zhang *et al.*, 2001) are included in this group. Most SVTLEs could also act upon factor V, factor VIII, and factor XIII in the coagulation pathway (Amiconi *et al.*, 2000, Rosing *et al.*, 2001), but they have a much lower specific activity than that of thrombin and do not activate factors involved in the coagulation cascade. Although, SVTLEs are resemble with thrombin to an extent, they are different structurally and functionally in terms of the activation to coagulants. These properties of SVTLEs enable to use as defibrinogenating agents like Ancrod (Au *et al.*, 1993) and Batroxobin (Lochnit *et al.*, 1995).

Gloydius halys ussuriensis snake venom is venomous pit viper species found in far east Russia, northeastern China and Korean peninsula. Many kinds of enzymes such as thrombin-like enzyme, L-amino acid oxidase, and phospholipase A_2 have been found from *Gloydius halys ussuriensis* snake venom (Liu *et al.*, 2002, Zhang *et al.*, 2007, Yang *et al.*, 2002). However, a sort of thermostable serine proteases that can cleave fibrinogen have not yet been studied until now. In this study, a thermostable thrombin-like serine protease designated to as TLSP was purified and characterized from *Gloydius halys ussuriensis* snake venom (Russian viper).

II. MATERIALS AND METHODS

II-1. Materials

The crude venom of *Gloydius halys ussuriensis* was obtained as a lyophilized powder from Latoxan (Valence, France). Hiprep 16/10 Q FF column, Source 15 Q 4.6/100 PE column, Superdex 75 10/300 column, Mono Q^{TM} HR 5/5, and PD-10 column were obtained from Amersham Biotech Co. (Uppsala, Sweden). The Synthetic chromogenic substrates such as VPR-*p*NA (Boc-Val-Pro-Arg-*p*NA) and LGR-*p*NA (Boc-Leu-Gly-Arg-*p*NA) were from Seikagaku corp. (Tokyo, Japan), and S-2222 (Co-Ile-Glu-(-OR)Gly-Arg-*p*NA), S-2238 (H-D-Phe-Pip-Arg-*p*NA), S-2251 (H-D-Val-Leu-Lys-*p*NA), S-2288 (H-D-Ile-Pro-Arg-*p*NA), S-2765 (Z-D-Arg-Gly-Arg-*p*NA), and S-2444 (Glu-Gly-Arg-*p*NA) were obtained from Chromogenix Co. (Washington, USA). EDTA (ethylenediamintetraacetic acid), EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), TLCK (tosyl-L-lysine chloromethyl ketone), TPCK (tosyl-L-phenylalanine chloromethyl-ketone), 1,10-PT (1,10-phenanthroline), β-mercaptoethanol, PMSF (phenylmethanesulphonylfluoride), and DTT (dithiothreitol) were obtained from Sigma (St. Louis, MO, USA). Prothrombin was obtained from CalBiochem (Darmstadt, Germany). Plasminogen and Plasmin were obtained from Roche Applied Science (Mannheim, Germany). Protein molecular weight markers were obtained from Fermentas (Darmstadt, Germany). N-glycosidase F was obtained from New England BioLabs (Beverly, MA, USA). Schiff's reagent was obtained from sigma (St. Louis, MO, USA). Human fibrinogen and other chemicals were obtained from Sigma (St. Louis, MO, USA).

II-2. Purification of TLSP from *Gloydius halys ussuriensis* snake venom

The lyophilized crude venom (103.26 mg) was dissolved in 3 ml of 20 mM Tris-HCl (pH 8.0) and centrifuged at 13,000 x*g* for 1 min to remove insoluble materials. The supernatant was applied to a Hiprep 16/10 Q FF column equilibrated with the same buffer. The bound proteins were eluted with a linear gradient of 0 to 0.5 M NaCl at a flow rate of 2.5 ml/min and 1.5 ml fractions were collected. The fractions containing amidolytic activity against Boc-VPR-*p*NA as a substrate were pooled and concentrated by ultra filtration using Amicon YM 10 membrane (Millipore, MA, USA). The concentrated proteins were applied to a Source 15 Q 4.6/100 PE column equilibrated with 20 mM Tris-HCl (pH 8.0). The bound proteins were eluted with a linear gradient of 0 to 0.25 M NaCl at a flow rate of 0.7 ml/min and 1.0 ml fractions were collected. The active fractions were pooled and concentrated as described above. The fractions were applied to a Superdex 75 10/300 GL column equilibrated with 20 mM Tris-HCl (pH 8.0) containing 200 mM NaCl at flow rate 0.5 ml/min and 0.5 ml fractions were collected. The active fractions were pooled and concentrated, and then applied to a Mono Q^{TM} HR 5/5 column equilibrated with the 20 mM Tris-HCl (pH 8.0). The bound proteins were eluted with a linear gradient of 0 to 0.2 M NaCl at a flow rate of 0.7 ml/min and 0.5 ml fractions were collected. To obtain a pure enzyme, the fractions containing the major protein were again applied to a Mono Q^{TM} HR 5/5 column. The active fractions were pooled and analyzed on a 12% SDS-polyacrylamide gel under reducing condition, and then used as the purified protease. Protein concentrations were determined

with Bradford reagent (Sigma, USA) according to the manufacturer's instructions.

II-3. SDS-PAGE analysis

SDS-PAGE was performed according to the method of Laemmli (1970). Samples to be analyzed were mixed an equal volume of 2 x SDS-PAGE sample buffer, heated at 100℃ for 2 min, and then loaded onto 8, 10 or 12% gel. After electrophoresis, protein bands were visualized by staining the gel with Coomassie brilliant blue (CBB) or periodic acid-Schiff (PAS). Molecular weight markers used consisted of β-galactosidase (*E. coli*, 116 kDa), ovalbumin (chicken egg white, 45 kDa), lactate dehydrogenase (porcine muscle, 35 kDa), restriction endonuclease Bsp98I (*E. coli*, 25 kDa), β-lactoglobulin (bovine milk, 18.4 kDa), lysozyme (chicken egg white, 14.4 kDa).

II-4. Deglycosylation of TLSP

To identify N-linked glycoprotein through the removal of carbohydrates from TLSP, the 25 μl reaction sample of TLSP (2 μg) was deglycosylated by N-glycosidase F (NEB, USA) followed manufacturer's instructions and incubated at 37℃ for 16 hr. The reaction was stopped by the addition of a 5 μl of 6 x SDS-PAGE sample buffer followed by heating at 100℃ for 2 min, and then electrophoresed on a 10% SDS-polyacrylamide gel.

II-5. Amidolytic activity of TLSP

The amidolytic activity of TLSP (1 μg) was assayed in 100 μl of reaction mixture composed of 20 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 0.1 mg/ml BSA and 0.4 mM of chromogenic substrates in a 96-well plate. The substrates were used Boc-VPR-*p*NA and S-2238 for thrombin; Boc-LGR-*p*NA, S-2222 and S-2765 for factor Xa; S-2251 for plasmin and plasminogen; S-2288 for t-PA and a broad serine proteases; S-2444 for urokinase. After the addition of the chromogenic substrates, the reaction mixtures were incubated at 37℃ for 20 min. The hydrolysis of substrates by TLSP was determined by measuring the increased absorbance at 405 nm with 96-well plate reader (Molecular Device Corporation, California, USA).

II-6. Fibrinolytic activity assay

Fibrinolytic activity was measured the turbidity of fibrin polymer in a 96-well plate using a spectrophotometer. Ninety microliters of 1 mg/ml fibrinogen in 20 mM Tris-HCl (pH 8.0) was incubated with 10 μl thrombin (10 U/ml), and the fibrin polymer was allowed to form at room temperature for 1 hr. Thereafter, 40 μl of TLSP (5 μg) and plasmin (1 μg) was added to the fibrin polymer and incubated for 2, 4, and 16 hr at room temperature. The decrease in absorbance at 350 nm was recorded with 96-well plate reader (Molecular Devices). Fibrinolytic activity was also measured by using fibrin plates. The fibrin plate was prepared by mixing an equal volume of 1% fibrinogen and 1% agarose in 20 mM Tris-HCl (pH 8.0) with 500 μl of thrombin (6 U/ml). The plate was allowed to polymerize at room temperature

for 1 hr. Samples were spotted into the plate, and the plate was incubated at 37℃ for 20 hr.

II-7. Fibrinogenolytic activity assay

To determine the fibrinogenolytic activity of TLSP, human fibrinogen (200 μg) was incubated with different concentrations (1, 3, 5, or 10 μg) of TLSP in a total reaction volume of 200 μl containing 20 mM Tris-HCl (pH 8.0) at 37℃ for 2 hr. To analysis time-dependent fibrinogenolytic activity, TLSP (12.5 μg) was incubated with human fibrinogen (250 μg) in a total reaction volume of 250 μl containing 20 mM Tris-HCl (pH 8.0) at 37℃. Tweenty five microliters aliquots were taken from reaction mixture at various times (0.5 to 24 hr) and electrophoresed on 12% SDS-polyacrylamide gel to examine the cleavage pattern.

II-8. Effect of TLSP on prothrombin and plasminogen

To analysis the proteolytic activity of TLSP on prothrombin and plasminogen, TLSP (3 μg) was incubated with prothrombin or plasminogen (40 μg) in a total reaction volume of 80 μl containing 20 mM Tris-HCl (pH 8.0) at 37℃. Twenty microliters aliquots were taken from reaction mixture at various times of 4, 8 and 24 hr and electrophoresed on 10% SDS-polyacrylamide gel. To measure proteolytic activity on the fragments of prothrombin generated by TLSP, prothrombin (40 μg) was incubated with or without TLSP (3 μg) at 37℃ for 24 hr, and then the thrombin activity was

assayed with the reaction samples and thrombin (0.05 U) mixed with 150 μl of 20 mM Tris-HCl (pH 8.0) containing 100 μg of fibrinogen. For the detection of fibrin polymerization, the change in turbidity was measured at 350 nm with 96-well plate reader (Molecular Devices).

II-9. Effect of protease inhibitors and divalent cations on TLSP enzyme activity

TLSP (1 μg) was pre-incubated with various protease inhibitors such as EGTA, PMSF, aprotinin, bestatin, 1,10-PT, EDTA, TPCK, TLCK, DFP (1 mM), β-mercaptoethanol, and DTT (500 mM) or various divalent cations (1 mM) such as Ni^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, and Ca^{2+} in a reaction volume of 50 μl containing 50 mM Tris-HCl (pH 8.0) at 37℃ for 10 min. To determine their effects on the proteolytic activity of TLSP, the chromogenic substrate Boc-VPR-*p*NA (0.4 mM) was incubated with the reaction mixtures in a final reaction volume of 100 μl at 37℃ for 20 min. Residual activities were assayed as described above and compared with that of control without inhibitors.

II-10. Effect of temperature and pH on TLSP enzyme activity

The optimal pH for TLSP activity was determined by buffers having pHs of 4 to 12. TLSP was incubated with the chromogenic substrate Boc-VPR-*p*NA at 37℃ for 20 min with the following buffer system: 50 mM Sodium acetate (pH 4 to 5); 50 mM Sodium phosphate (pH 6 to 7); 50 mM

Tris-HCl (pH 8 to 9); 50 mM Glycine-NaOH (pH 10 to 11); 50 mM Sodium carbonate (pH 12). Temperature dependency of TLSP was determined under standard conditions at different temperatures. TLSP was pre-incubated at 4 to 100℃ for 30 min in 50 mM Tris-HCl (pH 8.0), and then assayed with Boc-VPR-*p*NA (0.4 mM) as described above. To analysis the thermal stability of TLSP, Samples containing TLSP (4 μg) were each pre-incubated at 37℃ or 100℃ for 30 min in a total reaction volume of 40 μl containing 20 mM Tris-HCl (pH 8.0), and then incubated with fibrinogen (30 μg) at 37℃ for 1 and 2 hr. Fibrinogenolytic acitvity was measured as described above.

II-11. N-terminal sequencing

TLSP was subjected to SDS-PAGE on 12% gel. After electrophoresis, proteins were transferred to PVDF membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11.0) containing 10% methanol. The membrane was stained with Coomassie brilliant blue and followed by destaining. Target bands were excised from the membrane and The analysis of N-terminal amino sequence was requested to Korea Basic Science Institute (KBSI, Seoul, Korea).

III. RESULTS AND DISCUSSION

III-1. Purification of TLSP

A thermostable thrombin-like serine protease was purified from *Gloydius halys ussuriensis* snake venom by five-chromatographic procedures (Fig. 2). The crude venom was fractionated by an anion exchange chromatography on a Hiprep 16/10 Q FF column (Fig. 2A). Amidolytic activity was found in the fractions of third peak when Boc-VPR-*p*NA was used as a chromogenic substrate. Futher chromatography of the pooled fractions was perfomed on an anion exchange chromatography of a Source 15 Q 4.6/100 PE column (Fig. 2B). The active fractions containing activity were also applied to a gel filtration chromatography of a Superdex 75 10/300 GL (Fig. 2C), and then an anion exchange chromatography of a Mono Q^{TM} HR 5/5 (Fig. 2D). Finally, The active fractions pooled were again applied to a Mono Q^{TM} HR 5/5 (Fig. 2E). The purification of TLSP is summarized in Table 1. The proteins obtained from each purification steps were electrophoresed on 12 % SDS-polyacrylamide gel (Fig. 3). The purification yield and the specific activity of TLSP were 2.3% and 1,000 U/mg, respectively. The apparent molecular weights were corresponded to 35 kDa and 26 kDa under reducing and non-reducing conditions, respectively, on SDS-polyacrylamide gel (Fig. 4). TLSP could be stained with periodic acid-Schiff (PAS) (Fig. 5) and the size was reduced to about 33 kDa after digestion with *N*-glycosidase F (Fig. 6) on the reducung SDS-polyacrylamide gel. These aspects of TLSP were different apparently from other known thrombin-like enzymes (Bao *et al.*, 2005, Cho *et al.*, 2001, Hahn *et al.*, 1996, Yang *et al.*, 2002) from *Gloydius*

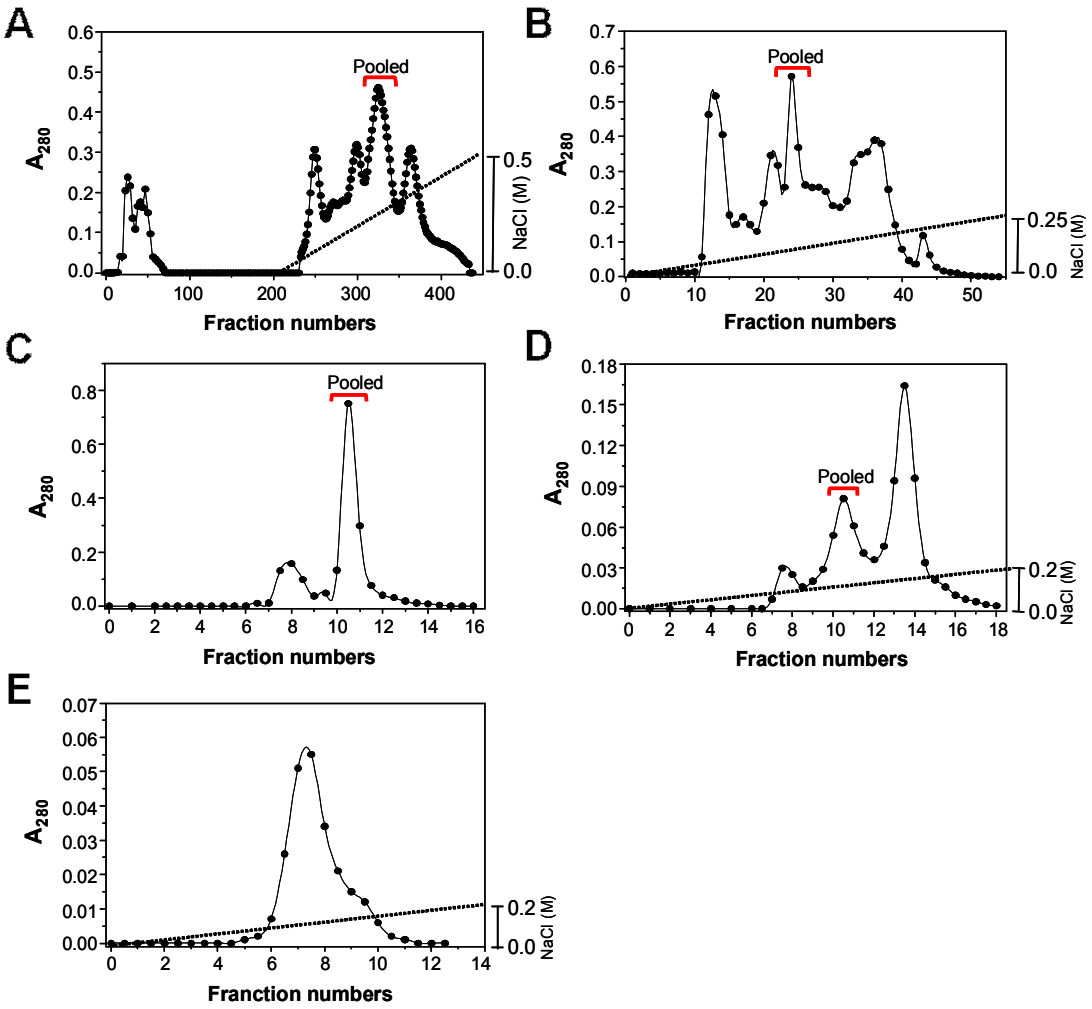

Fig. 2. Purification steps of TLSP. First step, (A) an anion exchange column chromatography on a Hiprep 16/10 Q FF. Second step, (B) an anion exchange column chromatography on Source 15 Q 4.6/100 PE. Third step, (C) a gel filtration column chromatography on a Superdex 75 10/300 GL. Fourth and fifth step, (D) and (E) an anion exchange column chromatography on a Mono Q^{TM} HR 5/5. The brackets indicate the pooled fractions that showed amidolytic activity when Boc-VPR-*p*NA was used as a substrate.

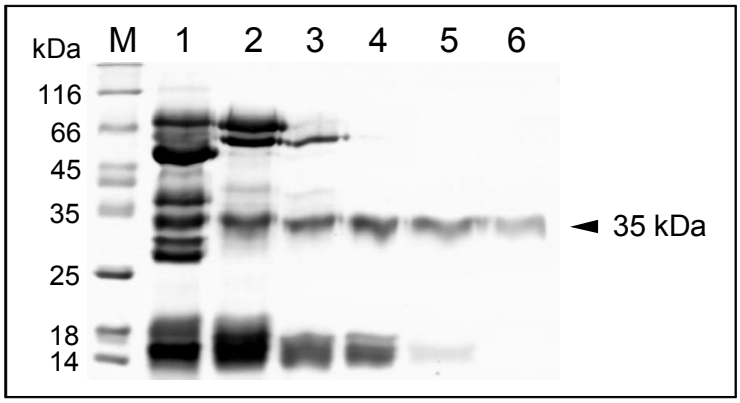

Fig. 3. SDS-PAGE analysis of the proteins obtained from the purification steps. The proteins from each purification steps were electrophoresed on 12% SDS-polyacrylamide gel under reducing condition and stained with Coomassie brilliant blue. Lanes M, molecular weight markers; 1, crude snake venom; 2, Hiprep Q; 3, Source Q; 4, Superdex 75; 5 and 6, Mono Q.

Table 1. Purification summary of TLSP.

Steps	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)[b]
Crude venom	103.26	6,195	60	100
Hiprep 16/10 Q FF	17.60	1,584	90	25.5
Source 15 Q 4.6/100 PE	4.06	609	150	9.8
Superdex 75 10/300 GL	0.98	196	200	3.2
Mono Q HR 5/5 (1)	0.39	192	492	3.1
Mono Q HR 5/5 (2)	0.14	140	1,000	2.3

[a] One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 0.25 mmol of the chromogenic substrate (Boc-VPR-*p*NA) in 15 min at 37℃.
[b] Total activity in crude venom was assigned the value of 100%.

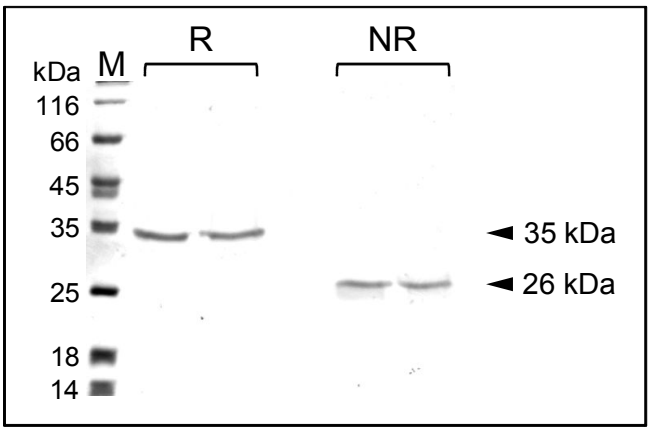

Fig. 4. SDS-PAGE analysis of the purified TLSP. TLSP was electrophoresed on 12% SDS-polyacrylamide gel under reducing (R) and non-reducing conditions (NR). Proteins were stained with Coomassie brilliant blue. Lane M, molecular weight markers.

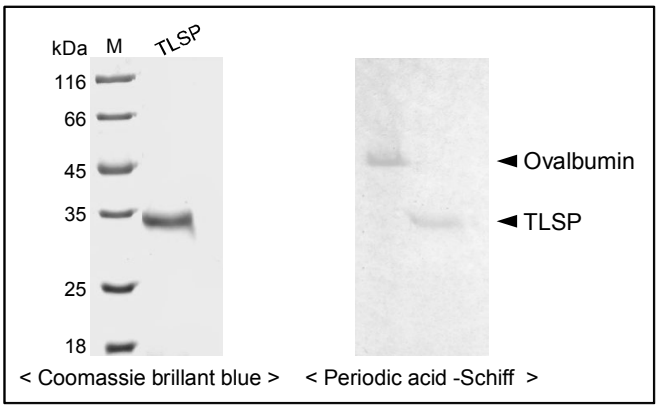

Fig. 5. SDS-PAGE analysis of TLSP stained with Coomassie brilliant blue or periodic acid-Schiff. TLSP and molecular weight markers separated by 12% SDS-PAGE were stained with PAS as described in Materials and Methods. Lane M, molecular weight markers, in which 45 kDa band corresponds to ovalbumin.

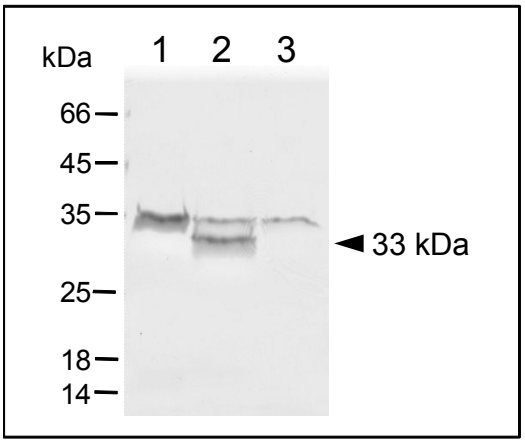

Fig. 6. SDS-PAGE analysis of the deglycosylated TLSP. TLSP was incubated with N-glycosidase F as described in Materials and Methods, separated on 12 % SDS-polyacrylamide gel, and stained with Coomassie blue. Lanes 1, TLSP only; 2, TLSP treated with N-glycosydase F; 3, N-glycosydase F only.

halys ussuriensis (*Agkistrodon caliginosus*) snake venom.

III-2. Enzyme kinetics of TLSP

To characterize the enzyme kinetics of TLSP, K_M and K_{cat} were determined with Boc-VPR-*p*NA as a substrate, K_M = 0.3 mM and K_{cat} = 8.69 s^{-1} (Table 2). This result indicates that Boc-VPR-*p*NA is a favorable substrate for TLSP when the kinetic values was compared with those of a thrombin-like enzyme from *Lachesis muta muta* (Magalhaes *et al.*, 1997), K_M = 0.23 and K_{cat} = 2.80 with Bz-Arg-Nan as a substrate and Stejnobin from *Trimeresurus stejnegeri* (Zhang *et al.*, 1998), K_M = 0.125 K_{cat} = 0.098 with S-2266 as a substrate.

III-3. N-terminal amino acid sequence of TLSP

Many kinds of enzymes such as thrombin-like enzyme, L-amino acid oxidase and phospholipase A_2 have been found in the snake venom of *Gloydius halys ussuriensis* (Liu *et al.*, 2002, Zhang *et al.*, 2007, Yang *et al.*, 2002).

The N-terminal amino acid sequence of TLSP was determined to be NH_2-VVGGDEHNIN-COOH, showing a significant similarity to those of thrombin-like serine proteases as shown in Table 3. The N-terminal amino acid sequence showed 90%, 80%, 70%, and 30% identities with VLβF isolated from *vipera lebetina,* Calobin isolated from *Agkistrodon caliginosus,* Contortrixobin isolated from *Agkistrodon contortrix contortrix* snake venom, α -thrombin from human plasma, respectively (Table 3). Calobin isolated from

Table 2. Kinetic values of TLSP.

K_M (mM)	K_{cat} (s^{-1})	K_{cat}/K_M ($mM^{-1}s^{-1}$)
0.3	8.69	28.8

Table 3. Comparison of N-terminal amino acid sequence of TLSP with those of α-thrombin and other thrombin-like serine proteases.

Source (Enzyme name)	N-terminal amino acid sequence *	Identity (%)
▪ *Gloydius halys ussuriensis* (TLSP)	V V G G D E H N I N	100
▪ *Vipera lebetina* (VLβF)	V V G G D E C N I N	90
▪ *Agkistrodon caliginosus* (Calobin)	V I G G D E C N I N	80
▪ *Agkistrodon contortrix contortrix* (Contortrixobin)	V V G G D E C N I N	70
▪ Human plasma (α – thrombin)	I V E G S D A E I G	30

* Shadowed boxes indicate the conserved residues.

Agkistrodon caliginosus have been characterized as a thrombin-like serine protease (Cho *et al.*, 2001). VLβF isolated from *vipera lebetina* and Contortrixobin isolated from *Agkistrodon contortrix contortrix* snake venom are thrombin-like serine β-fibrinogenases (Amiconi *et al.*, 2000, 1994, Samel *et al.*, 2002). It is been known that thrombin-like enzymes share a high degree of sequence homology (~67%), but they share a low sequence homology with thrombin (26-33%) (Castro *et al.*, 2004). As sown in Table 3, TLSP shared more than 70% sequence homology with thrombin-like serine proteases, but only 30% with α-thrombin.

III-4. Enzymatic properties of TLSP

III-4-1. Amidolytic activity of TLSP

Substrate specificity of TLSP was examined by observing its amidolytic activity using various synthetic chromogenic substrates. As shown in Table 4, TLSP showed the highest amidolytic activity for Boc-Val-Pro-Arg-*p*NA (VPR-*p*NA), thrombin specific substrate, giving rise to 100%. However, H-D-Phe-Pip-Arg-*p*NA (S-2238) for thrombin was not susceptible as below as 27%. The Boc-Leu-Gly-Arg-*p*NA (LGR-*p*NA) for factor Xa and H-D-Ile-Pro-Arg-*p*NA (S-2288) for t-PA showed about 85% and 81% activities relative to that of VPR-*p*NA. However, TLSP could not hydrolyze the following substrates: Co-Ile-Glu-(-OR)Gly-Arg-*p*NA (S-2222), Z-D-Arg-Gly-Arg-*p*NA (S-2765) for factor Xa, and H-D-Val-Leu-Lys-*p*NA (S-2251) for plasmin and plasminogen.

These results indicate that TLSP hydrolyzes specifically Boc-VPR-*p*NA like

Table 4. Substrate specificity of TLSP.

Chromogenic substrates	Specific for	Activity (%)*
VPR (Boc-Val-Pro-Arg-*p*NA)	Thrombin	100
LGR (Boc-Leu-Gly-Arg-*p*NA)	Factor Xa	85
S-2288 (H-D-Ile-Pro-Arg-*p*NA)	t-PA and a broad serine proteases	81
S-2444 (Glu-Gly-Arg-*p*NA)	Urokinase	40
S-2765 (Z-D-Arg-Gly-Arg-*p*NA)	Factor Xa	28
S-2238 (H-D-Phe-Pip-Arg-*p*NA)	Thrombin	27
S-2251 (H-D-Val-Leu-Lys-*p*NA)	Plasmin and Plasminogen	16
S-2222 (Co-Ile-Glu-(-OR)Gly-Arg-*p*NA)	Factor Xa	14

* The amidolytic activity of TLSP was measured as described in Materials and Methods. Enzyme activity was expressed as a relative value compared to that of Boc-VPR-*p*NA as control.

thrombin.

III-4-2. Proteolytic activity of TLSP

Thrombin is able to cleave both α- and β-chains of fibrinogen. However, most thrombin-like enzymes from snake venom preferentially release only one of α- and β-fibrinogen chains (Castro *et al.*, 2004, Kini, 2005). As shown in Fig. 7, The β-chain of human fibrinogen could be cleaved by TLSP (Fig. 7). However, the α-chain was resistant to TLSP treatments during the duration time period. Thrombin-like serine proteases such as pallabin (Fan*et al.*, 1999), halystase (Matsui *et al.*, 1998), bilineobin (Komori *et al.*, 1993), and contortrixobin (Amiconi *et al.*, 2000) also preferentially hydrolyze the β-chain of fibrinogen. To further analyze the cleavage pattern of human fibrinogen by TLSP, human fibrinogen was incubated with TLSP for 24 hr at 37℃. Although TLSP completely cleave the β-chain of human fibrinogen within 4 hr, it could also digest the α-chain of fibrinogen after 12 hr incubation (Fig. 8A). In addition, TLSP could not induce the formation of fibrin polymer (Fig. 8B). These results indicate that TLSP does not have a fibrin-clotting activity. The same results have been obtained from halystase (Matsui *et al.*, 1998).

The proteolytic activity of TLSP was also examined on plasminogen and prothrombin. As shown in Fig. 9, TLSP could cleave both plasminogen and prothrombin (Fig. 9A). The proteolytic fragments of prothrombin generated by TLSP showed similar with that of factor Xa, but it did not have thrombin activity (Fig. 9B). The fibrinolytic activity of TLSP was also observed on fibrin

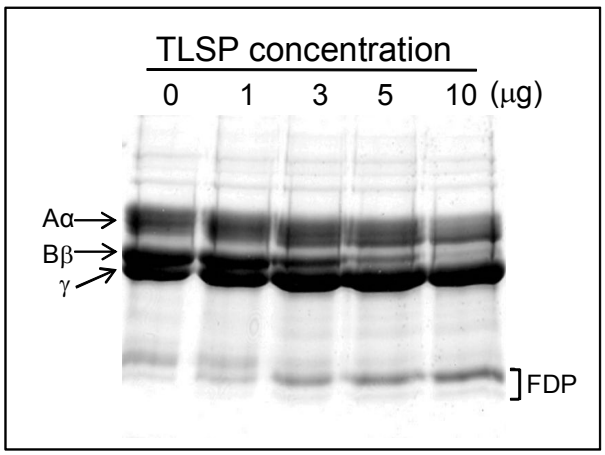

Fig. 7. Degradation of fibrinogen by TLSP. Different concentrations (1, 3, 5 or 10 μg) of TLSP were incubated with fibrinogen (200 μg) for 2 hr at 37℃. Each samples was electrophresed on 10% SDS-polyacrylamide gel as stained with Coomassie brilliant blue. FDP, fibrinogen degrading products.

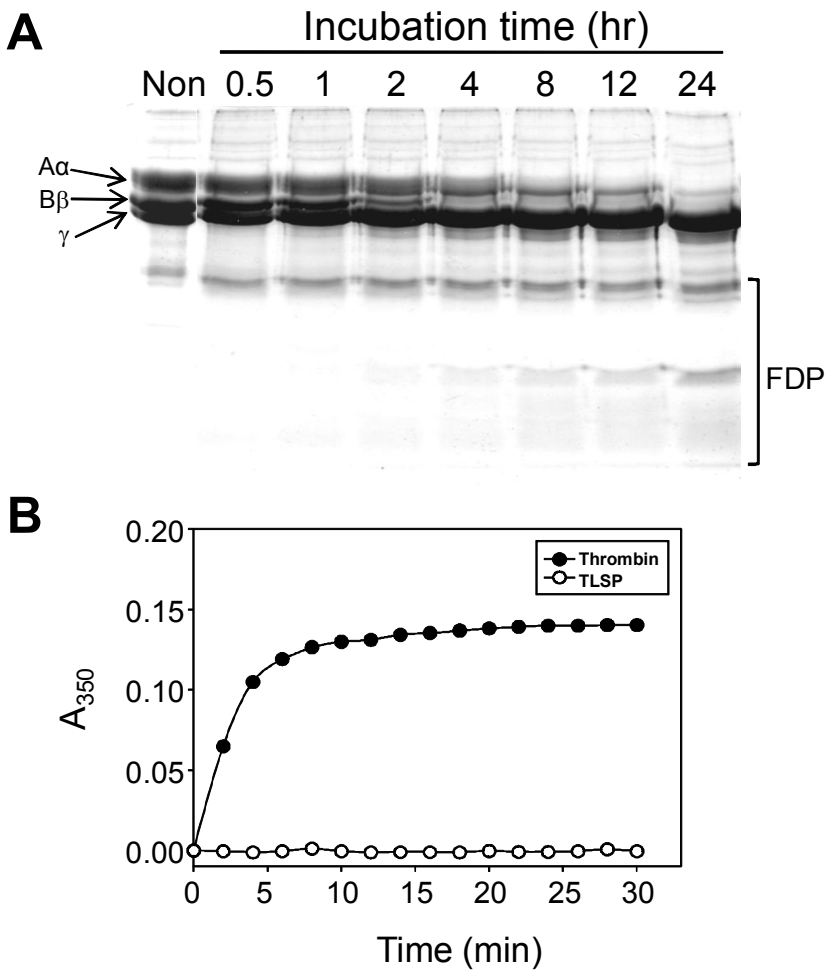

Fig. 8. Time-dependent degradation of fibrinogen and formation of fibrin polymer by TLSP. (A) TLSP was incubated with fibrinogen for different time periods at 37℃ and the samples were electrophoresed on 12% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue. Non, fibrinogen only; FDP, fibrinogen degrading products. (B) Thrombin activity was assayed by measuring the degree of fibrin formation as described in Materials and Methods. TLSP or thrombin was incubated with fibrinogen at 37℃ for 30 min and the formation of fibrin polymer was monitered as an increase in turbidity at 350 nm.

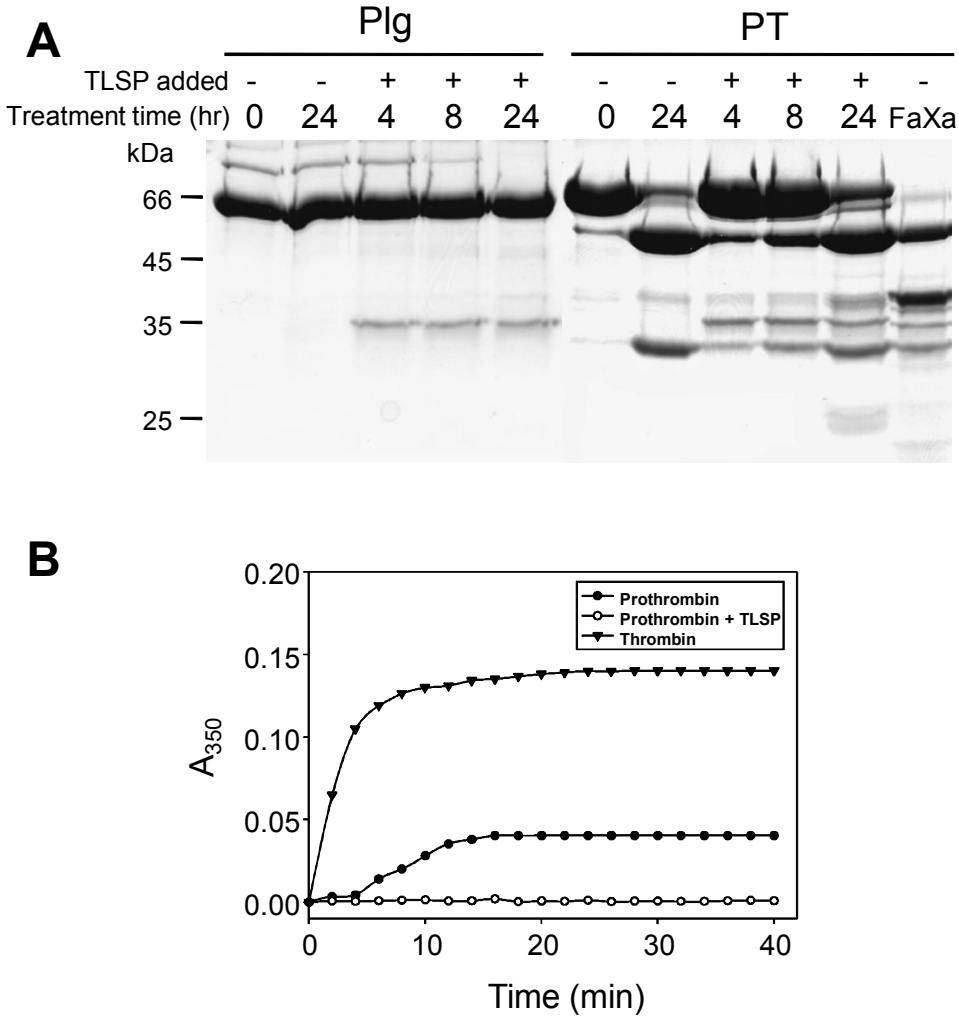

Fig. 9. Cleavage of plasminogen and prothrombin by TLSP. (A) Plasminogen and prothrombin were incubated with or without TLSP at 37℃ for different times periods and the samples were electrophoresed on 12% SDS-polyacrylamide gel. Plg, plasminogen; PT, prothrombin; FaXa, factor Xa. (B) The fragments of prothrombin generated by TLSP was incubated with fibrinogen for 24 hr and the increase of turbidity was measured as described in Materials and Methods.

plate (Fig. 10A) and measured changing of turbidity on fibrin polymer at 350 nm (Fig. 10B). However, it could not cleave fibrin polymer and cross-linked fibrin formed by factor XIIIa (Fig. 10C).

III-4-3. Effect of protease inhibitors and divalent cations on TLSP enzyme activity

The effect of various protease inhibitors on TLSP activity were investigated. As shown in Table 5, the proteolytic activity of TLSP was inhibited by serine protease inhibitors such as PMSF, aprotinin and DFP. The enzyme activity of TLSP was strongly inhibited by aprotinin, a typical serine proteases inhibitor. However, Metalloprotease inhibitors such as EDTA, EGTA, and 1,10-PT showed no effect on TLSP. These results suggest that TLSP may be a serine protease. TLSP was also inhibited by some divalent cations such as Ni^{2+}, Zn^{2+} and Cu^{2+} (Table 6.).

Ca^{2+} and Mg^{2+} have been known to act as important factors related to the activity of the snake venom proteins (Shikamoto *et al.*, 2003). However, they did not affect the activity of TLSP.

III-4-4. Effect of pH and temperature on TLSP enzyme activity

The Effect of pHs on the proteolytic activity of TLSP was examined by various buffer systems. As shown in Fig. 11, the optimal pH for the enzyme activity was determined to be 9.0 and the stable range of pH was from 8.0 to 10.0. In addition, the enzyme activity of TLSP remained

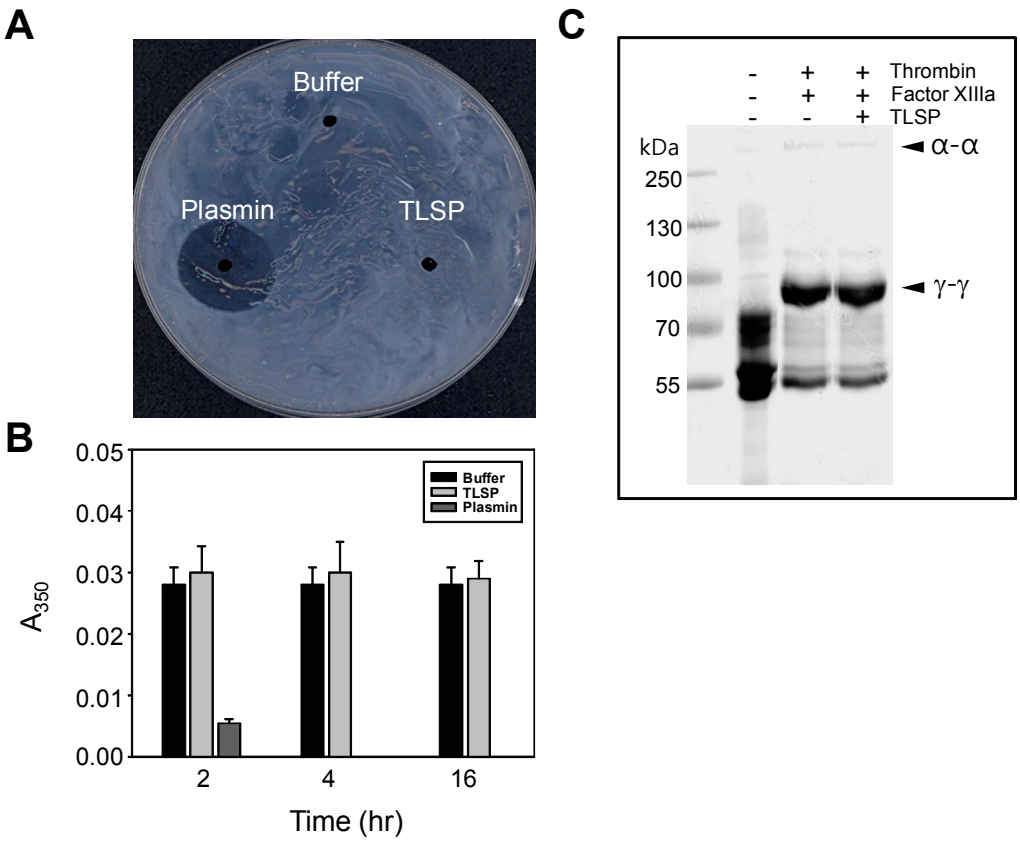

Fig. 10. Effect of TLSP on fibrin polymer and cross-linked fibrin. (A) Fibrinolytic activity was assayed on fibrin plate. Samples were applied on the plate and allowed to incubate for 20 hr at 37℃. (B) Fibrinolytic activity was also measured by a change in the turbidity of fibrin polymer. Fibrinogen was added to thrombin and the fibrin polymer was allowed to form at room temperature for 1 hr. Thereafter, plasmin or TLSP was added to the polymer and incubated for the indicated time periods. Buffer was used instead of enzyme as control. (C) The enzyme activity of TLSP on cross-linked fibrin was analyzed by SDS-PAGE. Polymerization of fibrin was initiated by the addition of fibrinogen, thrombin, and factor XIIIa and $CaCl_2$ followed by incubation at room temperature for 1 hr. The cross-linked fibrin was reacted with TLSP (2 μg) at room temperature for 2 hr followed by SDS-PAGE using 8% gel.

Table 5. Effect of various inhibitors on TLSP enzyme activity.

Additive[a]	Activity (%)[b]
Control	100
EGTA	98.8
PMSF	61.4
β-mercaptoethanol	31.6
DTT	15.5
Aprotinin	0.6
Bestatin	104.1
1,10-PT	108.3
EDTA	96.3
TPCK	109
TLCK	82.8
DFP	99.5

[a] One mM each of EGTA, PMSF, aprotinin, bestatin, 1,10-PT, EDTA, TPCK, TLCK, and DFP was used. β-mercaptoethanol and DTT were used at a final concentration of 500 mM.

[b] Enzyme activity was expressed as a relative value compared to that of Boc-VPR-*p*NA as control.

Table 6. Effect of divalent cations on TLSP enzyme activity.

Additive[a]	Activity (%)[b]
Control	100
$NiCl_2$	9.1
$ZnCl_2$	1.4
$MgCl_2$	99.3
$MnCl_2$	98.5
$CuCl_2$	17.5
$CaCl_2$	97.2

[a] The final concentration for each additive was 1 mM.
[b] Enzyme activity was expressed as a relative value compared to that of Boc-VPR-*p*NA as control.

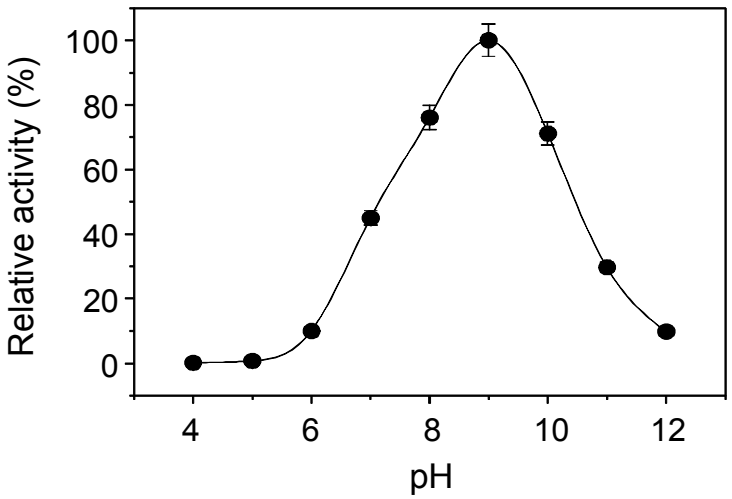

Fig. 11. Effect of pH on TLSP enzyme activity. TLSP enzyme activity was assayed with Boc-VPR-*p*NA as substrate at various pHs using the buffer systems as described in Materials and Methods.

59% at 100℃, showing stability on high temperature (Fig. 12). The stability of TLSP enzyme activity was also confirmed (Fig. 13). As shown in Fig. 13, the pre-incubated TLSP at 100℃ for 30 min showed clearly an enzyme activity that completely cleave the β-chain of fibrinogen within 2 hr. This result suggest that TLSP is a very thermostable enzyme and has a β-fibrinogenase activity that is resistance to inactivation by heat and pH extremes (Samel *et al.*, 2002, Siigur *et al.*, 1991, Swenson *et al.*, 2005).

There is a general agreement that resistance to denaturation is provided by covalently bound carbohydrates (Mahar *et al.*, 1987, Siigur *et al.*, 1991). As shown in Fig. 14, TLSP treated with N-glycosidase to remove the carbohydrate moieties show no enzyme activity when Boc-VPR-*p*NA was used as a substrates. This result strongly suggests that carboxylation of TLSP is an important for the enzyme activity of TLSP.

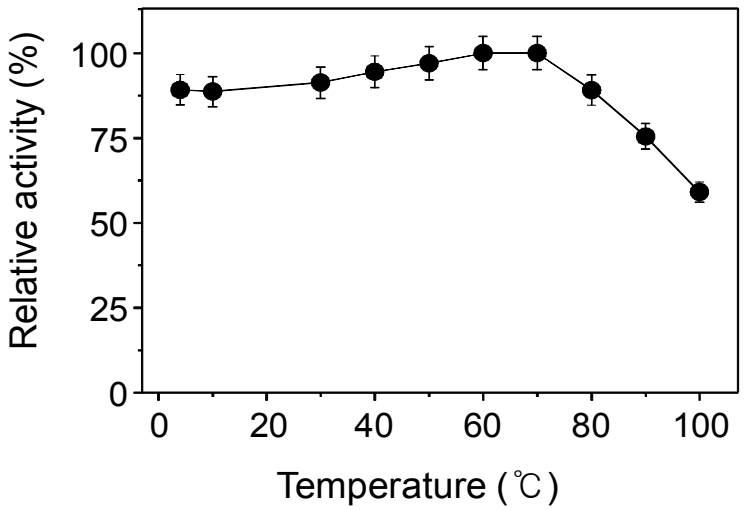

Fig. 12. Effect of temperature on TLSP enzyme activity. TLSP enzyme activity was assayed with Boc-VPR-*p*NA as substrate at various temperatures as described in Materials and Methods.

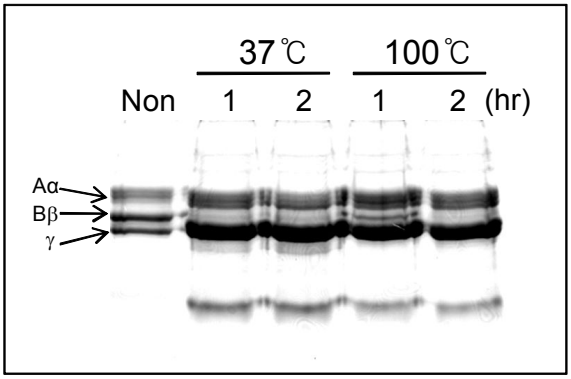

Fig. 13. Effect of temperature on the fibrinogenolytic activity of TLSP. Samples containing 2 μg of TLSP were pre-incubated for 30 min at 37℃ or 100℃, and then the samples were incubated for 1 and 2 hr at 37℃ with 30 μg of fibrinogen. Non, fibrinogen only.

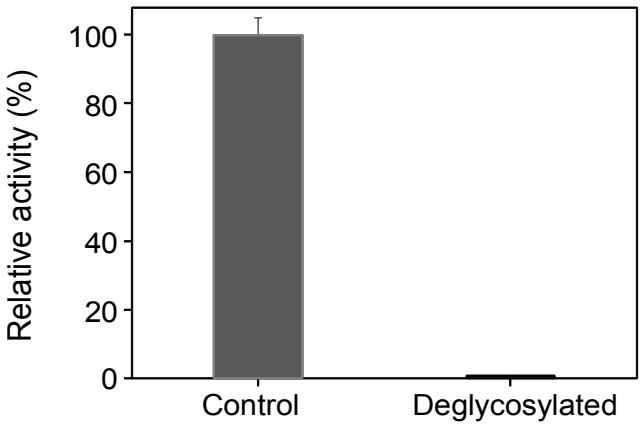

Fig. 14. Effect of the removal of carbohydrates on TLSP. TLSP was deglycosylated by *N*-glycosidase-F at 37℃ for 2 hr. TLSP enzyme activity was assayed with Boc-VPR-*p*NA as a substrate and expressed as a relative value compared to that of non-treated control.

IV. 적 요

뱀독으로부터 열 안정성 트롬빈-유사 단백질분해효소의 정제 및 특성분석

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뱀독에 포함되어 있는 단백질분해효소들은 혈소판 응집, 혈액응고, 피브린 분해, 혈압, 신경계 등에 다양한 영향을 미친다. 본 연구에서는 *Gloydius halys ussuriensis* 뱀독으로부터 열 안정성 트롬빈-유사 단백질분해효소를 분리 및 정제하고 그 특성을 분석하였다. TLSP(thrombin-like serine protease)라 명명한 이 단백질분해효소는 이온교환 수지인 Hiprep 16/10 Q FF, Source 15 Q 4.6/100 PE 및 Mono Q^{TM} HR 5/5와 gel filtration 수지인 Superdex 75 10/300 GL을 이용한 크로마토그래피법으로 분리 및 정제하였다. SDS-PAGE를 이용하여 확인한 결과, TLSP는 단일 폴리 펩타이드로 구성되어 있었으며, N-말단의 아미노산 서열분석결과 "NH_2-VVGGDEHNIN-COOH"임을 확인하였다. 이 아미노산 서열은 다른 뱀독으로부터 분리된 VLβF, Calobin 및 Contortrixobin과 같은 트롬빈-유사 세린계열 단백질분해효소들의 N-말단서열과 80%이상 유사하였다. SDS-polyacrylamide gel에서 TLSP의 분자량은 35 kDa이었다. PAS 염색과 N-glycosidase 처리 후 분자량 감소를 분석하여 확인한 결과, TLSP의 분자량이 약 33 kDa으로 감소함을 확인함으로써 이 효소가 당단백질임을 알 수 있었다. TLSP는 피브리노겐의

베타사슬을 37℃에서 2시간 이내에 완전히 절단하였지만, 알파사슬 절단능은 미약하였다. TLSP는 피브리노겐을 절단하여 피브린 중합체 형성을 유도하지 못할 뿐만 아니라 프로트롬빈 활성화와 피브린 중합체 분해활성능을 모두 갖고 있지 않았다. 이러한 결과는 TLSP가 피브리노겐의 베타사슬을 비교적 선택적으로 분해하는 베타사슬-특이적 피브리노겐 분해효소임을 시사하는 것이다. TLSP는 Boc-VPR-*p*NA, Boc-LGR-*p*NA, S-2288 등, 여러 가지 발색기질들을 가수분해하는 특성을 지니고 있었으며, 그 중 트롬빈-특이 기질로 알려진 Boc-VPR-*p*NA를 가장 선호하였다. Boc-VPR-*p*NA를 기질로 이용하여 TLSP의 효소활성을 분석한 결과, K_M과 k_{cat}값은 각각 0.3 mM과 8.69 s^{-1}이었다. TLSP의 효소활성은 세린계열 단백질분해효소 억제제인 aprotinin, PMSF 및 DFP 등에 의해 억제되었지만, metalloprotease들의 억제제인 EDTA 및 1,10-PT 등에 의해서는 억제되지 않았다. 이러한 결과들은 TLSP가 세린계열 단백질 분해효소임을 시사하는 것이다. TLSP의 효소활성은 Ni^{2+}, Zn^{2+} 및 Cu^{2+} 등과 같은 2가 이온들에 의해 억제되었다. TLSP의 최적 pH는 약 9.0이었으며, 100℃에서도 59%의 효소활성을 지니는 열에 매우 안정한 효소임을 확인하였다. N-glycosidase를 처리하여 TLSP부터 탈당화(deglycosylation)를 시킨 결과, 효소활성이 완전히 사라짐을 확인함으로써 당화가 효소활성과 열안정성에 매우 중요함을 알 수 있었다. 본 연구에서 얻은 연구결과들은 TLSP가 피브리노겐의 베타사슬을 비교적 선택적으로 자르는 열 안정성 트롬빈-유사 단백질 분해효소임을 시사하는 것이다.

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	영어 : **Purification and characterization of a thermostable thrombin-like serine protease from a snake venom**				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2009년 8월 일

저작자 : 조 으 리 (서명 또는 인)

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