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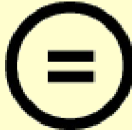

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Biochemical and molecular characterization of fibrinolytic serine proteases from a marine polychaete *Cirriformia tentaculata*

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August 2014
Doctoral Dissertation

Biochemical and molecular characterization of fibrinolytic serine proteases from a marine polychaete *Cirriformia tentaculata*

Graduate School of Chosun University

Department of Life Science

JONG WOO PARK

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Biochemical and molecular characterization of fibrinolytic serine proteases from a marine polychaete *Cirriformia tentaculata*

갯지렁이 유래 세린계열 단백질분해효소의 혈전분해 특성 및 유전자 발현

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이 論文을 理學 博士學位申請 論文으로 提出함

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Biochemical and molecular characterization of fibrinolytic serine proteases from a marine polychaete *Cirriformia tentaculata*

A dissertation submitted in partial fulfillment of

the requirement for the degree of

DOCTOR OF PHILOSOPHY

To the Faculty of

Department of Life Science

at

CHOSUN UNIVERSITY

by

JONG WOO PARK

Date Approved:

June 26, 2014

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ABBREVIATIONS

1,10-PT ······ 1,10-phenanthroline
A. marina ······ *Arenicola marina*
AOX ······ Alcohol oxidase
ATIII ······ Anti-thrombin III
BMMY ······ Buffered Methanol-Complex Medium
BSA ······ Bovine serum albumin
C_1-inh ······ C_1-inhibitor
CAPS ······ 3-(cyclohexylamino)-1-propanesulfonic acid
C. teleta ······ *Capitella teleta*
C. tentaculata ······ *Cerriformia tentaculata*
CTSP ······ *Cerriformia tentaculata* serine protease
DFP ······ Diisopropyl fluorophosphate
DTT ······ Dithiothreitol
E. coli ······ *Escherichia coli*
EDTA ······ Ethylenediamine tetraacetic acid
E. fetida ······ *Eisenia fetida*
E. japonensis ······ *Enchytraeus japonensis*
EtBr ······ Ethidium bromide
FV ······ Factor V
FVII ······ Factor VII
FVIII ······ Factor VIII
FIX ······ Factor IX
FX ······ Factor X
FXI ······ Factor XI
FXII ······ Factor XII
FXIII ······ Factor XIII

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GSP ············ Gene specific primer
HMWK ············ High molecular weight kininogen
LB ············ Luria-Bertani medium
L. bimastus ············ *Lumbricus bimastus*
M-MLV RT ············ M-MLV reverse transcriptase
PBS ············ Phosphate buffered saline
PAGE ············ Polyacrylamide gel electrophoresis
P. aibuhitensis ············ *Perinereis aibuhitensis*
PAI ············ Plasminogen activator inhibitor
PCR ············ Polymerase chain reaction
PDB ············ Protein data bank
PEG ············ Polyethylene glycol
PK ············ Prekallikrein
PL ············ Phospholipid
P. leucophryna ············ *Periserrula leucophryna*
PMSF ············ Phenylmethanesulfonyl fluoride
*p*NA ············ *p*-nitroanilide
P. pastoris ············ *Pichia pastoris*
PVDF ············ Polyvinylidene fluoride
RACE ············ Rapid amplification of cDNA end
SDS ············ Sodium dodecyl sulfate
TCA ············ Trichloroacetic acid
TF ············ Tissue factor pathway inhibitor
TLCK ············ Tosyl-lysine chloromethyl ketone
TPCK ············ Tosyl-phenylalanyl chloromethyl ketone
tPA ············ Tissue-type plasminogen activator
uPA ············ Urokinase-type plasminogen activator
YNB ············ Yeast nitrogen base

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YPD ········· Yeast peptone dextrose
YPG ········· Yeast peptone glycerol
YPM ········· Yeast peptone methanol
WHO ········· World Health Organization

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ABSTRACT

Biochemical and molecular characterization of fibrinolytic serine proteases from a marine polychaete *Cirriformia tentaculata*

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Many proteases belonging to serine protease family play important roles in blood homeostasis. These include factor Xa (FXa), thrombin, and plasmin. These proteases are circulating in blood stream as the forms of zymogens. During the course of coagulation, thrombin (activated proteolytically from prothrombin by FXa) cleaves fibrinogen to fibrin, leading to the formation of fibrin clot. However, an abnormal accumulation of blood clot in the circulatory system can evoke lethal diseases such as myocardial infarction and stroke. Clinically, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and streptokinase are often being used for clearing thrombotic occlusions. However, it has been reported that the administration of tPA or uPA can evoke various side effects, including bleeding and allergic reaction, which are related to their indirect fibrinolytic activities resulted from the activation of plasminogen to plasmin. Therefore, it is still necessary to search for a safe and direct-acting fibrinolytic agent from various biological

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resources. In this study, novel three distinct kinds of alkaline serine proteases (named CTSP-1, -2, and -3) exhibiting fibrinolytic activities were purified from the polychaete *Cirriformia tentaculata* and characterized in terms of their enzymatic properties and kinetics. The estimated molecular masses of purified CTSP-1, -2, and -3 enzymes were found to be 28.8, 30.9, and 28.4 kDa, respectively. The enzymes were active at the temperature range of 50-60°C under pH 8.5-9.0. In addition, their proteolytic activities could be completely inhibited by PMSF and DFP, but not by 1,10-PT and bestatin. These results suggest that they are all typical serine proteases, not metalloproteases or cysteine proteases. CTSP-1 and -2 cleaved arginine, whereas CTSP-3 digested tyrosine residue at the carboxyl sides in their peptide substrates. Amino acid sequencing results showed that the N-termini of CTSP-1, -2, and -3 were composed of Ile-Met-Asn-Gly-Ser-Pro-Ala-Ala, Ile-Met-Tyr-Gly-Gln-Glu-Ala-Ala, and Ile-Ile-Gly-Gly-Thr-Glu-Ala-Asp, respectively. A typical hepta-sequence (I-X-X-G-X-X-A) conserved in serine proteases from annelid species was also found in N-termini of all CTSPs. The enzymes could cleave all chains (Aα, Bβ, and γ) of fibrinogen within 20 min and also hydrolyze actively fibrin polymer as well as cross-linked fibrin. The enzymes also could actively digest the fibrin clot in blood plasma milieu. Meanwhile, three cDNA clones capable of encoding the three CTSPs could be obtained by 5´- and 3´-rapid amplification of cDNA ends (RACEs). The sequencing results showed that the open reading frames (ORFs) of CTSP-1, -2, and -3 clones were composed of 804, 738, and 756 bp, respectively. In addition, the deduced amino acid sequences of CTSP-1, -2, and -3 were composed of 267, 246, and 252 amino acids, respectively. When the amino acid sequences of three CTSPs were compared, significant sequence homologies were found between them as follows: 73.3% identity between CTSP-1 and -2, 39.9% between CTSP-1 and -3, and 44.2% between

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CTSP-2 and -3. The comparison of amino acid sequences also showed that all the three CTSP enzymes contained the same catalytic triads composed of His, Asp, and Ser residues, which are conserved in serine protease family. Among the cDNAs obtained, *CTSP-3* gene was cloned into a yeast secretory vector (pPICZαA) and expressed in a methyltrophic yeast *Pichia pastoris* X-33 (*P. pastoris* X-33) under the control of the *AOX1* promotor. An active recombinant CTSP-3 enzyme (named yrCTSP-3) could be successfully expressed from the yeast harboring a recombinant plasmid pPICZαA-*CTSP-3* by treatment with 0.5% methanol for 72 h. Western blot analysis with anti-His tag antibody revealed that yrCTSP-3 could be secreted into culture medium as a processed form, with an appearent molecular mass of 32 kDa in size. From a total 11.85 mg proteins of culture supernatant, 40 μg of active yrCTSP-3 enzyme could be purified with 85.03% in yield by using an ammonium sulfate precipitation (0-70%) and a His-tag affinity chromatography in order. The kinetic parameters including K_M, K_{cat}, and K_{cat}/K_M for the purified yrCTSP-3 enzyme were found to be 0.225 mM, 7.13 sec^{-1}, and 31.49 $mM^{-1}sec^{-1}$, respectively, which could be comparable to those of native enzyme. The purified recombinat yrCTSP-3 enzyme exhibited a similar fibrinolytic activity to native enzyme, as judged by fibrin plate assay. These results suggest that the yeast expression system can be used for obtaining a functional yrCTSP-3 enzyme. Taken together, the results obtained demonstrate that CTSP enzymes have potential of becoming therapeutic agents for thrombus dissolution.

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I. INTRODUCTION

Hemostasis is the physiologic system, which supports the blood in the fluid state. A normal hemostatic system suppresses the formation of blood clot in 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. 1). However, the accumulation of blood clot in blood vessels of the circulatory system can lead to vascular blockage. This phenomenon of thrombotic occlusions encompasses a range of various lethal diseases, such as myocardial infarction, stroke, and pulmonary thrombo embolism (Becattini *et al*., 2005). For example, 70% of sudden cardiac deaths are caused by thrombosis, and the cardiovascular diseases are composed of top 4 causes of death in the world, according to a report of the World Health Organization (WHO) (Davies, 2000; Lloyd-Jones *et al*., 2009). However, cardiovascular disease treatment is not easy to patients, since the development of thrombosis diagnostics and thrombolytic therapy is influenced by the complexity of the hemostatic system comprising of coagulation factor and platelet signaling. In recent year, many anti-coagulant and fibrinolytic enzymes have been developed for treatment of thrombosis by the many researchers. Anti-coagulants can reduce the ability of the blood to clot, on the other hand, fibrinolytic enzyme is dissolution of blood clot (Blann *et al*., 2002; Perler, 2005). These therapeutic agents had been developed after the identification of blood coagulation and fibrinolysis mechanisms. These pathways are schematically represented in Figs. 2 to 4.

Blood coagulation cascade is divided into extrinsic (also called tissue factor) and intrinsic (also called contact activation) pathway. The initiation of either pathway results in activation of factor X (FX), leading to the formation of thrombin, which is referred to as common pathway (Nesheim, 2003).

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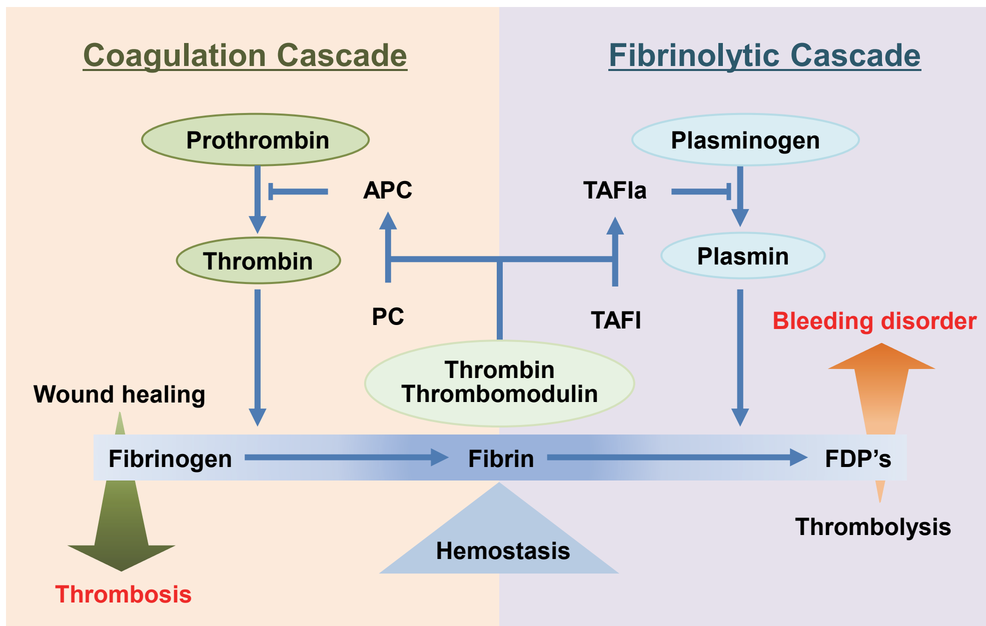

Fig. 1. The balance between coagulation and fibrinolysis. PC, protein C; APC, activated protein C; TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa, activated thrombin activatable fibrinolysis inhibitor; FDP's, fibrin degradation products.

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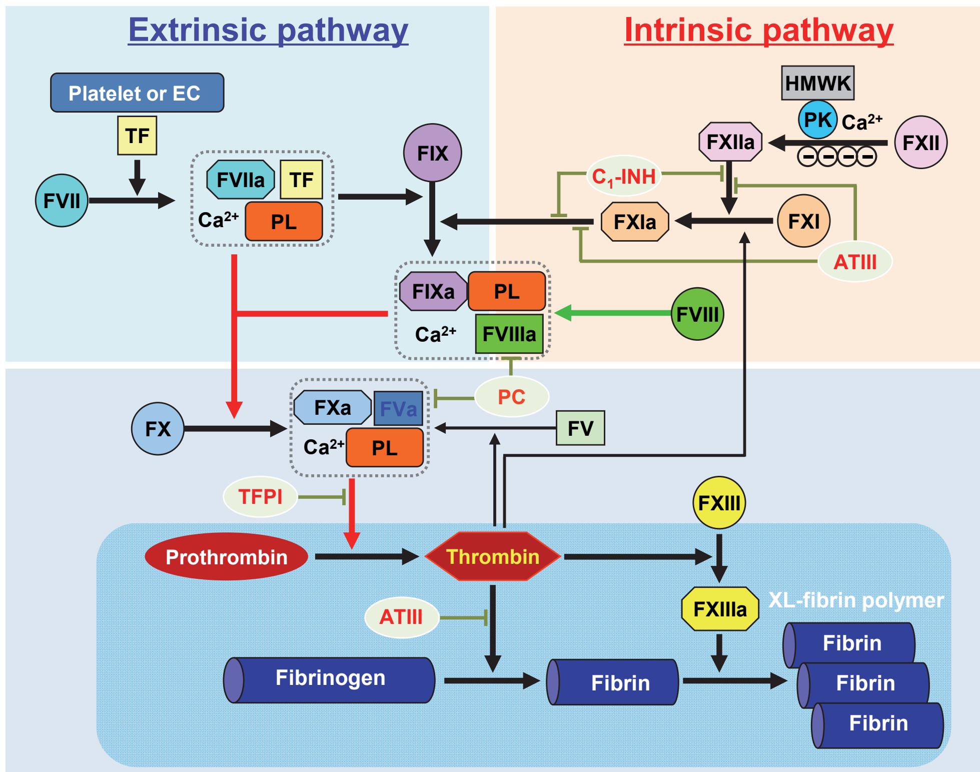

Fig. 2. Blood coagulation cascade. EC, Endothelial cell; TF, Tissue factor; PL, Phospholipid; HMWK, High molecular weight kininogen; PK, Prekallikrein; C_1-INH, C_1-inhibitor; TFPI, tissue factor pathway inhibitor; ATIII, anti-thrombin III, respectively. The action of natural inhibitors ATIII, C_1-INH, and protein C are indicated by blunt-ended line. The width of the arrow lines represented the strength of induction or activation.

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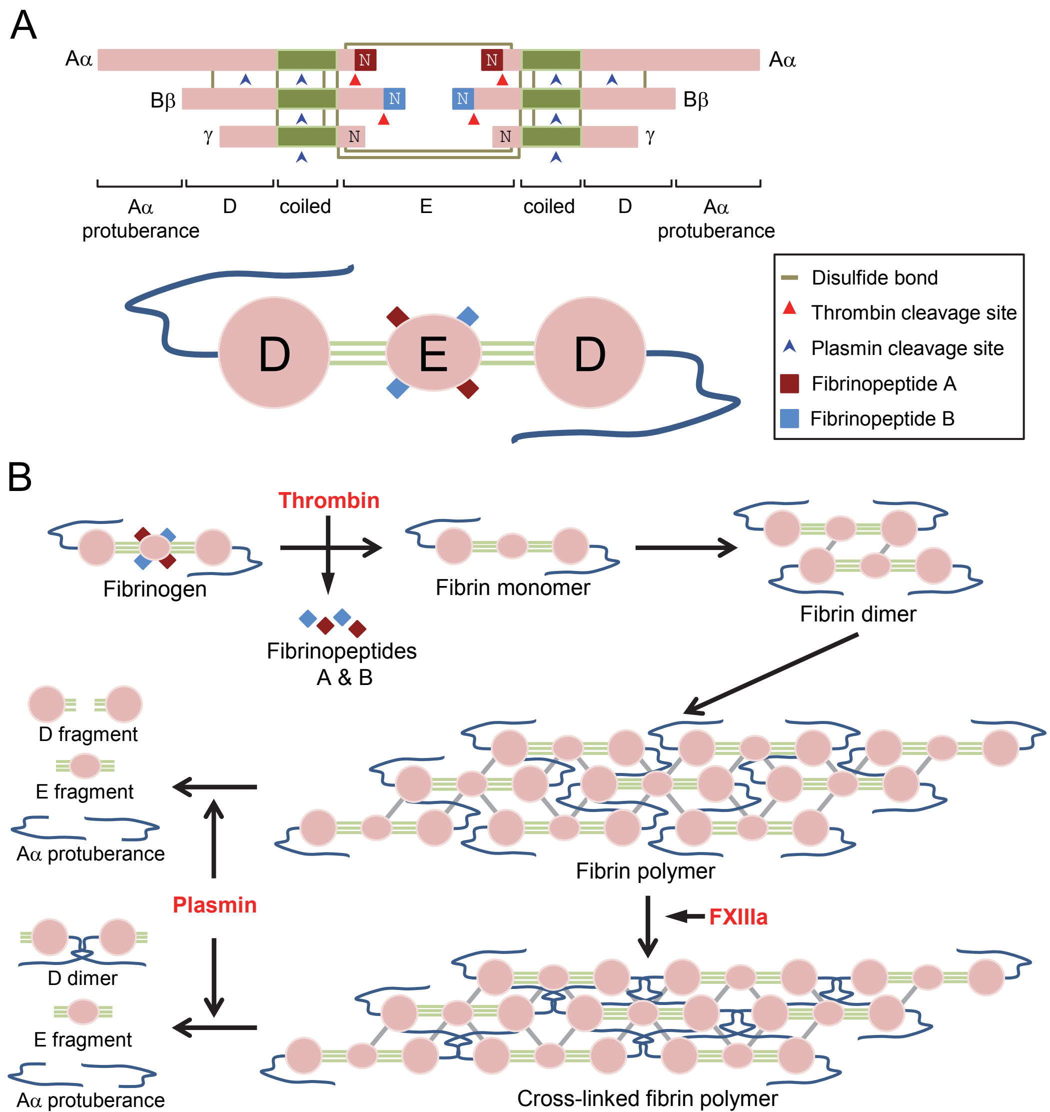

Fig. 3. The conversion of fibrinogen to fibrin. (A) The polypeptide and domain composition of fibrinogen. (B) Pathway of fibrin polymerization and degradation.

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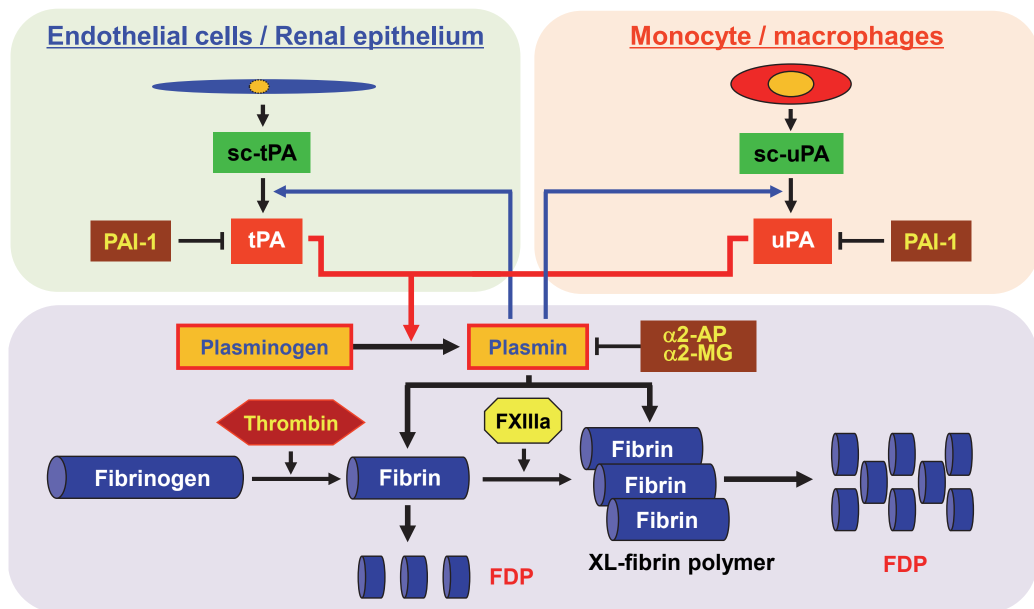

Fig. 4. Overview of the fibrinolytic system. tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; sc, single-chain; PAI, plasminogen activator inhibitor-1; α_2-AP, α_2-antiplasmin; α_2-MG, α_2-macroglobulin; FDP, fibrin degradation products, respectively.

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In the extrinsic pathway, vascular damage can lead to the platelet or endothelial cell activation with the exposure of tissue factor (TF). TF binds to the clotting factor VII (FVII) and initiates the TF-FVII activation complex formation. The TF-FVIIa complex can activate factor IX (FIX) via proteolysis. The activated FIX (FIXa) also can activate FX, in the presence of its activated cofactor VIII (FVIIIa) (Hoffman and Monroe, 2007).

The intrinsic pathway is triggered by factor XII (FXII, also called Hageman factor) when it contacts with a negatively charged surface such as glass or membrane of an activated platelet, provided the prekallikrein and its cofactor high molecular weight kininogen (HMWK) is present. Activated FXII (FXIIa) proteolytically cleaved factor XI (FXI) and then factor IX is activated by the FXIa (Riddel *et al*., 2007; Tanaka *et al*., 2009).

The common pathway begins with the activation of FX by either the extrinsic or intrinsic pathway. Proteolytically activated FXa required calcium binding phospholipid and cofactor V (FV) for prothrombin activation. After cleavage by FXa, prothrombin is converted to thrombin and then it catalyzes the proteolysis of the soluble plasma protein fibrinogen to fibrin. The fibrin monomers polymerize to form insoluble fibrin. Thrombin also can activates factor XIII (FXIII) and the activated FXIII (FXIIIa) mediates the cross-linking of the fibrin polymer (Riddel *et al*., 2007; Tanaka *et al.*, 2009).

The coagulation pathway can be regulated by enzymatic inhibition or modulation of cofactors. For examples, tissue factor pathway inhibitor (TFPI) binds to FXa and neutralizes its activity. The TFPI and FXa complex then inhibits FVIIa bound to TF. A serine protease inhibitor anti-thrombin is the most important inhibitor of coagulation. Anti-thrombin inactivates thrombin, FXa, and other serine protease in a reaction that is accelerated by heparin. Moreover, C_1-inhibitor (C_1-INH) is the most important physiological inhibitor of plasma kallikrein, FXIa, and FXIIa (Davis, 2004). Thrombomodulin serves as

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a receptor for thrombin. Thrombomodulin and thrombin complex no longer converts fibrinogen to fibrin, or amplifies its generation. Furthermore, thrombin and thrombomodulin complex can activate vitamin K-dependent protein C in the presence of protein S as cofactor. Activated protein C degrades FVa and FVIIIa, blocking the amplification of the coagulation systems (Riddel *et al*., 2007; Tanaka *et al*., 2009).

Fibrinogen and fibrin play essential roles in blood clotting, wound healing, cell-to-cell interactions, and inflammatory responses (Jennewein *et al*., 2011). As shown in Fig. 3A, the fibrinogen is composed of six polypeptide chains: two each of Aα, Bβ, and γ, joined by disulfide bonds and the dimeric structure can be categorized into four major domains: central E domain, two identical terminal D domain, and coiled coil C domain (Mosesson, 2005). The N-terminus of central E domain is composed of the fibrinopeptides A and B. In the process of coagulation (Fig. 3B), the two Aα and two Bβ chains of fibrinogen are cleaved by thrombin and the resulting fibrinopeptides A and B are released. The fibrin monomers devoid of the fibrinopeptides have ($\alpha\beta\gamma$)2 structures and then exposed polymerization site of E domain that interaction to D domain of fibrin monomer. As the fibrin polymer continues to make entangled long fibers, the fibrin clot. In the presence of FXIIIa, the fibrin clot is stabilized to form γ-γ dimer and α-α polymers. These cross-links help strengthen the fibrin clot, making it more resistant to physical and chemical damage (Mosesson, 2005). The fibrin deposits formed in arteries cause an intravascular thrombosis, which can induce cardiovascular diseases, such as myocardial infarction (Acharya and Dimichele, 2008). Therefore, fibrinolysis is essential for degrading a blood clot to maintain hemostasis (Jennewein *et al*., 2011). The formation of fibrin from fibrinogen is summarized in Fig 3B. The fibrin clot is subsequently digested by fibrinolytic system (Fig. 4). Single-chains of urokinase and tissue type plasminogen activator (sc-uPA and

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sc-tPA) are expressed on monocyte and endothelial cells. These inactive zymogens are converted to uPA and tPA by locally derived plasmin. Activated uPA and tPA convert plasminogen to plasmin and then the active plasmin catalyzes the proteolysis of fibrinogen and fibrin. Plasmin is itself activated from the plasminogen by uPA and tPA generation, however, this conversion is tightly regulated by plasminogen activator inhibitor-1 and -2 (PAI-1 & -2). Furthermore, activated plasmin is inhibited by α_2-antiplasmin and α_2-macroglobulin (Shanmukhappa *et al*., 2005).

In general, plasminogen activators and plasmin-like enzymes can be used as thrombolytic agents. Clinically, tPA and uPA, endogenous serine proteases secreted by vascular endothelium, are now being used for treating not only stroke but also myocardial infarction and atherosclerosis, since they dissolve effectively fibrin clot that are deposited in blood vessels (Lansberg *et al*., 2012). However, they do not dissolve fibrin thrombus directly but just catalyze the proteolytic activation of plasminogen to plasmin, the major enzyme responsible for clot breakdown (Murciano *et al*., 2003).

Especially, tPA therapy for dissolving fibrin clot often causes a bleeding in the brain or in other parts of the body and evokes an allergic reaction (ex., rashes, hives, leg swelling, and throat swelling) (Gurwitz *et al*., 1998; Kase *et al*., 1992). The side effects caused by these enzymes, in general, seem to be related to the facts that they are not only indirect fibrinolytic enzymes, but also activate matrix metalloproteases, a family of endopeptidases that degrade extracellular matrix proteins (Adibhatla and Hatcher, 2008).

On the other hand, one clinically important thrombolytic agent, which is streptokinase, and its production from *Streptococcus equisimilis* (Christensen, 1945; Medved *et al*., 1996). Streptokinase has a indirect fibrinolytic activity and it clot dissolving activity is via the activation of plasminogen. Unlike uPA and tPA, which are proteases, streptokinase possesses no proteolytic activity

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of its own (Castellino, 1981). Streptokinase acquires its plasminogen activating property by complexing with plasminogen. Streptokinase is as effective as tPA in treatment to acute myocardial infraction, but this microbial protein can illicit severe anaphylactic shock (White, 1990). Therefore, there is still a need to obtain direct thrombolytic agents, not showing the side effects displayed by the general plasminogen activators (Lansberg *et al.*, 2012).

To date, many fibrinolytic enzymes have been purified and characterized from various sources (Choi *et al.*, 2011), including snake venoms (De-Simone *et al.*, 2005; Leonardi *et al.*, 2002), marine green alga (Matsubara *et al.*, 2000), and insects (Amarant *et al.*, 1991; Hellmann and Hawkins, 1964; Matsushima *et al.*, 1993). Another fibrinolytic enzyme was produced by fungi, such as Chinese traditional medicinal mushroom (Choi *et al.*, 2011). Furthermore, fermented foods are attracting more interest in the development of fibrinolytic enzyme. Many fibrinolytic enzymes were purified from Asian traditional foods, such as a Korean fermented soybean sauce (Chungkook-jang) (Kim *et al.*, 1996), Chinese fermented soybean food (Douchi) (Peng *et al.*, 2003), and fermented soybean food in Japan (Natto) (Fujita *et al.*, 1993).

The details of the biological and physiological properties of fibrinolytic enzymes are summarized in Table 1. In particular, a great deal of effort has been made to identify and purify fibrinolytic enzymes from various earthworm species (Hrzenjak *et al.*, 1998; Mihara *et al.*, 1991; Nakajima *et al.*, 1993). Previous reports show that earthworms living under various environments such as wetlands and mud flats possess different isotypes of fibrinolytic enzymes (Kim *et al.*, 1998; Mihara *et al.*, 1992). Clinical trials also have shown that the fibrinolytic enzymes from earthworms have a great potential of becoming effective therapeutic agents for specific thrombus dissolution (Hwang *et al.*, 2002; Mihara *et al.*, 1992). Even though earthworm fibrinolytic

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Table. 1. Comparison of the characteristics of various fibrinolytic enzymes.

Source	Enzyme	M.W. (kDa)	N-terminal sequence	Function (Chain specificity)	Type of Proteases	General peptide substrate	Optimal pH / °C	References
Human	Plasmin	88.4	ECKTGNGKN (VVGGCVAHP)	Fibrinolytic ($A\alpha > B\beta >> \gamma$)	Serine protease	Gly-Pro-Lys	8.5 / 37	Forsgren *et al*., 1987 Greig and Cornelius, 1963
	tPA	70	SYQVICRDE (IKGGLFADI)	Plasminogen activator	Serine protease	Ile-Pro-Arg	8.5 / 41	Camiolo et al., 1971 Itagaki *et al*., 1991
	uPA	55	SNELHQVPS (IIGGEFTTI)	Plasminogen activation	Serine protease	Glu-Gly-Arg	8.5 / 41	Barlow, 1976 Itagaki *et al*., 1991
Streptococcus equisimilis H46A	Streptokinse	47	MKNYLSFGM (VAGTVEGTN)	Plasminogen activation	Non protease	NA[a]	7.5 / 37	Christensen, 1945 Malke & Ferretti, 1984
Lonomia achelous	Achelase	24	IVGGSVTTI	Fibrinolytic ($A\alpha > B\beta >> \gamma$)	Serine protease	Glu-Gly-Arg	NA[a]	Amarant, 1991
Lumbricus rubellus	F-III-1 F-II F-I-1 F-I-0	27 36 40 43	IVGGIEARP VIGGTNASP IIGGSNASP VVGGSDTTI	Fibrinolytic ($A\alpha > B\beta >> \gamma$)	Serine protease	Phe-Pip-Arg Ala-Pro-Val Ile-Pro-Arg Val-Leu-Arg	9-11 / 60	Nakajima, 1993
Bacillus subtilis sub sp. natto	Nattokinase	27.7	AQSVPYGIS	Fibrinolytic (NA[a])	Serine protease	Ala-Pro-Phe	8.0 / 40	Fujita *et al*., 1993

[a] Data not available.

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Table 1. Continued.

Source	Enzyme	M.W. (kDa)	N-terminal sequence	Function (Chain specificity)	Type of Proteases	General peptide substrate	Optimal pH / °C	References
Bacillus sp. strain CK 11-4	CK	28.8	AQTVPYGIP	Fibrinolytic & Plasminogen activator	Serine protease	Val-Leu-Lys	10.5 / 70	Kim *et al.*, 1996
Codium divaricatum	CDP	31	NA[a]	Fibrinolytic (Aα)	Serine protease	Glu-Gly-Arg	9 / ND[a]	Matsubara *et al.*, 2000
Vipera ammodytes ammodytes	VaH	70	MVTKYSSI	Fibrinolytic (Aα)	Metallo protease	Ala-/-Leu-Tyr-Leu	7.5 / ND[a]	Leonardi *et al.*, 2002
Bacillus amyloliquefaciens DC-4	Subtilisin DFE	28	AQSVPYGVS	Fibrinolytic (NA[a])	Serine protease	Ala-Pro-Phe	9 / 48	Peng *et al.*, 2003
Eisenia fetida	EFE-a EFE-b EFE-d EFE-f	24.6 29.5 24.2 23.0	VIGGTNASP IVGGIEARP IIGGSNASP VVGGSDTTK	Fibrinolytic (Aα>Bβ>>γ)	Serine protease	Phe-Gly-Arg Phe-Gly-Arg Ala-Pro-Phe Ala-Pro-Phe	NA[a]	Wang *et al.*, 2003
Cordyceps sinensis	CSP	31	ALATNHGAP	Fibrinolytic (Aα>Bβ>>γ)	Serine protease	Ala-Pro-Trp	7.0 / 40	Li *et al.*, 2007
Perenniporia fraxinea	NA[a]	42	ASYRVLPIT	Fibrinolytic (Aα>Bβ)	Metallo protease	Arg-Pro-Tyr	6 / 35-40	Kim *et al.*, 2008

[a] Data not available.

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Table 1. Continued.

Source	Enzyme	M.W. (kDa)	N-terminal sequence	Function (Chain specificity)	Type of Proteases	General peptide substrate	Optimal pH / °C	References
Streptomyces sp. CS684	FP84	35	GTQENPPSS	Fibrinolytic (Bβ)	Metallo protease	NA[a]	7 / 45	Simkhada *et al*., 2009
Periserrula leucophryna	PLFP	30	IVGGQNARQ	Fibrinolytic (Aα>Bβ)	Serine protease	Val-Leu-Lys	NA[a]	Koo *et al*., 2010
Neanthes japonica (*Iznka*)	NJF	28-32	NA[a]	Fibrinolytic (Aα=Bβ>γ)	Serine protease	Phe-Pip-Arg	9.0 / 60	Deng *et al*., 2010
Cordyceps militaris	NA[a]	34	APVEQCDAP	Fibrinolytic (Aα>Bβ>γ)	Serine metallo protease	Ala-Pro-Phe	7.0 / 40	Choi *et al*., 2011
Virgibacillus halodenitrificans SK1-3-7	NA[a]	26-36	ELLEFERNA/ EAQTLVDFL	Fibrinolytic (Aα>Bβ)	Serine protease	Ala-Pro-Phe	7 / 40	Montriwong *et al*., 2012

[a] Data not available.

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enzymes has advantages in clinically application, there are some problem as used for therapeutic agent. It hydrolyzes other proteins *in vivo* and the broad specificity may lead to hemorrhage. For those reasons, it is still worthwhile to screen a highly specific fibrinolytic enzyme(s) from earthworms and polychaete worms.

Proteolytic enzymes are useful for biochemical research, industry and therapeutic purpose. However, in most case, proteases are difficult to purify, because they are expressed in low levels from host or contaminated with other materials. For these reasons, many researchers have been fascinated to obtain them as recombinant proteases. As a result, many recombinant proteases are produced from various host cells, such as bacterial cells, insect cells, yeast cells, and mammalian cells. Large-scale expression in mammalian systems are often difficult, expensive and time consuming. Unlike eukaryotic expression systems, bacterial expression systems are easy and inexpensive. However, almost all bacterial cells are not able to post-translational modification, such as glycosylation, acetylation, and formation of disulfide bridges. Futhermore, only some proteins are soluble upon overexpression in *Escherichia coli* (*E. coli*), as most of the expressed proteins are insoluble (Hammarstrom *et al*., 2002; Yang *et al*., 2003). Therefore, yeast production systems have been designed to take an advantage for post-translational modification and economic efficiency (Cregg *et al*., 2009).

Among several yeast systems, methylotrophic yeast such as *Pichia pastoris* (*P. pastoris*) system can be successful for the production of soluble recombinant proteins. Several recombinant proteins have been produced in *P. pastoris.* They include a G-protein coupled receptors (Hori *et al*., 2010; Sarramegna *et a*l., 2003), bovine enterokinase light chain (Peng *et al*., 2004), and *E. coli* phytase (Chen *et al*., 2004). The successful expression of

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recombinant proteins provides a way to obtain single component with objective function.

This study describes the purification and characterization of three novel alkaline serine fibrinolytic enzymes from a polychaete species, *Cirriformia tentaculata* (*C. tentaculata*), with a focus on their biochemical properties and fibrino(geno)lytic activities in terms of the cleavage of thrombosis components. Together with these biochemical studies, three cDNA clones capable of encoding CTSP-1, -2, and -3 enzymes were obtained by using 5´- and 3´-RACEs and their nucleotide sequences were analysed. Among the cDNA clones obtained, *CTSP-3* gene was expressed in a yeast *P. pastoris* cells and the recombinant protease (named yrCTSP-3) was purified using one step affinity chromatography.

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II. MATERIALS AND METHODS

II-1. Materials

Diisopropyl fluorophosphate (DFP), azocasein, ammonium sulfate, bovine serum albumin (BSA), trizma base, trichloroacetic acid (TCA), biotin, and other chemicals were purchased from Sigma (St. Louis, MO, USA). Chromatographic columns, including HiPrep 16/10 Q FF, Mono Q 4.6/100 PE, Superdex 75 10/300 GL, HiTrap Chelating HP, and PD-10 were from Amersham Pharmacia Biotech Co. (Uppsala, Sweden). Yeast extract, tryptone, peptone, yeast nitrogen base (YNB), and bacto agar were purchased from Becton Dickinson (Baltimore, MD, USA). T_4 DNA ligase, T_4 RNA ligase, RNase H, restriction enzymes *Xho* I and *Xba* I were purchased from New England BioLabs (Beverly, MA, USA). Agarose gel extraction kit, PCR purification kit and i*pfu* polymerase were obtained from iNtRON Biotechnology (Seonnam, Korea). Plasmid extraction kit, *Taq* polymerase, Hotstart *Taq* polymerase, *Moloney Murine leukemia virus* (M-MLV) reverse transcriptase, λ /*Hind* III marker, and 100 bp DNA ladder were purchased from BiONEER (Daejeon, Korea). Synthetic chromogenic substrates, including H-D-Phe-Pip-Arg-*p*NA (S-2238), H-D-Val-Leu-Lys-*p*NA (S-2251), H-D-Ile-Pro-Arg-*p*NA (S-2288), Pyro-Glu-Gly-Arg-*p*NA (S-2444), MeO-Suc-Arg-Pro-Tyr-*p*NA (S-2586), and N-α-Z-D-Arg-Gly-Arg-*p*NA (S-2765) were obtained from Chromogenix (Milan, Italy). Z-Arg-Arg-*p*NA (L-1225) and H-Leu-*p*NA (L-1305) were from Bachem (Bubendorf, Switzerland). Boc-Val-Pro-Arg-*p*NA and Boc-Leu-Gly-Arg-*p*NA were from Seikagaku (Tokyo, Japan). Protein molecular mass markers were obtained from Fermentas (Opelstrasse, Germany) and Geneaid (Taiwan). The plasmids pGEM-T easy and pJET1.2/blunt were obtained from Promega (Madison, WI, USA) and

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Fermentas. The pPICZαA plasmid was obtained from Invitrogen (Carlsbad, CA, USA). The Anti-his tag monoclonal antibody was purchased from Novagen (Madison, WI, USA). The peroxidase conjugated anti-mouse secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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 3,000 x*g* to remove blood cells. The resulting plasma was stored at -70°C until used. The organism *C. tentaculata* was identified on the basis of its morphological characteristics, including the dorsal transverse tentacles inserted on a single anterior segment, and the notopodia and neuropodia with capillary and acicular setae (Imajima, 1964; Paik, 1989) (Fig. 5). The polychaete worm individuals were collected from the coast of Wando island (34°20´27˝N, 126°49´5˝E, Republic of Korea), immediately washed, frozen, and stored at -70°C until used.

II-2. Cultivation of *E. coli* and yeast cells

E. coli cells were cultured in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) overnight at 37°C under aerobic conditions. Yeast *P. pastoris* X-33 cells were grown in YPD (2% peptone, 1% yeast extract, and 2% dextrose), YPG (2% peptone, 1% yeast extract, and 1% glycerol), YPM (2% peptone, 1% yeast extract, and 0.5% methanol), or BMMY (100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 2% peptone, 1% yeast extract, and 0.5% methanol) media at 28 or 30°C under aerobic conditions.

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Fig. 5. Photograph of *Cirriformia tentaculata*. This organism has orange-yellow colored semi-cylindrical body with filamentous gill and palpus. It grows up to 10 cm and normally lives in subtidal zone.

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II-3. Purification of proteolytic enzymes

For the purification of proteolytic enzymes, the frozen worms (approximately 200 g) were thawed in 500 ml of ice-cold 25 mM phosphate buffer (pH 7.0), homogenized three times at two intervals of 5 min at the maximal speed using an electric homogenizer (Pro Scientific, Oxford, UK), and stirred for 12 h at 4°C. Cell debris were removed from the homogenate by centrifuging at 8,000 x*g* and 4°C for 30 min and the supernatant was subjected to ammonium sulfate precipitation. The salt was added to the supernatant at a saturation concentration of 40%, stirred for 6 h at 4°C, and then centrifuged for 40 min at 12,000 x*g*. The resulting protein precipitate was discarded, since the fraction still contained a large amount of white floating lipid-like materials even with a small amount of proteins. Soluble proteins remained in the supernatant were further fractionated with 60% ammonium sulfate in a saturation concentration, in which the sample was stirred for 6 h at 4°C and the resulting protein precipitate was collected by centrifuging for 40 min at 12,000 x*g* and 4°C. The proteins obtained were dissolved in 25 mM phosphate buffer (pH 7.0) and desalted on a PD-10 column equilibrated with the same buffer. The desalted proteins (about 660 mg) were loaded onto a HiPrep 16/10 Q FF column equilibrated with 25 mM phosphate buffer (pH 7.0) and the bound proteins were eluted by a NaCl linear gradient ranging from 0-0.6 M in the same buffer. The active fractions were pooled, concentrated by ultrafiltration using an YM 10 membrane (Millipore, Billerica, MA, USA), and then desalted on a PD-10 column equilibrated with 25 mM phosphate buffer (pH 7.0). The desalted proteins (total 40 mg) were loaded onto a Mono Q 4.6/100 PE column equilibrated with 25 mM phosphate buffer (pH 7.0) and the bound proteins were eluted by a NaCl linear gradient of 0-0.3 M in the same buffer. Three protein pools

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in three peaks (peaks I, 3.8 mg; II, 5 mg; III, 10 mg) were concentrated, desalted, and loaded separately onto Superdex 75 10/300 GL gel filtration columns equilibrated with 25 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. The active fractions were collected, pooled, and stored at −70°C as purified enzymes. Protein concentrations were determined by Bradford method as described elsewhere.

II-4. Protease activity assay

Azocasein assay was routinely used for examining protease activity in chromatographic steps and for investigating temperature and pH requirements for the purified enzyme as described previously (Chang *et al*., 2005). In typical azocasein assay, a reaction mixture (total 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°C for 30 min. The reaction was stopped by the addition of 100 μl of 10% TCA and centrifuged at 10,000 x*g* for 10 min. 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 a protease digesting 1 μg of azocasein per min. The buffer systems used in the azocasein assay for the pH requirement of enzyme were as follows: 50 mM citrate (pH 5.5-6.5); 50 mM Tris-HCl (pH 7.0-9.0); 50 mM glycine-NaOH (pH 10.0-11.0). The effects of temperature on enzyme activity in the assay were examined for 30 min at various temperature conditions. The thermo-stability of the purified enzyme was also investigated using the azocasein assay, in which 0.5 μg of enzyme was pre-incubated for 20 min at 60°C and further incubated for 20 min at 37°C. On the other hand, the amidolytic activity of enzyme towards various chromogenic substrates was determined as follows: typically 80 μl of 50 mM

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Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 0.1 mg/ml BSA were mixed with 10 μl of purified enzyme (typically 0.5 μg) and then the mixture was incubated at 37°C for 10 min. The reaction was initiated by adding 10 μl of 4 mM chromogenic substrate and the absorbance at 405 nm was continuously monitored for 20 min in a 96-well plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The effects of various protease inhibitors and divalent cations on enzyme activity were examined using a chromogenic substrate at 37°C for 20 min, in which one unit of protease activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of *p*-nitroanilide (*p*NA) per min.

II-5. Fibrino(geno)lytic activity assay

For fibrinogenolytic activity assay, a total 200 μl of reaction mixture consisting of fibrinogen (100 μg) and purified enzyme (5 μg) in 25 mM Tris-HCl buffer (pH 7.5) in a microcentrifuge tube was incubated at 37°C, and 20 μl of reactants were withdrawn at the intervals of 0, 1, 3, 5, 10, 20, 30, 60, and 90 min. The reaction was stopped by the addition of 4 μl of 6x SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heated at 100°C for 3 min. The resulting products (10.5 μg) were analyzed by SDS-PAGE as described previously (Chang *et al*., 2005). Fibrinolytic activity of enzyme was measured on fibrin plate and also by turbidity assay as described previously. Fibrin plate was made by mixing 2 ml of 1% fibrinogen, 2 ml of 1% agarose, and 70 μl of thrombin (17.7 U/ml) in 25 mM sodium phosphate buffer (pH 7.5). Into the wells (3 mm in diameter) made in the plate, 20 μl each of phosphate buffered saline (PBS) (pH 7.4), purified enzyme (2 μg), and plasmin (0.04 U) were inoculated and incubated at 37°C for 6 h to visualize halo zones. The turbidity assay was performed by

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measuring the decrease in turbidity of fibrin polymer formed by the enzyme in a 96-well plate. Typically 90 μl of 1 mg/ml fibrinogen in 25 mM phosphate buffer (pH 7.5) was added to 10 μl thrombin (17.7 U/ml) and incubated for 1 h at 25°C to allow the forming of fibrin polymer. Thereafter, 10 μl of purified enzyme (2 μg) or plasmin (0.04 U) were added and incubated for 2 h at 37°C. 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 produced by incubating a total 15 μl of reaction mixture consisting of 10 μg fibrinogen, 0.02 U thrombin, 0.002 U FXIIIa, and 1 mM $CaCl_2$ in 50 mM Tris-HCl buffer (pH 7.5) at 25°C for 1 h. For the detection of cross-linked fibrin cleavage, 0.5 μg of purified enzyme was added to the cross-linked fibrin sample and incubated at 37°C for 30 min. The reaction was stopped by the addition of 3 μl of 6x SDS-PAGE sample buffer and heated at 100°C for 3 min. The reaction products (approximately 11 μg of proteins) were electrophoresed onto 8% SDS polyacrylamide gel and visualized by staining the gel with Coomassie brilliant blue.

II-6. Analysis of the N-terminal sequence of purified enzyme

Purified enzyme was subjected to electrophoresis on 12% SDS-polyacrylamide gel and transferred to PVDF membrane in 10 mM CAPS buffer (pH 11) containing 10% methanol. The blot was stained with Coomassie brilliant blue, followed by destaining. Target bands were excised from the membrane and subjected to N-terminal sequencing with a Precise 491 HT protein sequencer (Applied Biosystems, Foster City, CA, USA) as described elsewhere. The N-terminal sequencing was performed by the Korea Basic Research Institute (Seoul, Korea) using Edman degradation as described elsewhere.

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II-7. Effects of serum albumin and human plasma on enzyme activity

The reaction mixture (typically 90 μl) was composed of 0.5 μg enzyme to be tested and 6.7 mg/ml BSA or 6.7 mg/ml human plasma proteins in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. After adding 10 μl of chromogenic substrate (4 mM), the absorbance at 405 nm was monitored at 37°C for 20 min. In this assay, enzyme activity was expressed as a relative activity, compared to that of non-enzyme treated control.

II-8. Turbidimetric lysis assay of human plasma clot

Human plasma clot formation and lysis assay was performed with the modified method of Carter *et al* (Carter *et al*., 2007). Briefly, 90 μl of human blood plasma and 10 μl of activation solution consisting of 3.54 U/ml thrombin and 5 mM $CaCl_2$ in 25 mM phosphate buffer (pH 7.4) were mixed, and the mixture was incubated for 2 h at 25°C to allow the formation of plasma clot. Thereafter, 20 μl each of purified enzyme (15 μg) and plasmin (0.1 U) were added and further incubated at 37°C, during which the decrease in absorbance at 350 nm was recorded with a 96-well plate reader every hour for 8 h. The relative turbidity was expressed as a percentage of a decrease in turbidity, relative to that at the beginning of incubation.

II-9. Total RNA extraction and mRNA isolation

For preparation of total RNA, fresh *C. tentaculata* samples were

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homogenized using a tissue filter with homogenizer instrument (BiONEER, Daejeon, Korea) in the presence of Trizol reagent (iNtRON, Seonnam, Korea). Total RNA was obtained according to manufacturer’s protocols using the easy-spin total RNA extraction kit (iNtRON, Seonnam, Korea). Typically, 100 μl of the oligo(dT)-magnetic beads (Agilent, Santa Clara, CA, USA) were added and incubated at room temperature for 20 min with agitation every 5 min. The tubes were placed in a magnet specific to the separation of the magnetic beads containing the Poly(A)$^+$ RNA, and the supernatant was removed. Two hundred μl of wash solution was then added, and again the tubes were vortexed to wash the magnetic beads. The tube was again transferred to the magnet to separate the beads containing the captured Poly(A)$^+$ RNA. A second wash was performed with 200 μl, after which the beads were resuspended with 50 ml of elution buffer. The isolated mRNA was used in the cDNA synthesis.

II-10. 3´-rapid amplification of cDNA ends

The 3´-ends of three CTSP cDNAs were obtained using 3´-RACE PCRs. A M-MLV reverse transcriptase was used to generate cDNAs for the 3´-RACE. A total 100 pmol of oligo dT-anchor primer (Table 2) was added to 80 ng of mRNA to hybridize. The solution was incubated at 70°C for 5 min to disrupt to RNA secondary structures and immediately chilled on ice for 5 min. The cDNA synthesis mixture (20 μl of 5x reverse transcriptase reaction buffer, 10 μl each of 10 mM 4 dNTP’s, 10 μl of 100 mM DTT, and 5 μl of 200 U/μl M-MLV reverse transcriptase) was added and incubated for 5 min at 25°C. cDNA synthesis was carried out at 42°C for 60 min and then heat inactivated at 90°C for 5 min. The reactions were purified through a MEGAquick-spin total purification kit and eluted in 35 μl of elution buffer

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Table 2. Primers used for amplifying three *CTSP* genes.

Description	Name of oligomer	Sequence (5´ to 3´)	Length (bp)	Ta* (C)
Universal primers used for 3´-RACE	Anchor-dT	ATTCTAGAGGCCGAGGCGGCCGATTTTTTTTTTTTTTTTTTTTTT	45	42
	Anchor	ATTCTAGAGGCCGAGGCGGCCGA	23	58
	His-F	YTNACYGCYGCYCAYTGT	18	58
	Ser-R	CANKTNCCWCTKAGNCCWCCWGGT	24	58
Gene specific primers used for 3´-RACE	GSP1-1	ATYATGMANGGWTCNCCAGCY TCY	24	58
	GSP1-2	ATYATGTAYGGNCARGARGCYGCY	24	58
	GSP1-3	ATYATYGCWGCWCANGARGCYGAY	24	58
Gene specific primers used for 5´-RACE	GSP-RT1	ppp-GAGTCTGGCACTCACTGT	18	42
	CT1 F1	TGGCGATGGCGATCACGCTGGAGCAGCCTG	30	64
	CT1 R1	GCTGCTGAGGTCAGCGGAGGAGGACAGATG	28	64
	CT1 F2	ATGTACGCTAACACCCAAGTCATCAGCA	28	64
	CT1 R2	TCAATGCTTGAGACATCATGAGTGGACTCG	30	64
	GSP-RT2	ppp-GGTATCGCCAGTGAATTC	18	42
	CT2 F1	GTTCTGCATCTGGCAAGTTCTGCTGATACT	30	62
	CT2 R1	CTCAGACTCAGGACTGCCCTGGACGTG	27	62
	CT2 F2	TGAGCCAATCAGGATTGCTAGGGCTGCT	28	64
	CR2 R2	CGACAAGGACAGCACCACAGGTGTGGTA	28	64
	GSP-RT3	ppp-GACACAGTTGTCATAAGG	18	42
	CT3 F1	AGCAGCCCATGGCCCAATGACATTGC	26	62
	CT3 R1	TAGGCAGTTCCGTCAGTCAGT CTGTTGTG	29	62
	CT3 F2	GTCGTTGGCATCTCATCTGACCCCATAG	28	63
	CT3 R2	CAGCGTCAACGAGGACACAACCACA	25	63

*Ta, annealing temperature. Mixed bases code, Y (C/T); R (A/G); M (A/C); W (A/T); K (T/G); N (A/T/G/C).

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according to the manufacturer's instruction (iNtRON, Seonnam, Korea). Double-strand cDNA was amplified by PCR from single-strand cDNA using the Anchor primer and GSP1 primer (Table 2). Reaction was performed using a 10 pmol of GSP1 and Anchor primer (Table 2) in the following 50 μl reaction: 2 μl first-strand cDNA, 5 μl 10x polymerase reaction buffer, 5 μl 10 mM 4 dNTP's, and 5 U HotStart *Taq* DNA Polymerase. Reactions were processed in a DNA thermal cycler (Eppendrof, Hauppauge, NY, USA) through 5 min of pre-denaturation at 95°C and then 35 cycles of 40 sec denaturation at 94°C, 40 sec of annealing at 58°C and followed by 1 min of elongation at 72°C. The reactions were chilled on ice and the PCR products were purified using a MEGAquick-spin total purification kit. To increase the specificity of the amplified PCR products, a total 2 μl of purified PCR products were employed in second round PCR reaction. The reaction mixture was composed of 50 μl as follows: 2 μl of 1st PCR product, 1 μl of 10 pmol forward (GSP1 or Ser-F) and reverse (Anchor or His-R) primers (Table 2), 5 μl of 10x polymerase reaction buffer, 5 μl of 10 mM 4 dNTP's, and 5 U of *Taq* DNA Polymerase. PCR was performed for 2 min of pre-denaturation at 95°C and then 40 sec at 94°C, 40 sec at 58°C, and 1 min at 72°C for 35 cycles. PCR products were electrophoresed on a 1% agarose gel, and the expected DNA band was purified using a MEGAquick-spin total purification kit. The PCR products were cloned into a pGEM-T easy vector. The recombinant plasmid DNA was transformed into *E. coli* strain DH5α. DNA sequences of positive clones were analysed on both strands, using overlapping clones. The DNA sequencing was performed by the BiONEER (Daejeon, Korea) using dideoxy chain termination method as described elsewhere.

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II-11. 5´-rapid amplification of cDNA ends

The 5´-ends of three CTSP clones were obtained using 5´-RACE PCRs. First strand cDNA was synthesized using a 2 μg total RNA (or 15 ng mRNA) in a 20 μl reaction containing 10 pmol of GSP RT primers phosphorylated at 5´ end (Table 2), 2 μl of 10 mM 4 dNTP's, 2 μl of 100 mM DTT, 4 μl of 5x reaction buffer, and 1 μl of 200 U/μl M-MLV RT. The reactions were incubated at 42°C for 60 min, followed by heat inactivation M-MLV RT at 90°C for 5 min. One unit of RNaseH was added to the reaction and incubated at 37°C for 60 min to remove the mRNA. The reaction was purified through a MEGAquick-spin total purification kit. A 25 μl of the purified cDNA was ligated in a 50 μl reaction containing 5 μl of 10x ligase reaction buffer, 5 μl of 10 mM ATP, 13 μl of 50% PEG 8000, and 2 μl of 10 U/μl T_4 RNA ligase at 16°C for overnight. PCR amplification was performed using 5 μl of ligated cDNA with CT-F1 and CT-R1 primers (Table 2) under the following reaction conditions: 5 min of pre-denaturation at 95°C, followed by 35 cycles of 40 sec at 94°C, 40 sec at 62~64°C, and 2 min at 72°C. The PCR performed in 50 μl of reaction mixture containing 5 μl of ligated cDNA, 1 μl of 10 pmol each primer, 5 μl of 10x polymerase reaction buffer, 5 μl 10 mM 4 dNTP's, and 5 U HotStart *Taq* DNA Polymerase. To increase the specificity of the amplified PCR products, different volume of 1st PCR products were employed in second round PCR reaction. The reaction mixture was 50 μl as follows: 1~10 μl of 1st PCR product, 1 μl of 10 pmol CT-F2, CT-R2 primer (Table 2), 5 μl of 10x polymerase reaction buffer, 5 μl of 10 mM 4 dNTP's, and 5 U of *Taq* DNA Polymerase. PCR was performed for 2 min of pre-denaturation at 95°C and then 40 sec at 94°C, 40 sec at 63~64°C, and 1 min at 72°C for 35 cycles. PCR products were electrophoresed on a 1% agarose gel, and the expected DNA band was

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purified using a MEGAquick-spin total purification kit. The PCR products were cloned into a pGEM-T easy vector. The recombinant plasmid DNA was transformed into *E. coli* strain DH5α. DNA sequences of positive clones were performed by the BiONEER.

II-12. Identification and cloning of full length *CTSP* genes

cDNA was made from 1 μg of total RNA using oligo (dT)18 primer and M-MLV reverse transcriptase. Cycling conditions were 25°C for 50 min, 42°C for 1 h and 72°C for 15 min. The cDNA products were then used as PCR templates to amplify full length *CTSP* genes. The PCR reaction mixture was 50 μl as follows: 0.5 μg of cDNA, 1 μl of 10 pmol forward and reverse primers (Table 3), 5 μl of 10x polymerase reaction buffer, 5 μl of 10 mM 4 dNTP's, and 2.5 units *ipfu* polymerase. Reactions were processed in a thermal cycler through 35 cycles of 40 sec of denaturation at 94°C, 30 sec of annealing at different temperature (Table 3) and followed by 60 sec of elongation at 72°C. PCR products were electrophoresed on a 1% agarose gel, and the expected DNA bands were purified. Each PCR product was cloned into a pJET1.2/blunt vector. The resulting vectors were named pJET-*CTSP-1, -2*, and *-3,* respectively.

II-13. Computer-based modeling of three dimensional structures of CTSPs

Modeling (computation and analysis) of the three-dimensional structure of CTSP enzymes was performed using SWISS-MODEL computative modeling program (http://swissmodel.expasy.org/) (Arnold *et al*., 2006).

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Table 3. The primers used for cloning of *CTSP* genes.

Description	Name of oligomer	Primer sequence[a] (5´ to 3´)	Length (nt)	Ta[b] (°C)
Primers used for full gene amplification	CTSP-1 F	AAGACGACGACAAAGATGAAGGTTCTGTTG	30	54
	CTSP-1 R	CACCCTTTCCTACTGTACAAGGATTCTTGT	30	
	CTSP-2 F	AATCCGCTGGCGATACCGTCCAGGA	25	54
	CTSP-2 R	CGACTATGGTTTATTTGAACCGCTCGAAT	31	
	CTSP-3 F	TTAGACGGACCATCTCTTCTTGATCTACTG	30	52
	CTSP-3 R	GAACTATACTCAACCAAAACGGTTGT	26	
Primers used for cloning to pPICZαA	CTSP-3 Xho F	<u>CTCGAG</u>AAAAGAATCATTGGGGGAACTGAGGCT	33	64
	CTSP-3 Xba R	<u>TCTAGA</u>CAGCTGACGTCTCCGCCGCTGTTGGA	32	

[a] Underlined capitals mean restriction site for corresponding enzyme.
[b] Ta, annealing temperature.

Amino acid sequences of CTSPs were initially submitted to SWISS-MODEL for homology-based structure prediction. The set of structurally conserved regions was based on the solved crystal structure of Earthworm Fibrinolytic Enzyme (PDB code: 1m9u.1.A), Tryptase alpha-1 (PDB code: 2f9o.1.A), and Serine protease hepsin (PDB code: 1p57.1.B). Final structures were refined using the PyMOL molecular viewer (http://www.pymol.org/).

II-14. Construction of recombinant plasmid for the expression of recombinant CTSP-3 enzyme

The yeast expression vector pPICZαA was used for expression of CTSP-3 enzyme in *P. pastoris* X-33. pPICZαA vector has an alcohol oxidase (*AOX1*) promoter, which induced by methanol (Cregg *et al*., 2009). Interest target protein cloned under the control of the *AOX1* promoter is highly expressed when methanol is used as carbon source. The putative mature form of *CTSP-3* gene from the pJET-*CTSP-3* clone was generated by PCR using the primers listed in Table 2. The *Xho* I and *Xba* I restriction enzyme site were added to assist cloning into the pPICZαA vector in the frame. Mature form of *CTSP-3* gene amplification using pJET-*CTSP-3* DNA as a template was performed by adding 2.5 mM 4 dNTP's, 2.5 units *ipfu* polymerase, and 10 pmol each of forward and reverse primers (Table 3) in a reaction buffer. Reaction was processed in a thermal cycler through 35 cycles of 40 sec of denaturation at 94°C, 30 sec of annealing at 64°C and followed by 60 sec of elongation at 72°C. PCR products were electrophoresed on a 1% agarose gel, and the expected band was excised and purified. The amplified PCR product was doubly digested with *Xho* I and *Xba* I and then ligated with *Xho* I/*Xba* I-cut pPICZαA vector to give the construct pPICZαA-*CTSP-3.* Positive clones were selected and their DNA

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sequences were determined to confirm the expected reading frame.

II-15. Transformation of yeast *P. pastoris* X-33

P. pastoris vectors are designed for homologous integration into *AOX1* locus (Fig. 6). Linear DNA can generate stable transformant of *P. pastoris* via integration or homologous recombination between the construct DNA and homology regions within the yeast genome (Fig. 6) (Cregg *et al*., 2009). Furthermore, pPICZαA vector has a *Sh ble* (Zeocin antibiotic resistance) gene for selection markers. To prepare competent cells, yeast *P. pastoris* X-33 cells were cultured overnight in 30 ml of YPD medium at 30°C with shaking (OD_{600} = 0.8 to 1.0). The culture was centrifuged at 4,000 x*g* for 10 min at 25°C and then the pellet was resuspended in 10 ml of distilled water. The cells were pelleted by centrifugation at 4,000 x*g* for 10 min at 25°C, and then cell pellet was resuspended in 0.5 ml of 100 mM LiCl solution. After another centrifugation step as above, the resulting cell pellet was resuspended in 200 μl of 100 mM LiCl solution to yield the final competent cell suspension. The resulting 50 μl of prepared *P. pastoris* X-33 competent cells were collected by centrifugation at 4,000 x*g* for 1 min at 25°C and dissolved in 300 μl of DNA mixture (240 μl of 50% PEG, 36 μl of 1 M LiCl, 50 μg of single-stranded salmon DNA, and 2 μg of *Sac* I-linearized pPICZαA-*CTSP*-*3* plasmid). The mixture was incubated at 30°C for 30 min without shaking and then heat shock was performed at 42°C for 25 min in a heating block. The cells were harvested by centrifugation at 4,000 x*g* for 1 min at 25°C, then resuspended in 1 ml of YPD medium. After incubation for 3 h at 30°C with shaking, cells were plated in the YPD-zeocin agar medium (150 μg/ml of zeocin) for the selection of transformants. The plate was incubated at 30°C until colonies appeared.

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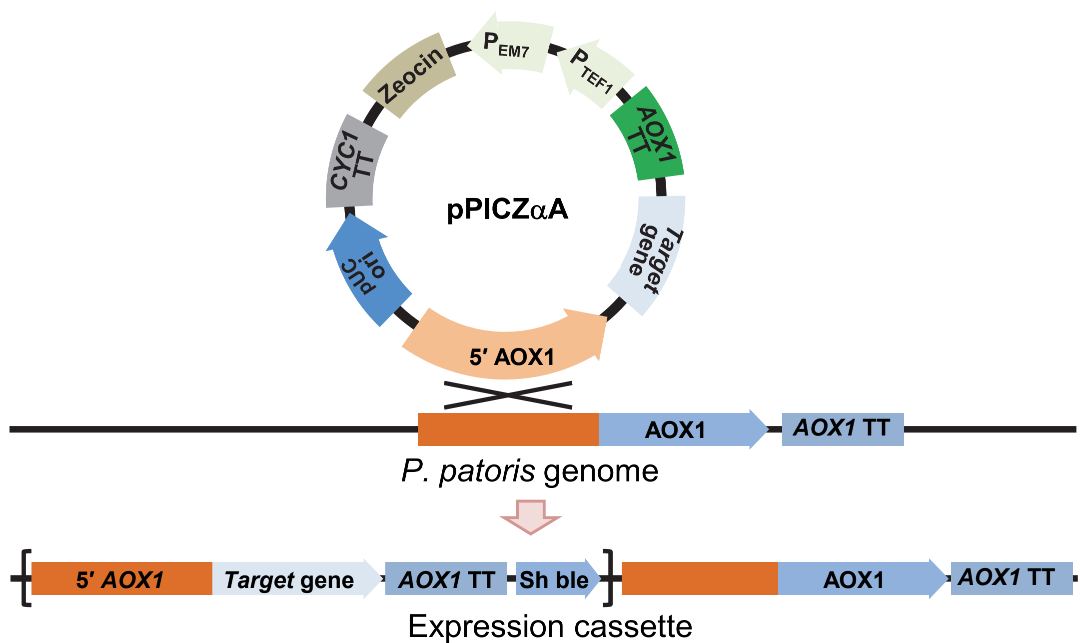

Fig. 6. Possible integration of the pPICZαA vector into the *P. pastoris* genome in which the gene insertion event can occur at AOX1 locus.

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II-16. Time-course expression of recombinant CTSP-3 enzyme in *P. pastoris* X-33

To analyse the protein expression levels of the selected clones, a small scale protein expression screen was performed. *P. pastoris* X-33 cells harboring pPICZαA-*CTSP-3* expression cassette was inoculated into 5 ml YPD medium containing 150 μg/ml zeocin and incubated at 30°C overnight. Two milliliters of this overnight culture was used to inoculate 100 ml of fresh YPG medium and grown at 30°C until the OD_{600} of about 2-3 was reached. Cells were harvested by centrifugation at 4,000 x*g* for 15 min at room temperature and then cell pellet was washed with 50 ml of 0.5x PBS buffer. The cell pellet was resuspended in 50 ml of YPM and BMMY media. These cultures were incubated at 28°C for 144 h with constant shaking in 250 ml flasks, supplemented with 0.5% methanol every day, with sampling performed daily. The samples were centrifuged and then supernatant was subjected to TCA precipitation. The precipitate was dissolved in 2x SDS page sample buffer.

II-17. TCA precipitation

Proteins were precipitated by addition of same volume of 20% trichloroacetic acid (TCA) and incubated for 30 min at 4°C. The samples were centrifuged at 10,000 x*g* for 30 min and the pellet was washed in 1 ml of 100% acetone, to reduce TCA contamination. The resulting precipitate was collected by centrifugation at 10,000 x*g* for 10 min at 4°C and the sample left to dry.

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II-18. 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 2x SDS-PAGE sample buffer, boiled at 100°C for 5 min, and then loaded onto 8-12% polyacrylamide gel. After electrophoresis, protein bands were visualized by staining the gel with Coomassie brilliant blue.

II-19. Western blotting

For Western blot analysis, samples were subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was washed in TBS-T buffer (20 mM Tris-HCl, pH7.5, 0.1% Tween 20) and non-specific binding sites were blocked in block reagent (5% skim milk in TBS-T) for 3 h at room temperature on a shaker. The membrane was incubated overnight at 4°C with anti-His tag antibody in the blocking solution. After incubation, the membrane was washed in TBS-T buffer incubated with a diluted peroxidase-conjugated anti-mouse secondary antibody for 2 h at room temperature and again washed. Bound antibody was detected by West-Zol plus detection reagent (iNtRON, Seonnam, Korea) and exposed on X-ray film.

II-20. Expression and purification of recombinant CTSP-3 enzyme from a yeast *P. pastoris* X-33

Single colony of transformant was initially grown in 50 ml of YPD medium containing 150 μg/ml zeocin media for 24 h at 30°C and 200 rpm.

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Ten milliliters of this overnight culture was used to inoculate 500 ml of YPG medium and cells were grown at 30^oC until the A_{600} reached 4-5. Four sets of this culture were prepared. Cells were harvested by centrifugation at 4,000 x*g* for 15 min at room temperature and then cell pellet was washed with 500 ml of 0.5x PBS buffer. The cell pellet was resuspended in 500 ml of YPM medium. This culture was incubated for 144 h at 28^oC with constant shaking in 2 L flasks, supplemented with 0.5% methanol every day. The culture supernatant was harvested by centrifugation at 8,000 x*g* for 20 min at 4^oC. The supernatant, which contained the expressed proteins, was subjected to ammonium sulfate precipitation at 70% saturation. The resulting protein pellet was collected by centrifugation at 12,000 x*g* for 30 min at 4^oC, dissolved in 25 mM Tris-HCl (pH 7.5) containing 10 mM imidazole, and then applied to a PD-10 column equilibrated in the same buffer to remove residual $(NH_4)_2SO_2$. The sample was applied to a HiTrap Chelating HP equilibrated with the same buffer. The CTSP-3 was eluted with a linear gradient of imidazole from 0 to 300 mM in the same buffer. Active fractions were pooled, concentrated by ultra-filtration using Amicon YM 10 membrane (Millipore, Billerica, MA, USA).

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III. RESULTS

III-1. Purification and characterization of native CTSP enzymes

III-1-1. Purification of CTSP enzymes

For the purification of the enzymes, the worm crude extract (approximately 660 mg) was subjected to various saturation concentrations of ammonium sulfate. The 40-60% saturation of ammonium sulfate could precipitate proteins showing high proteolytic activity (Fig. 7). The 40-60% ammonium sulfate precipitated proteins were applied onto a HiPrep 16/10 Q FF column (Fig. 8). The proteins bound on the column were eluted by a linear NaCl gradient of 0-0.6 M and the proteolytic activity of each fraction was assayed with azocasein as a substrate (Fig. 8). As shown in Fig. 8, fifteen fractions (17-31) eluted by 0.2-0.35 M range of NaCl showed protease activity and proteins were pooled as an active fraction. The proteins pooled were concentrated by ultrafiltration using an YM 10 membrane and then desalted on a PD-10 column equilibrated with 25 mM phosphate buffer (pH 7.0). The desalted proteins were loaded onto a Mono Q 4.6/100 PE column, and the bound proteins were eluted by a NaCl linear gradient of 0-0.3 M. As shown in the activity profile of Fig. 9, the anion exchanger separated proteases into at least three peaks (I, II, and III). The proteins contained in the peaks were pooled individually and further separated by a Superdex 75 10/300 GL column, from which three kinds of CTSP (stands for *C. tentaculata* serine protease) enzymes could be purified (Figs. 10-12). CTSP-1 (Fig. 10) and CTSP-2 (Fig. 11) were eluted at volumes corresponding

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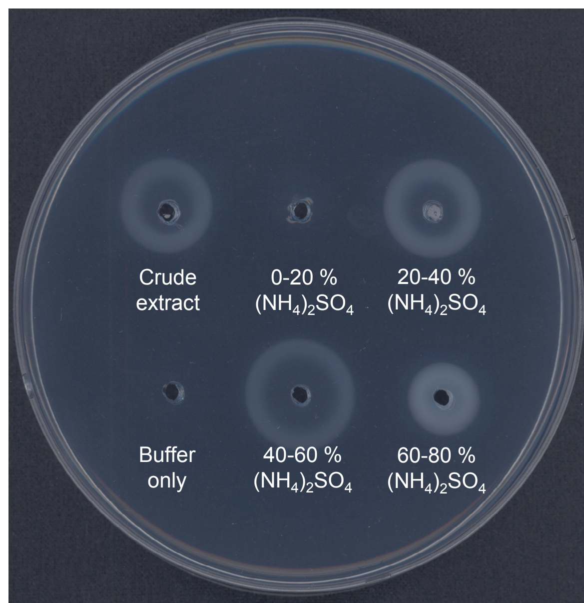

Fig. 7. Proteolytic activities of the ammonium sulfate-fractionated proteins on 1% casein plate. Twenty micrograms each of fractionated proteins was inoculated into the hole and then incubated for 8 h at 37°C to visualize halo zones.

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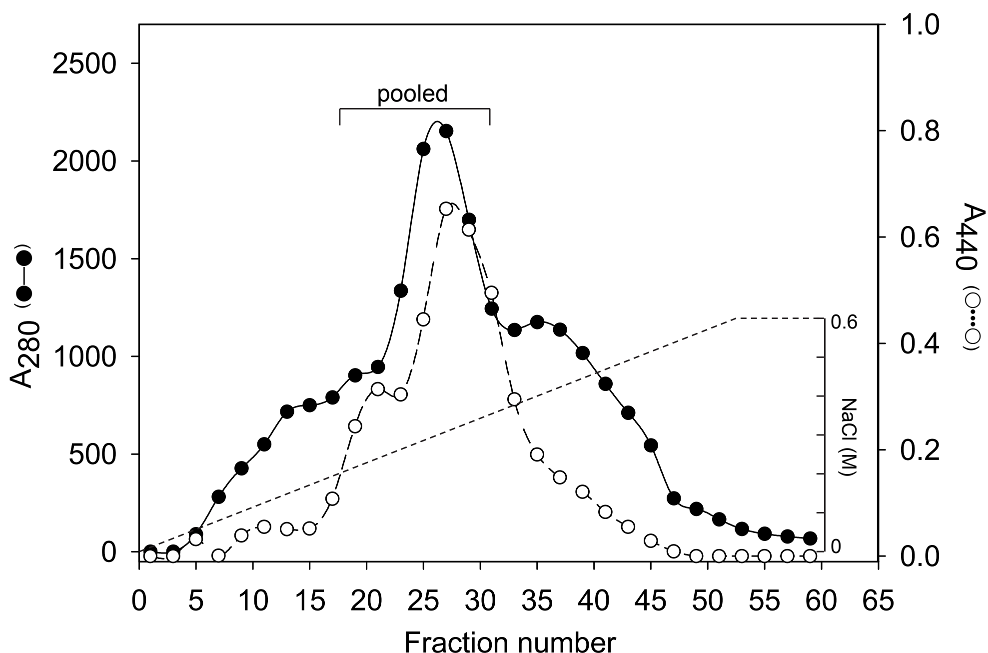

Fig. 8. Purification of CTSP enzymes from *C. tentaculata* using HiPrep Q column chromatography. Proteins fractionated with 40-60% ammonium sulfate were loaded onto an anion exchanger HiPrep Q column and the proteins bound were eluted by a NaCl linear gradient of 0-0.6 M. Active fractions were pooled from fraction numbers 17-31 as indicated. Protein elution was monitored by measuring the absorbance at 280 nm (●). The protease activity (○) was assayed with azocasein as a substrate, in which the absorbance was measured at 440 nm.

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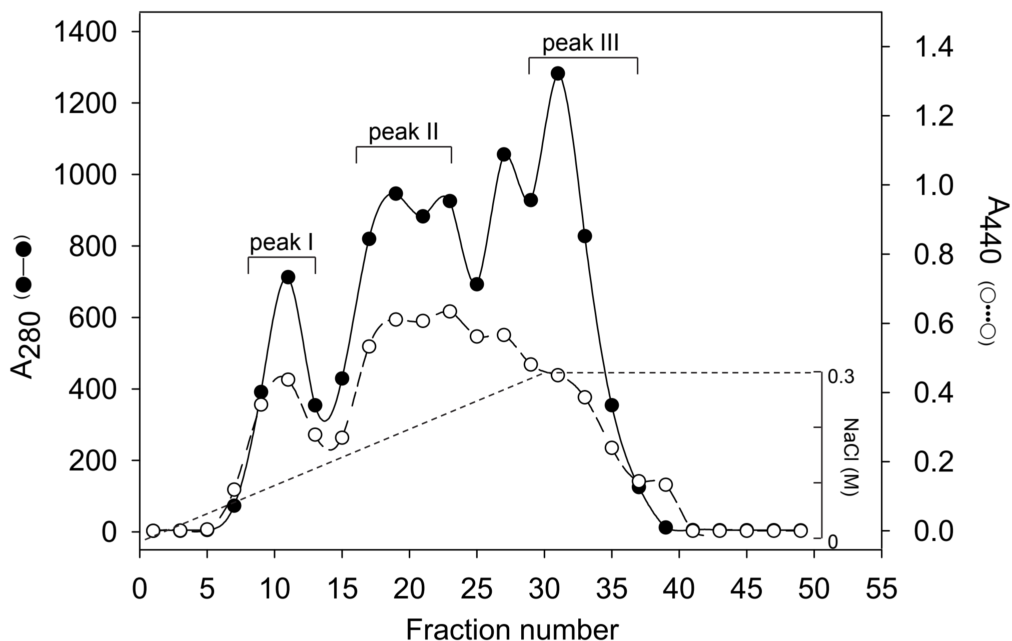

Fig. 9. Purification of CTSP enzymes from *C. tentaculata* using Mono Q column chromatography. Proteins collected from the HiPrep Q column chromatography were applied onto an anion exchanger Mono Q column and the proteins bound were eluted by a NaCl linear gradient ranging from 0-0.3 M. Three active fractions were obtained, from peaks I (fractions 7-13), II (fractions 14-27), and III (fractions 28-37) as indicated. Protein elution was monitored by measuring the absorbance at 280 nm (●). The protease activity (○) was assayed with azocasein as a substrate, in which the absorbance was measured at 440 nm.

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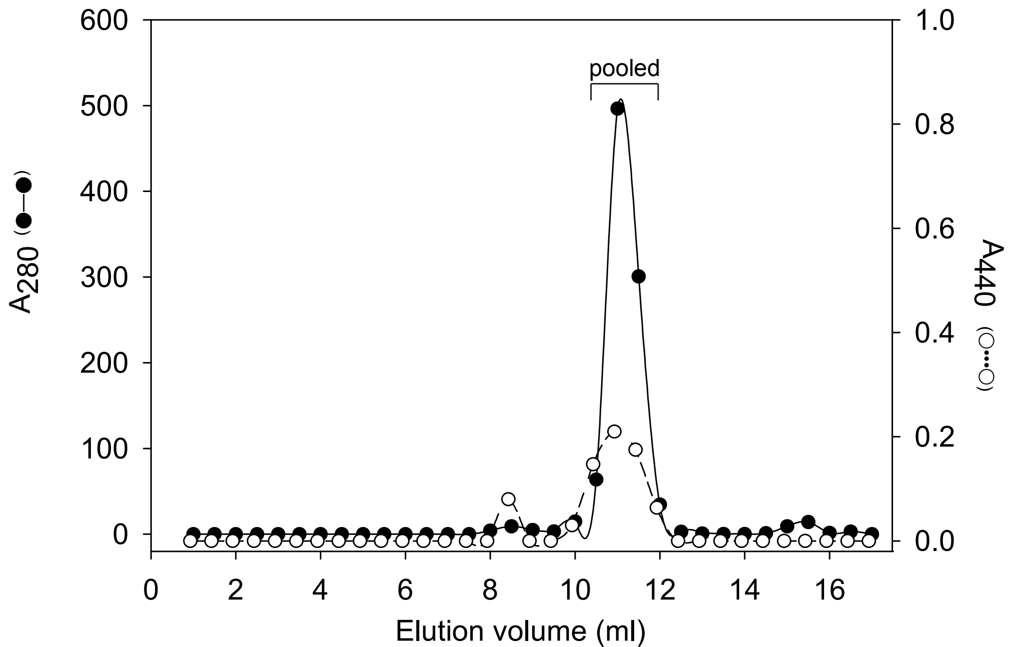

Fig. 10. Purification of CTSP-1 enzyme from *C. tentaculata* using Superdex 75 column chromatography. Proteins collected from the Mono Q column chromatography were applied onto a Size-exclusion column and the proteins were eluted with phosphate buffer. CTSP-1 enzyme appeared in the elution volume of 10.5-11.5 ml from the active peaks I. Protein elution was monitored by measuring the absorbance at 280 nm (●). The protease activity (○) was assayed with azocasein as a substrate, in which the absorbance was measured at 440 nm.

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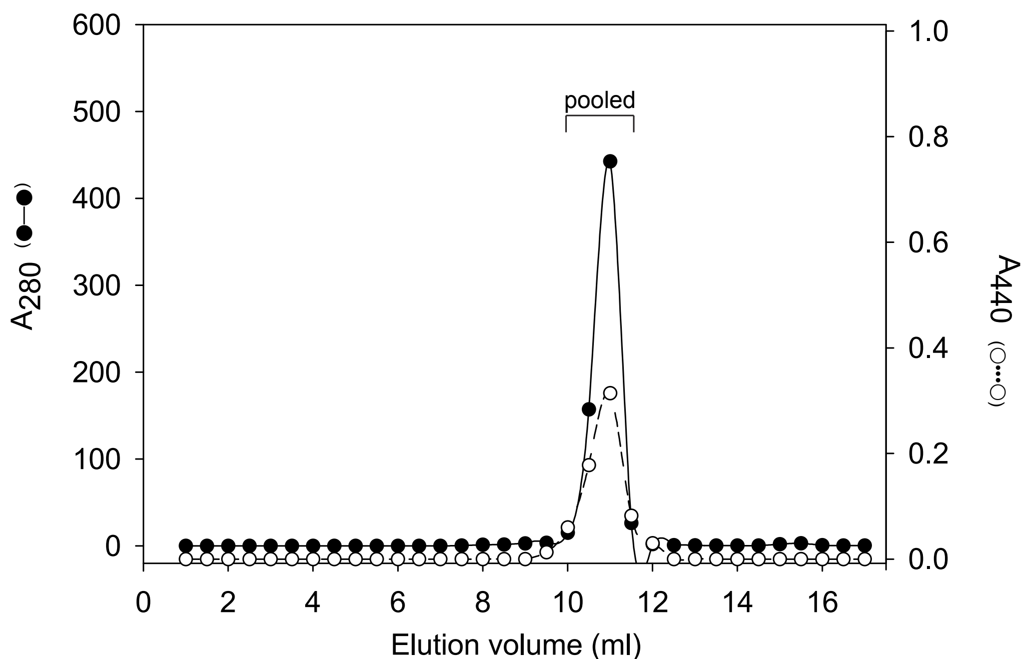

Fig. 11. Purification of CTSP-2 enzyme from *C. tentaculata* using Superdex 75 column chromatography. Proteins collected from the Mono Q column chromatography were applied onto a Size-exclusion column and the proteins were eluted with phosphate buffer. CTSP-2 enzyme appeared in the elution volume of 10.5-11.5 ml from the active peaks II. Protein elution was monitored by measuring the absorbance at 280 nm (●). The protease activity (○) was assayed with azocasein as a substrate, in which the absorbance was measured at 440 nm.

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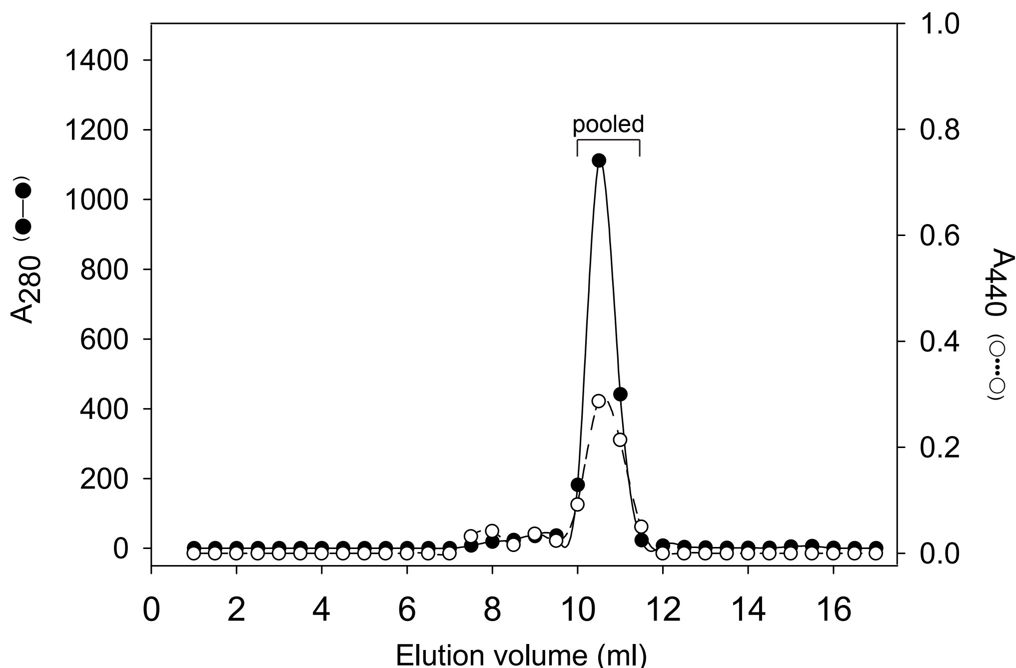

Fig. 12. Purification of CTSP-3 enzyme from *C. tentaculata* using Superdex 75 column chromatography. Proteins collected from the Mono Q column chromatography were applied onto a Size-exclusion column and the proteins were eluted with phosphate buffer. CTSP-3 enzyme appeared in the elution volume of 10-11 ml from the active peaks III. Protein elution was monitored by measuring the absorbance at 280 nm (●). The protease activity (○) was assayed with azocasein as a substrate, in which the absorbance was measured at 440 nm.

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to 10.5-11.5 ml, and CTSP-3 (Fig. 12) at 10-11 ml in the elution volumes. Purified CTSP enzymes appeared as single bands on an SDS-polyacrylamide gel stained with Coomassie brilliant blue and the molecular masses of CTSP-1, -2, and -3 were estimated to be 28.8, 30.9, and 28.4 kDa, respectively (Fig. 13). Each enzyme formed just one peak on gel filtration and appeared as single band on denaturing SDS-polyacrylamide gel, suggesting that all the enzymes purified are monomeric proteases. Table 3 summarizes the purification results. The specific activities of purified CTSP-1, -2, and -3 enzymes were estimated to be 1,044.4, 1,775.5, and 1,224 U/mg protein, respectively, and an average of 2.33 mg of enzymes could be obtained from 660 mg of total cell extracts (Table 4). The preliminary N-terminal amino acid sequencing results showed that the N-terminus of CTSP-1 was composed of Ile-Met-Asn-Gly-Ser-Pro-Ala-Ser. The N-termini of CTSP-2 and -3 were Ile-Met-Tyr-Gly-Gln-Glu-Ala-Ala, and Ile-Ile-Gly-Gly-Thr-Glu-Ala-Asp, respectively.

III-1-2. Substrate specificity and kinetic parameters of CTSP enzymes

CTSP enzymes were able to cleave various plasma protein substrates, including fibrinogen, bovine serum albumin, collagen type IV, plasminogen, γ-globulin, α_2-macroglobulin, prothrombin, high molecular weight kininogen, human prekallikrein, and factor XII (Figs. 14-16). The CTSP enzymes showed amidolytic activities towards various synthetic peptide substrates. Among the 10 chromogenic substrates tested, S-2238, S-2251, S-2288, S-2444, S-2586, and S-2765 were hydrolyzed by the enzymes at different rates, suggesting that they have different specificities (Figs. 17-19). The chromogenic substrate S-2444 was the most suitable for the enzymes

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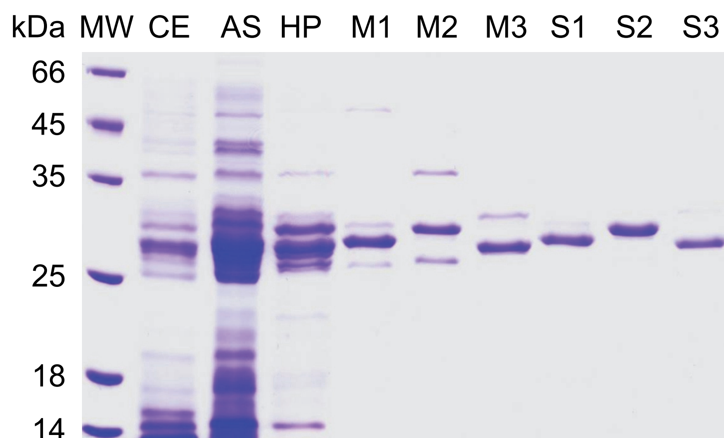

Fig. 13. SDS-PAGE of proteins obtained from various purification steps. Proteins collected from each purification step were electrophoresed on a 12% SDS polyacrylamide gel and stained with Coomassie brilliant blue to visualize. Lanes MW, protein molecular weight markers; CE, crude extracts; AS, ammonium sulfate fraction (40 to 60%); HP, HiPrep Q column chromatography; M1, Mono Q column chromatography peak I; M2, Mono Q column chromatography peak II; M3, Mono Q column chromatography peak III; S1, size exclusion chromatography of CTSP-1; S2, size exclusion chromatography of CTSP-2; S3, size exclusion chromatography of CTSP-3.

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Table 4. Summary of the purification of CTSP enzymes from the polychaete *C. tentaculata*.

Purification step	Pool/ Enzyme	Total protein (mg)	Total activity (U)	Specific activity[a] (U/mg)	Yield[b] (%)	Purification fold
Crude extracts	-	660	289093.3	438.0	100	1.0
40-60%$(NH4)_2SO_4$	-	110	55980.3	508.9	19.4	1.2
HiPrep Q	-	41.3	36621.9	886.7	12.7	2.0
Mono Q	I	4.2	3408.8	811.6	1.2	1.9
	II	9.0	10249.2	1138.8	3.5	2.6
	III	11.2	7599.1	678.5	2.6	1.5
Superdex 75	CTSP-1	1.4	1409.9	1044.4	0.5	2.4
	CTSP-2	1.1	1953.1	1775.5	0.7	4.1
	CTSP-3	4.5	5509.2	1224.3	1.9	2.8

[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%.

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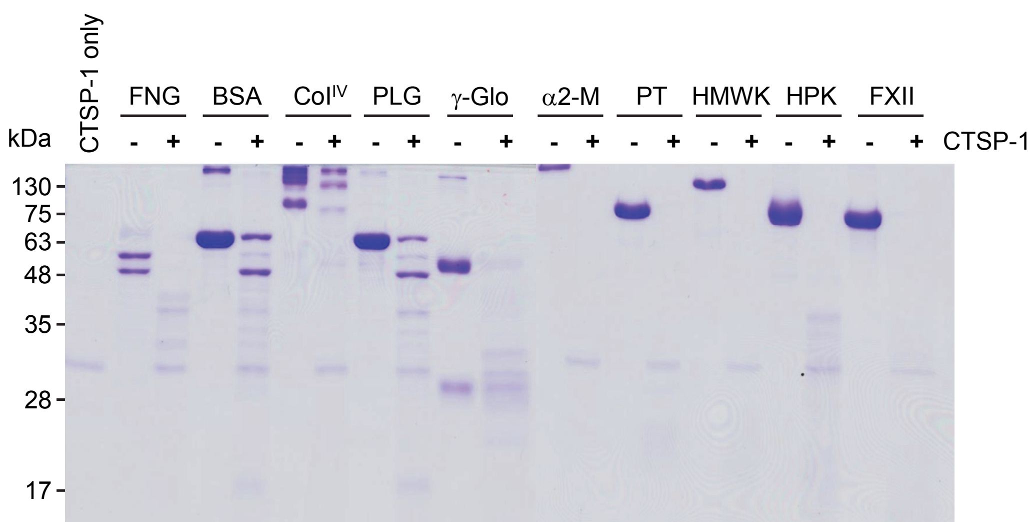

Fig. 14. Proteolytic cleavage activity of CTSP-1 enzyme against various plasma proteins. Five micrograms each of corresponding proteins was digested with 0.25 μg of purified CTSP-1 for 20 min at 37°C, electrophoresed on 12% SDS-polyacrylamide gel, and then stained with Coomassie Brillint Blue to visualize. FNG, fibrinogen; BSA, bovine serum albumin; Col^{IV}, collagen type IV; PLG, plasminogen; γ-Glo, γ-globulin; α_2-M, α_2-macroglobulin; PT, prothrombin; HMWK, high molecular weight kininogen; HPK, human pre-kallikrein; FXII, factor XII.

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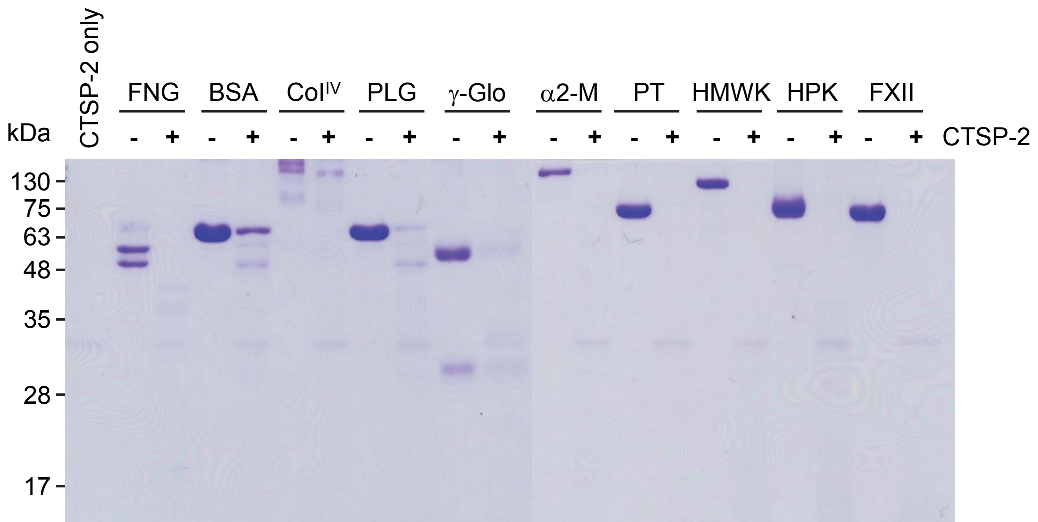

Fig. 15. Proteolytic cleavage activity of CTSP-2 enzyme against various plasma proteins. Five micrograms each of corresponding proteins was digested with 0.25 μg of purified CTSP-2 for 20 min at 37°C, electrophoresed on 12% SDS-polyacrylamide gel, and then stained with Coomassie Brillint Blue to visualize. FNG, fibrinogen; BSA, bovine serum albumin; Col^{IV}, collagen type IV; PLG, plasminogen; γ-Glo, γ-globulin; α_2-M, α_2-macroglobulin; PT, prothrombin; HMWK, high molecular weight kininogen; HPK, human pre-kallikrein; FXII, factor XII.

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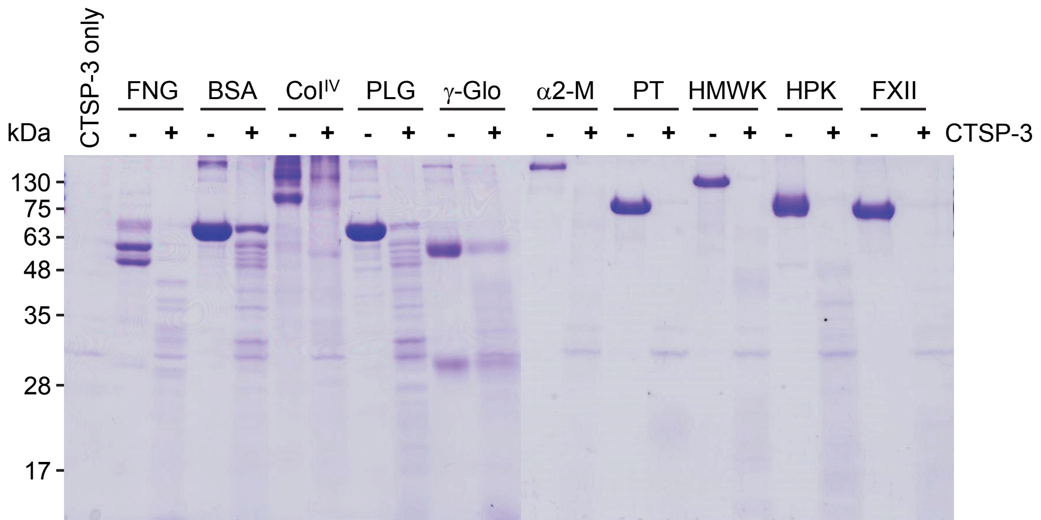

Fig. 16. Proteolytic cleavage activity of CTSP-3 enzyme against various plasma proteins. Five micrograms each of corresponding proteins was digested with 0.25 μg of purified CTSP-3 for 20 min at 37°C, electrophoresed on 12% SDS-polyacrylamide gel, and then stained with Coomassie Brillint Blue to visualize. FNG, fibrinogen; BSA, bovine serum albumin; Col^{IV}, collagen type IV; PLG, plasminogen; γ-Glo, γ-globulin; α_2-M, α_2-macroglobulin; PT, prothrombin; HMWK, high molecular weight kininogen; HPK, human pre-kallikrein; FXII, factor XII.

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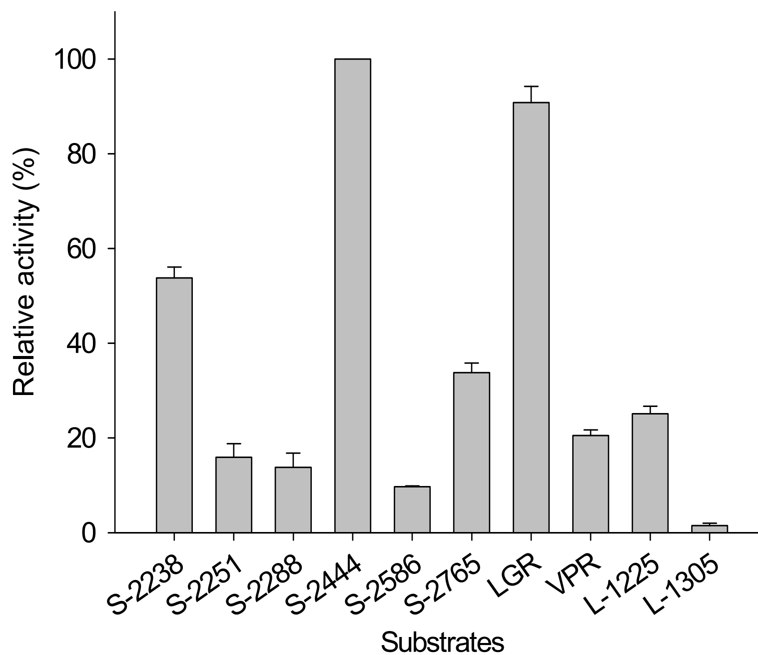

Fig. 17. Substrate specificity of the purified CTSP-1 enzyme. The relative activity was expressed as a percentage of the activity level in the S-2444 as a substrate. Data from six independent experiments were expressed as mean values ± S.D

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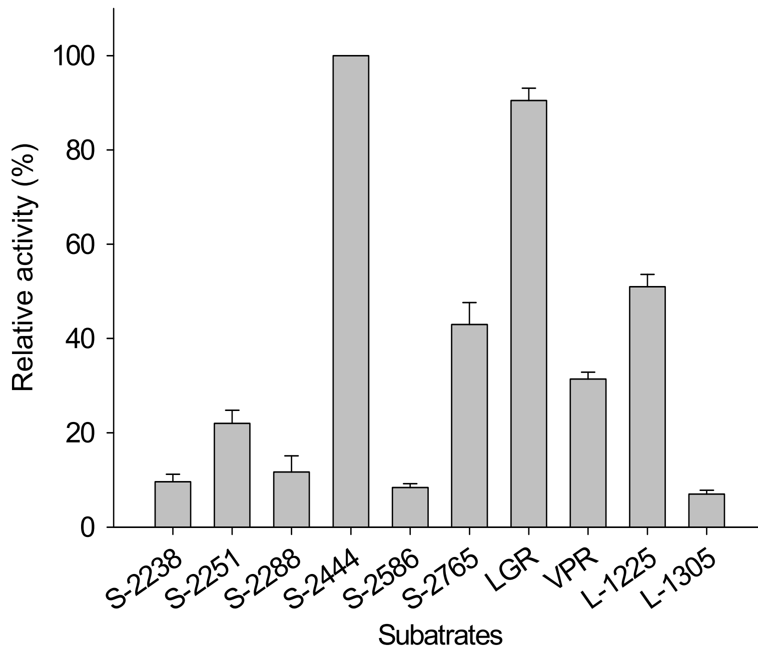

Fig. 18. Substrate specificity of the purified CTSP-2 enzyme. The relative activity was expressed as a percentage of the activity level in the S-2444 as a substrate. Data from six independent experiments were expressed as mean values ± S.D.

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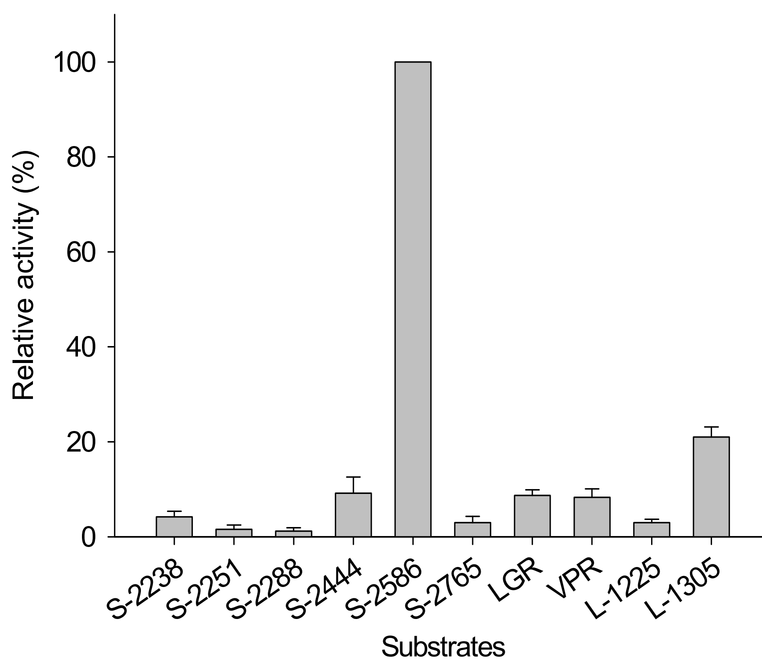

Fig. 19. Substrate specificity of the purified CTSP-3 enzyme. The relative activity was expressed as a percentage of the activity level in the S-2586 as a substrate. Data from six independent experiments were expressed as mean values ± S.D.

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CTSP-1 and -2, whereas S-2586 was for CTSP-3 (Table. 5). Therefore, the two chromogenic substrates (S-2444 and S-2586) were used in further studies, including the determination of cleavage site of enzyme and the analysis of enzyme kinetics. The specific activities of CTSP-1, -2, and -3 were approximately 2.11, 16.83, and 1.68 U/mg, respectively when their specific chromogenic substrates were used (Table 6). K_M values of CTSP-1, -2, and -3 were estimated to be 0.031, 0.038, and 0.942 mM, respectively. K_{cat} values of the same enzymes were found to be 1.30, 11.10, and 4.36 sec^{-1}, respectively. In particular, K_{cat}/K_M value of CTSP-2 was 6.96-fold higher than that of CTSP-1 when S-2444 was used as a substrate (Table 6). These results suggest that CTSP-2 is the most active enzyme among the purified proteases, as it has the highest turnover number and catalytic efficiency .

III-1-3. Proteolytic activities of CTSP enzymes

The effects of various protease inhibitors and divalent cations on the proteolytic activities of CTSP enzymes were examined (Table 7). The proteolytic activities of the three CTSP enzymes were completely inhibited by DFP and PMSF that are typical serine protease inhibitors, but not by EDTA and 1,10-PT that are known as metalloprotease inhibitors. As shown in Table 7, the three enzymes showed different sensitivities to serine protease inhibitors such as TLCK, TPCK, and aprotinin. The proteolytic activities of two enzymes CTSP-1 and -2 were strongly inhibited by TLCK and aprotinin, but not by TPCK. The enzyme activity of CTSP-3 was diminished by TPCK but not by TLCK and aprotinin (Table 7). It is well known that almost all serine proteases are inhibited by DFP and PMSF that covalently modify a catalytic serine residue in enzyme’s active site. As expected, the protease activities of all three enzymes were not inhibited by a cysteine protease

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Table 5. Amidolytic activities of CTSP enzymes towards various synthetic chromogenic substrates.

Substrate	Sequence	Typical substrate for	Specific activity(U/mg)[a]		
			CTSP-1	CTSP-2	CTSP-3
S-2238	H-D-Phe-Pip-Arg-*p*NA	Thrombin	0.93 ± 0.07	1.86 ± 0.45	0.33 ± 0.05
S-2251	H-D-Val-Leu-Lys-*p*NA	Plasmin	0.31 ± 0.01	3.46 ± 0.09	0.07 ± 0.00
S-2288	H-D-Ile-Pro-Arg-*p*NA	t-PA	0.47 ± 0.00	2.29 ± 0.08	0.03 ± 0.02
S-2444	pyroGlu-Gly-Arg-*p*NA	Urokinase	2.11 ± 0.03	16.83 ± 0.35	0.89 ± 0.04
S-2586	MeO-Suc-Arg-Pro-Tyr-*p*NA	Chymotrypsin	0.57 ± 0.02	4.49 ± 0.00	1.68 ± 0.01
S-2765	Z-D-Arg-Gly-Arg-*p*NA	FXa	1.11 ± 0.09	7.39 ± 0.51	0.18 ± 0.00

[a] One unit (U) of amidolytic activity was defined as the amount of a protease catalyzing 1 μmol of *p*NA/min. Data from six independent experiments were expressed as mean values ± S.D.

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Table 6. Kinetic parameters for CTSP enzymes to corresponding chromogenic peptide substrates.

Enzyme	Substrate used	K_M(mM)[a]	K_{cat} (sec^{-1})[a]	K_{cat}/K_M($mM^{-1}sec^{-1}$)
CTSP-1	S-2444	0.031 ± 0.003	1.30 ± 0.052	41.94
CTSP-2	S-2444	0.038 ± 0.009	11.10 ± 0.573	292.11
CTSP-3	S-2586	0.942 ± 0.024	4.36 ± 0.325	4.63

[a] Data from six independent experiments were expressed as mean values ± S.D.

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Table 7. Effects of various inhibitors and metal ions on CTSP enzyme activities.

Additive	Concentration (mM)	Relative activity (%)[a]		
		CTSP-1	CTSP-2	CTSP-3
Control	0	100 ± 1.7	100 ± 4.8	100 ± 3.4
TPCK	0.25	100 ± 3.7	104 ± 9.1	19 ± 5.4
TLCK	1	0 ± 0.2	0 ± 0.1	94 ± 0.5
PMSF	1	4 ± 0.3	24 ± 3.6	0 ± 7.5
Aprotinin	0.01	0 ± 0.0	0 ± 0.2	79 ± 5.6
Bestatin	1	101 ± 4.2	115 ± 8.5	98 ± 4.1
EDTA	1	102 ± 9.6	105 ± 1.5	95 ± 2.0
DTT	1	104 ± 1.6	105 ± 0.7	98 ± 7.0
DFP	1	1 ± 1.3	0 ± 0.3	0 ± 0.0
1,10-PT	1	99 ± 5.9	91 ± 2.8	89 ± 8.3
Ca^{2+}	1	107 ± 9.1	81 ± 0.8	100 ± 4.1
Cu^{2+}	1	94 ± 6.7	98 ± 9.1	93 ± 6.4
Mg^{2+}	1	99 ± 6.4	93 ± 2.6	102 ± 6.5
Mn^{2+}	1	93 ± 8.6	98 ± 2.1	106 ± 8.2
Ni^{2+}	1	102 ± 3.3	94 ± 9.2	93 ± 7.2
Zn^{2+}	1	80 ± 5.3	90 ± 1.8	88 ± 3.3

[a] In the activity assay, S-2444 was used as a substrate for CTSP-1 and -2, and S-2586 was for CTSP-3 with or without the corresponding additive at 37°C for 20 min. Data from six independent experiments were expressed as mean values ± S.D.

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inhibitor, bestatin (Table. 7). On the other hand, various divalent cations including $Ca^{2+}$and Zn^{2+} had no significant stimulatory or inhibitory effects on the enzyme activities (Table 7). The optimum pH range for CTSP-1, -2, and -3 enzyme activities were found to be 9.0, 8.5-9.0, and 8.5, respectively (Fig. 20). All these results suggest that the enzymes are alkaline serine proteases, not metalloproteases or cysteine proteases. The temperature-dependent experiments showed that the optimum temperatures for CTSP-1 and -2 enzymes were 60°C and that for CTSP-3 was 50°C, when azocasein was used as a substrate (Fig. 21). CTSP enzymes underwent a heat-induced degradation within 20 min at around 60°C, with a decrease (approximately 50-60%) in their proteolytic activities when they were pre-incubated for 20 min at temperatures above 50°C. However, the presence of 1 mM DFP could prevent the degradation event at 60°C (Fig. 22).

III-1-4. Fibrino(geno)lytic activity of CTSP enzymes

The three CTSP enzymes exhibited strong fibrinogenolytic and fibrinolytic activities, as they could cleave all three major chains of fibrinogen and cross-linked fibrin polymer as well (Figs. 23-27). As shown in Figs. 23 to 25, the Aα and Bβ chains of fibrinogen were totally degraded by the enzymes within 1 min at the mass ratio of 1:20 (enzyme versus fibrinogen). However, the γ chains were more resistant to be digested by the enzymes. CTSP-1 and -2 cleaved completely the chain within 10 min (Figs. 23A and 24A) whereas CTSP-3 required a longer time to digest it fully (Fig. 25A). In addition, the turbidity assay showed that the relative turbidities of fibrin polymers were decreased by the treatments with plasmin or CTSP enzymes in time-dependent manners (Fig. 26). These results suggest that the enzymes can actively cleave fibrin polymers that spontaneously formed from

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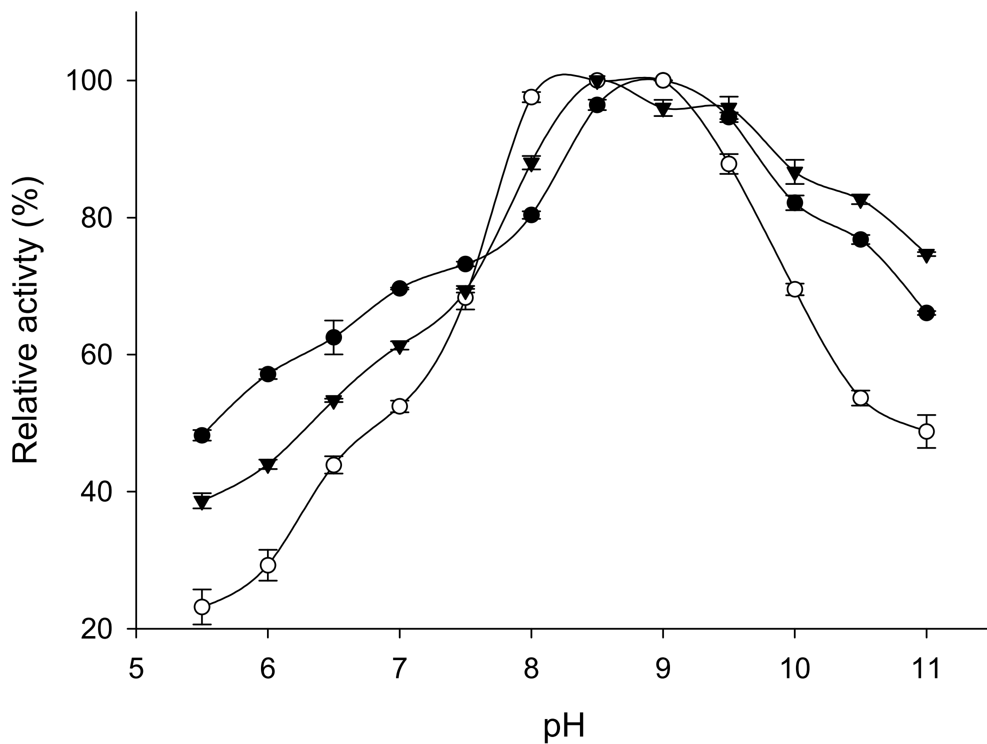

Fig. 20. Effect of pH on the enzyme activities of CTSP enzymes. Each CTSP enzyme (0.5 μg) was incubated at 37°C for 30 min with azocasein as a substrate under different pH conditions and the absorbance at 440 nm was measured. The enzyme activity was expressed as a relative activity, compared to that of control without enzyme added. Symbols ●, CTSP-1; ○, CTSP-2; ▼, CTSP-3.

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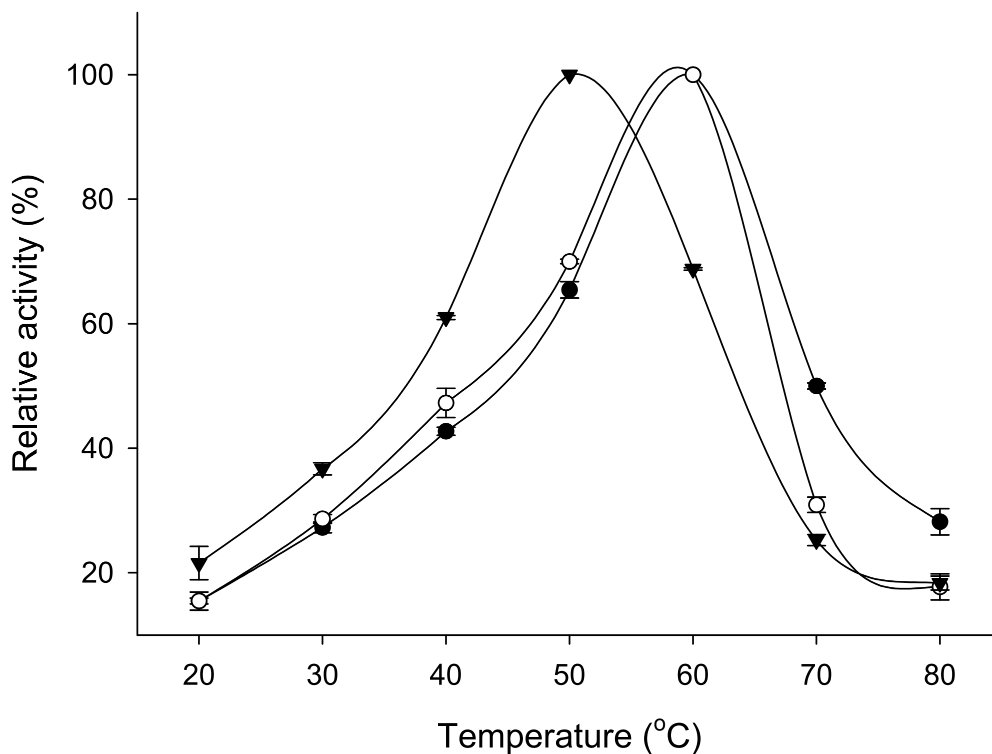

Fig. 21. Effect of temperature on the enzyme activities of CTSP enzymes. Each enzyme (0.5 μg) was incubated with azocasein as a substrate at various temperatures for 30 min as indicated, and the absorbance at 440 nm was measured. The enzyme activity was expressed as a relative activity, compared to that of control without enzyme added. Symbols ●, CTSP-1; ○, CTSP-2; ▼, CTSP-3.

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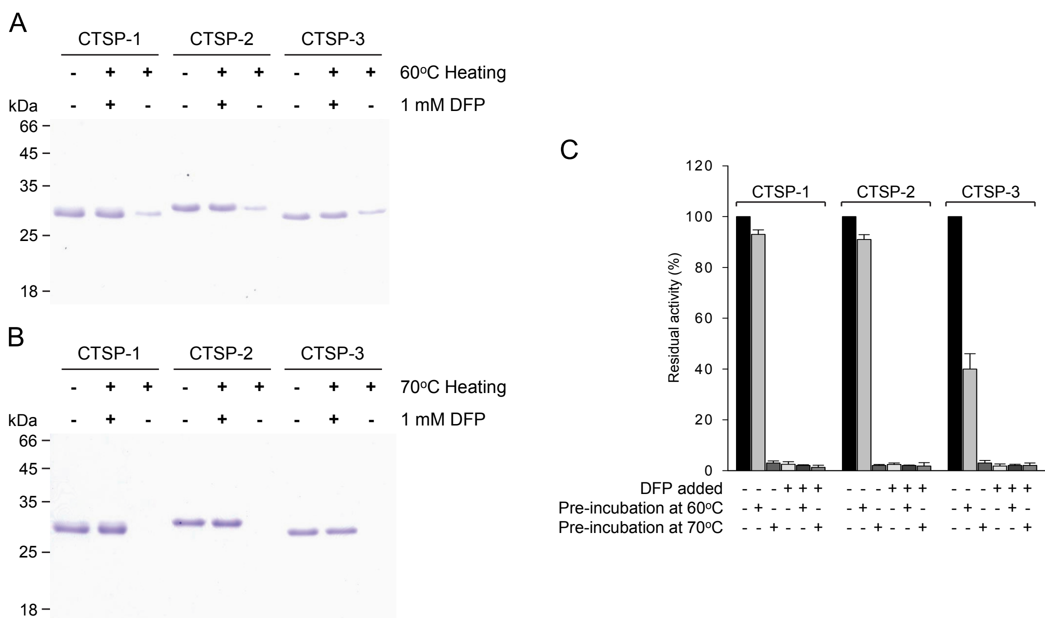

Fig. 22. Effect of DFP on the heat-induced degradation of CTSP enzymes. A total 10 μl of CTSP enzymes (5 μg) was pre-incubation at 60°C (A) or 70°C (B) for 20 min in the absence (-) or presence (+) of 1 mM DFP, from which 6 μl of aliquots (3 μg) were electrophoresed on 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue to visualize. The remaining samples (2 μg) were incubated for 20 min at 37°C with azocasein as a substrate and the residual protease activity was measured at A_{440} (C). In this assay, the proteolytic activity was expressed as a relative value compared to that of non pre-incubation control. Data from six independent experiments were expressed as mean values ± S.D. Symbols + and - mean the addition and omission of treatments, respectively.

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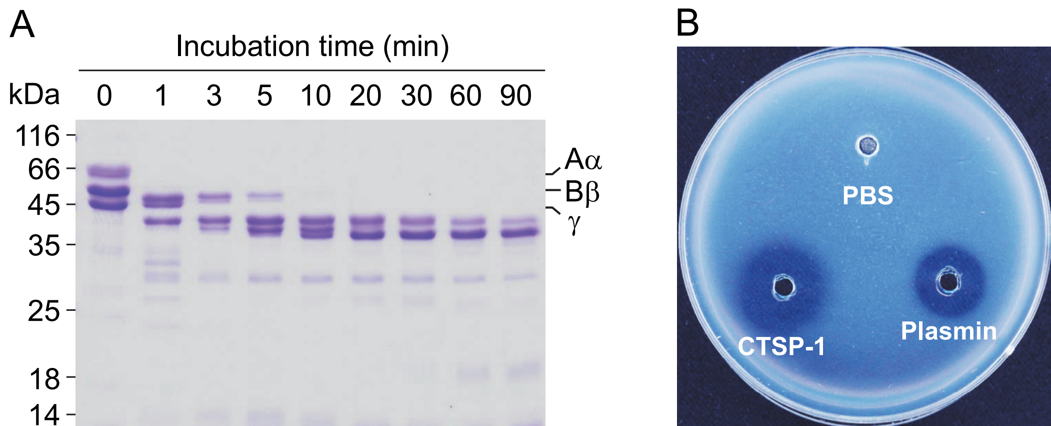

Fig. 23. Fibrino(geno)lytic activity of CTSP-1 enzyme. (A) Fibrinogenolytic activity of CTSP-1. In this assay, fibrinogen (10 μg) was incubated with 0.5 μg of CTSP-1 for various time periods at 37°C. The reaction products were electrophoresed on 12% polyacrylamide gel and stained with Coomassie brilliant blue. The positions of Aα, Bβ, and γ chains of fibrinogen on the gel are shown on the right side of the panel. (B) Fibrinolytic activity of CTSP-1 on fibrin plate. PBS, plasmin (0.04 U), and CTSP-1 enzyme (2 μg) were inoculated into the holes in the fibrin plate and incubated for 6 h at 37°C as indicated.

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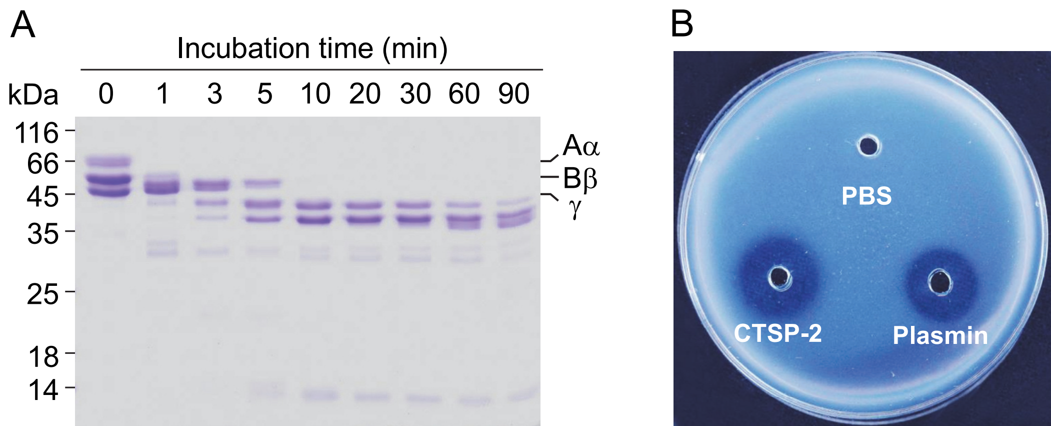

Fig. 24. Fibrino(geno)lytic activity of CTSP-2 enzyme. (A) Fibrinogenolytic activity of CTSP-2. In this assay, fibrinogen (10 μg) was incubated with 0.5 μg of CTSP-2 for various time periods at 37°C. The reaction products were electrophoresed on 12% polyacrylamide gel and stained with Coomassie brilliant blue. The positions of Aα, Bβ, and γ chains of fibrinogen on the gel are shown on the right side of the panel. (B) Fibrinolytic activity of CTSP-2 on fibrin plate. PBS, plasmin (0.04 U), and CTSP-2 enzyme (2 μg) were inoculated into the holes in the fibrin plate and incubated for 6 h at 37°C as indicated.

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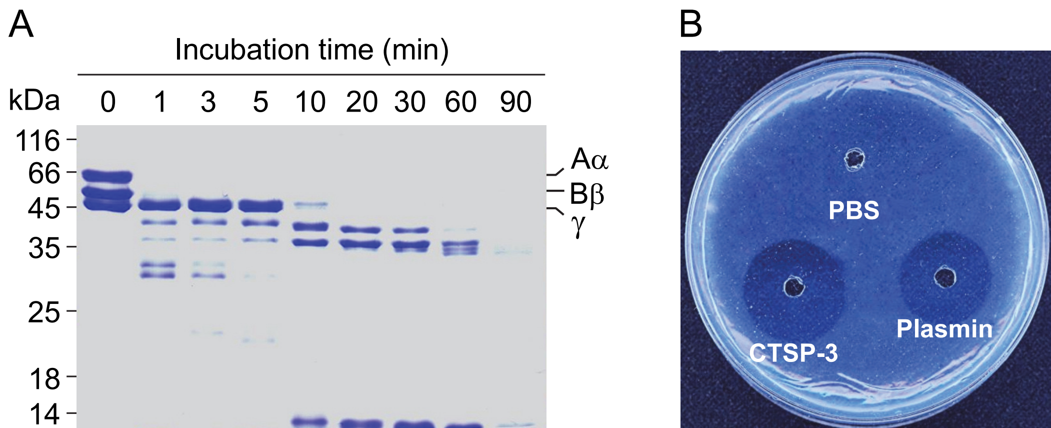

Fig. 25. Fibrino(geno)lytic activity of CTSP-3 enzyme. (A) Fibrinogenolytic activity of CTSP-3. In this assay, fibrinogen (10 μg) was incubated with 0.5 μg of CTSP-3 for various time periods at 37°C. The reaction products were electrophoresed on 12% polyacrylamide gel and stained with Coomassie brilliant blue. The positions of Aα, Bβ, and γ chains of fibrinogen on the gel are shown on the right side of the panel. (B) Fibrinolytic activity of CTSP-3 on fibrin plate. PBS, plasmin (0.04 U), and CTSP-3 enzyme (2 μg) were inoculated into the holes in the fibrin plate and incubated for 6 h at 37°C as indicated.

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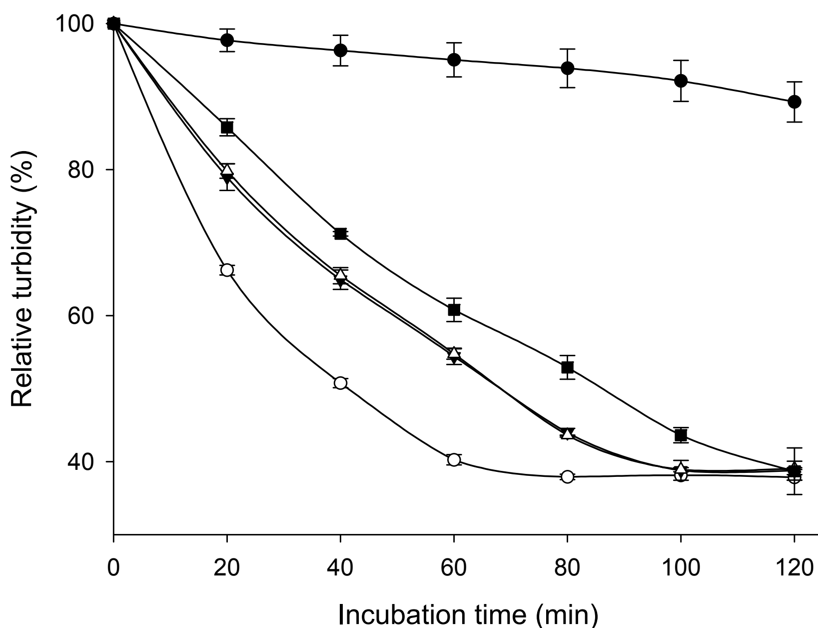

Fig. 26. Turbidity assay for the fibrinolytic activity of CTSP enzymes. In this assay, 90 μl of fibrinogen (1 mg/ml) were treated with 10 μl of thrombin (17.7 U/ml) for 1 h at 37°C and then 10 μl each of PBS, plasmin (0.04 U), CTSP-1 (2 μg), CTSP-2 (2 μg), or CTSP-3 (2 μg) were treated for 2 h at 37°C, during which the decrease in absorbance at 350 nm was then recorded in 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. Data from six independent experiments were expressed as mean values ± S.D. Symbols ●, PBS; ○, Plasmin ; ▼, CTSP-1; △, CTSP-2; ■, CTSP-3.

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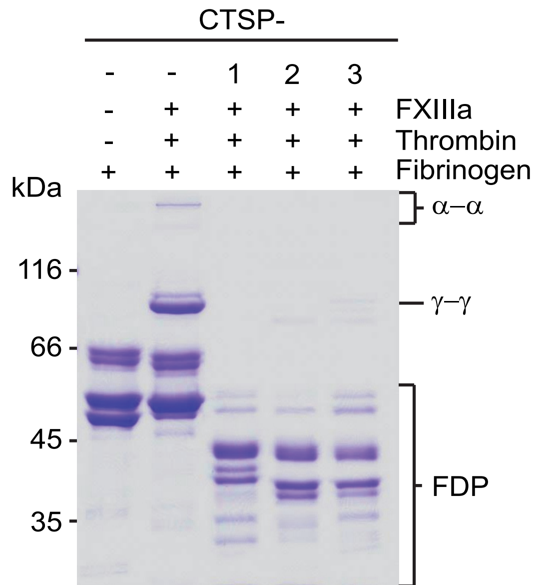

Fig. 27. Fibrinolytic activity assay for CTSP enzymes. The cross-linked fibrin formed by incubating fibrinogen (10 μg) and thrombin (0.02 U) in the presence of FXIIIa (0.002 U) was incubated with 0.5 μg each of CTSP enzymes for 30 min at 37°C. The reaction products were electrophoresed on an 8% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Symbols + and - represent the addition and the omission of corresponding additive, 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.

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fibrinogen by thrombin cleavage as plasmin does. These results suggest that the enzymes can actively cleave fibrin polymers that spontaneously formed from fibrinogen by thrombin cleavage as plasmin does. The fibrinolytic activities of CTSP enzymes against cross-linked fibrin, formed from fibrinogen by thrombin and FXIIIa in the presence of Ca^{2+}, were also observed by the cleavage assay on SDS-polyacrylamide gel (Fig. 27) and by fibrin plate assay (Figs. 23B, 24B, and 25B). As shown in Fig. 27, the α-α and γ-γ chains of cross-linked fibrin were susceptible to the cleavage by CTSP enzymes. The susceptibility of cross-linked fibrin cleavage to CTSP enzymes could be observed on fibrin plate as well (Figs. 23B, 24B, and 25B). When plasmin (0.04 U) and CTSP enzymes (each 2 μg) were applied into the wells made in the fibrin plate and incubated for 6 h at 37°C, halo zones appeared clearly (Figs. 23B, 24B, and 25B). In this assay, the radiuses of halo zones formed by the treatments with plasmin, CTSP-1, -2, and -3 enzymes were found to be 0.85, 0.99, 1.1, and 1.0 cm, respectively. Therefore, the adjusted plasmin units of CTSP-1, -2, and -3 enzymes were equivalent to 0.05, 0.07, and 0.06 units, respectively.

III-1-5. Efficacies of CTSP enzymes in cleaving fibrin clot in blood plasma milieu

The effects of serum albumin and blood plasma on the enzyme activities of plasmin, CTSP-1, -2, and -3 were examined (Fig. 28). The plasma itself only showed a basal level of intrinsic protease activity towards the chromogenic substrates used (Fig. 28). In the presence of BSA, the amidolytic activity of plasmin decreased to 91.1% and those of the three enzymes reduced to an average of 77.5%, compared to that of control without BSA (Fig. 28). These results suggest that a large amount of serum

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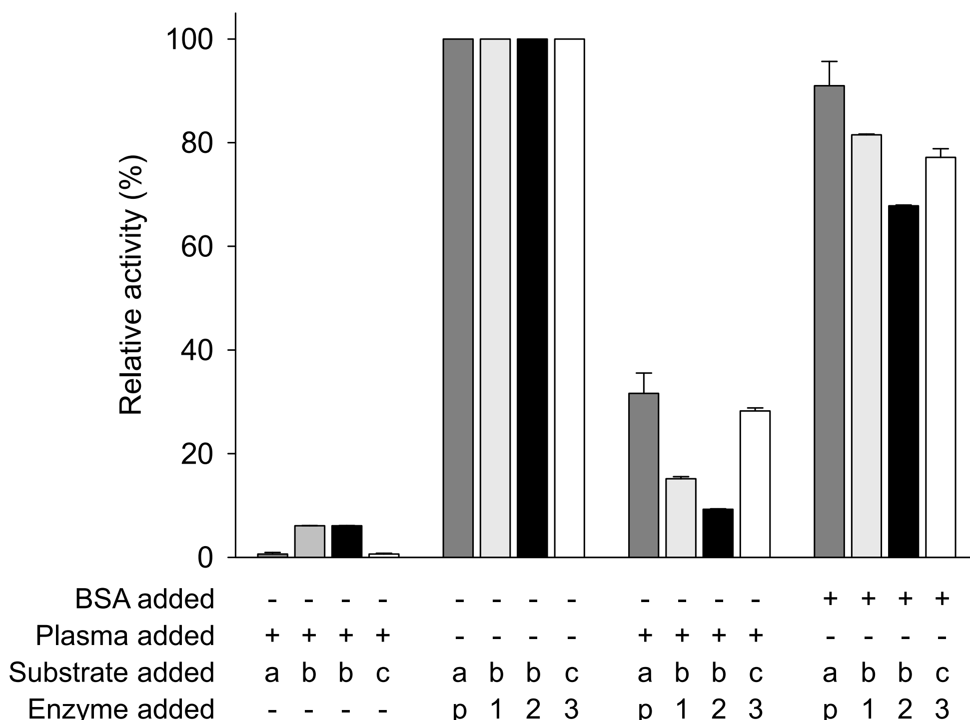

Fig. 28. Effects of BSA and plasma proteins on enzyme activity. In the assay, 6.7 mg/ml each of human plasma proteins or BSA were incubated with 0.5 mg enzyme to be tested and 0.4 mM chromogenic substrate, and the increase in absorbance at 405 nm was monitored at 37°C for 20 min. Symbols + and - indicate the addition and the omission of corresponding additive in the reaction, respectively. Data from six independent experiments were expressed as mean values ± S.D. a, S-2251; b, S-2444; c, S-2586; p, plasmin; 1, CTSP-1; 2, CTSP-2; 3, CTSP-3.

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albumin contained in blood affects the protease activity. The amidolytic activities of plasmin towards S-2251, CTSP-1 towards S-2444, CTSP-2 towards S-2444, and CTSP-3 towards S-2586 also significantly decreased to 31.6%, 15.2%, 9.3%, and 28.2%, respectively, in the presence of blood plasma that contained 6.7 mg of proteins per ml (Fig. 28). Even with the decreased proteolytic activities, the three CTSP enzymes could cleave actively the fibrin clot in plasma as shown by turbidimetric lysis method (Fig. 29). The enzymes plasmin (0.1 U), CTSP-1 (15 μg), CTSP-2 (15 μg), and CTSP-3 (15 μg) decreased the turbidity of plasma clot to 11, 71.3, 70.5, and 57.5% respectively, at the incubation time point of 7 h (Fig. 29), suggesting that the enzymes can cleave the fibrin clot in plasma milieu too. Interestingly, plasmin could not so effectively decrease the turbidity, even though it has stronger enzyme activity than CTSP enzymes in blood plasma (Fig. 28). Based on the data obtained by the turbidity assay (Fig. 29), the level of plasma clot dissolution by the enzymes could be evaluated in more detail, in which the value of maximal decrease in turbidity resulted by CTSP-1 at 8 h was set to 100% and then the increase in the dissolution of plasma clot was expressed (Fig. 30). In addition, it can be defined that the lag-time (also can be designated to reaction threshold, Rt) for an enzyme can be regarded as time required to reach a critical time point at which the dissolution process starts and the relative turbidity of plasma clots becomes measurable at 350 nm. As shown in Fig. 30, there could be found no lag times for CTSP-1 and -2, whereas those for CTSP-3 and plasmin were 3.9 and 5.0 h, respectively. In addition, apparent acceleration of reaction could be found in all types of the enzymes used. This apparent acceleration may be attributable to the fact that protein degradation leads to further proteolysis by exposing sterically hindered cleavage sites by destroying higher ordered structure (or clotted status) of plasma clots, representing increases in turbidity

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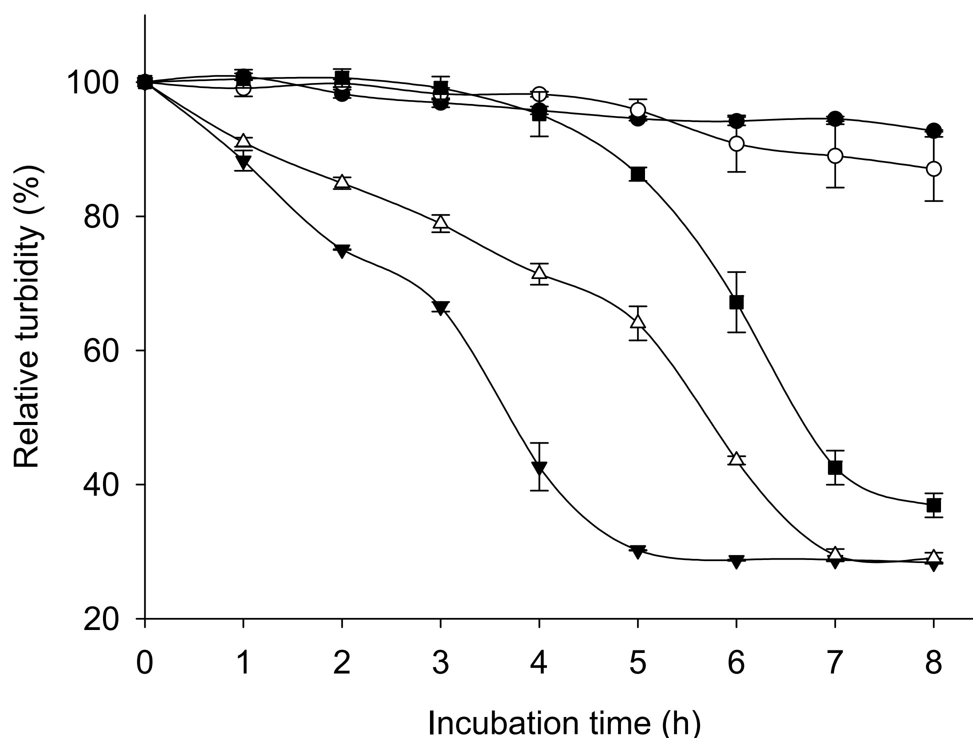

Fig. 29. Fibrinolytic activities of CTSP enzymes in blood plasma. Twenty microliters each of CTSP-1 (15 μg), CTSP-2 (15 μg), CTSP-3 (15 μg), plasmin (0.1 U), or PBS was mixed with 100 μl of human plasma clot and the reaction mixture was incubated at 37°C for 0-8 h, during which the decreases in absorbance at 350 nm were recorded in a 96-well plate reader every hour for 8 h. The relative turbidity was expressed as a percentage of a decrease in turbidity, relative to that at the beginning of incubation. Data from six independent experiments were expressed as mean values ± S.D. Symbols ●, PBS; ○, Plasmin ; ▼, CTSP-1; △, CTSP-2; ■, CTSP-3.

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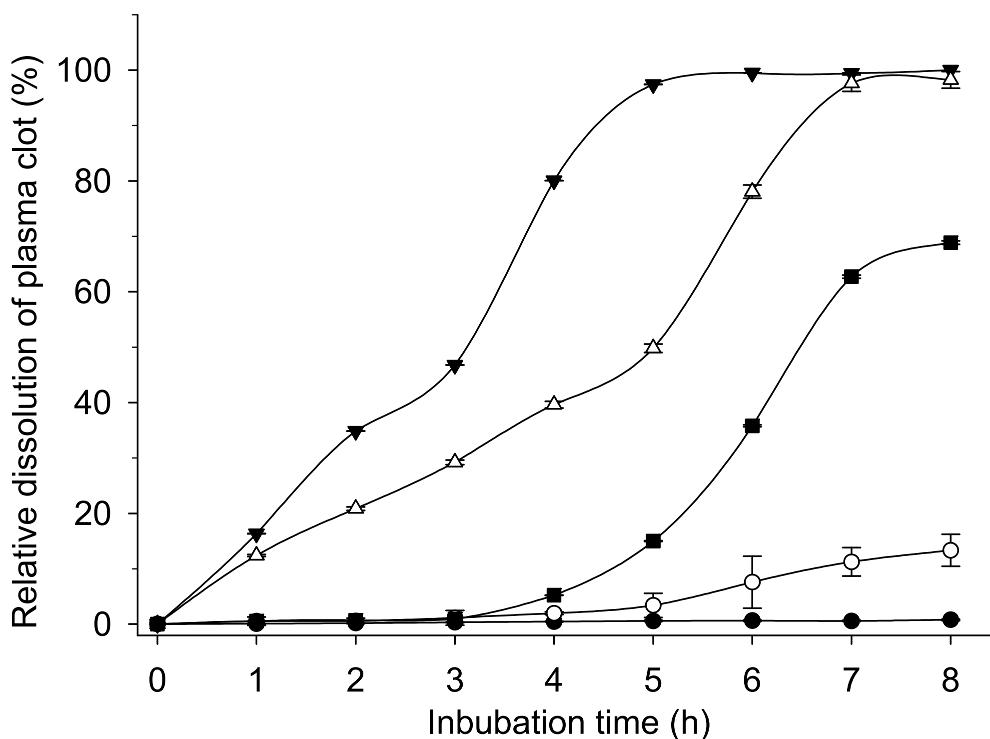

Fig. 30. Relative dissolution activity of CTSP enzyme against plasma clot. Using the data obtained by the turbidity assay (Fig. 29), the level of plasma clot dissolution are calculated, in which the value of maximal decrease in turbidity resulted by CTSP-1 enzyme at 8 h is set to 100%, and the increase in the relative dissolution of plasma clot was expressed. Data from six independent experiments were expressed as mean values ± S.D. Symbols ●, PBS; ○, Plasmin ; ▼, CTSP-1; △, CTSP-2; ■, CTSP-3.

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in the assay. On the other hand, 50% dissolution time for the enzymes can be defined as time required for dissolving 50% of plasma clots. As shown in Fig. 30, 50% dissolution times for CTSP-1, -2, and -3 were estimated to be 3.1, 5.0, and 6.6 h, respectively, when the same concentrations of enzymes (15 μg) were used; however, that for plasmin (0.1 U) could not be determined because of its too low enzyme activity.

III-2. Isolation and characterization of three *CTSP* genes

III-2-1. Design of oligonucleotide primers and isolation of *CTSP* genes

To obtain the cDNA clones encoding CTSP-1, -2, and -3 enzymes, degenerate primers were designed on the basis of N-terminal amino acid sequences of CTSP enzymes. Using the Protein Basic Alignment Search Tool (BLAST), two priming positions could be identified from the catalytic domains of several serine proteases, which are produced from *Periserrula leucophryna* (accession number: AAP51250), *Capitella teleta* (accession number: BAK20402), *Perinereis aibuhitensis* (accession number: ACL12061), and *Arenicola marina* (accession number: CAA64472) (Fig. 31). After multiple alignments, two consensus sequences (LTAAHC and CNGDSGGP) could be identified and universal degenerate primers, His-F and Ser-R were synthesized (Fig. 31). Gene specific degenerate primers (GSP1-1, GSP1-2, and GSP1-3) were designed on the basis of N-terminal eight amino acids of CTSP enzymes and the degenerate primers were synthesized (Fig. 31). The 3´-RACE PCRs were performed to identify 3´ ends of *CTSP* genes as shown in Fig. 32. In the 3´-RACE PCR, total cDNAs were synthesized by using the dT-Anchor primer from mRNA of

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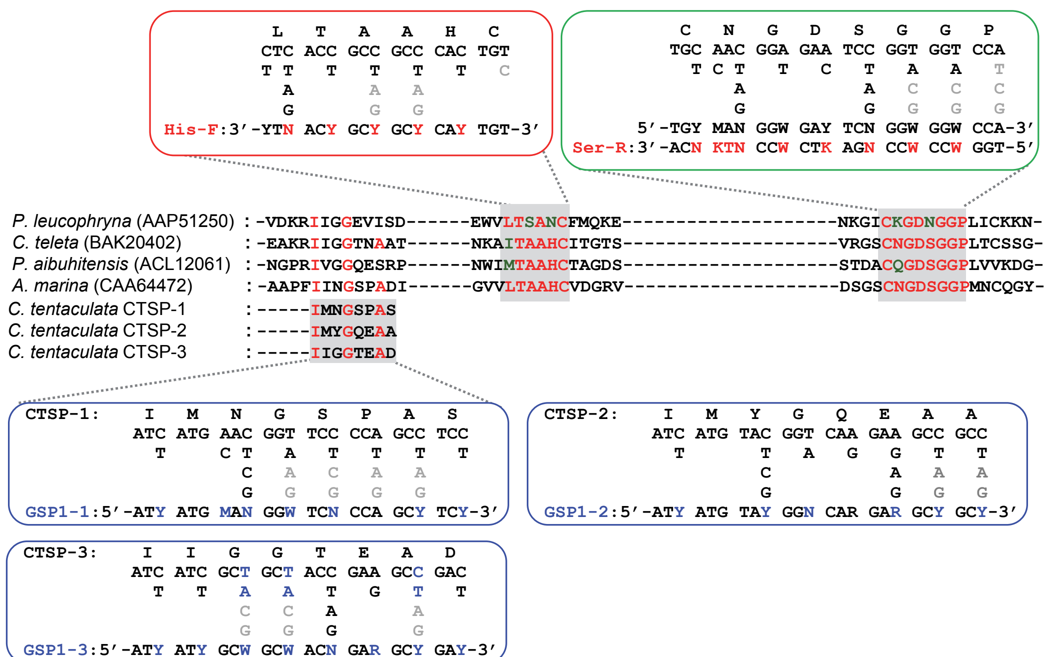

Fig. 31. Degenerate primers used for 3´-RACE. The degenerate primers were designed on the basis of the N-terminal sequences of CTSP-1 (IMNGSPAS), -2 (IMYGQEAA), and -3 (IIGGTEAD) and the two conserved catalytic domain sequences of other serine proteases from *P. leucophryna, C. teleta, P. aibuhitensis,* and *A. marina* (LTAAHC and CNGDSGGP). The degenerate primers were synthesized by incorporating 4 dNTPs as indicated. AAP51250, BAK20402, ACL12061, and CAA64472 represent the accession numbers of *P. leucophryna, C. teleta, P. aibuhitensis,* and *A. marina,* respectively. Mixed bases codes, Y, C + T; R, A + G; M, A + C; W, A + T; K, T + G; N, A + T + G + C.

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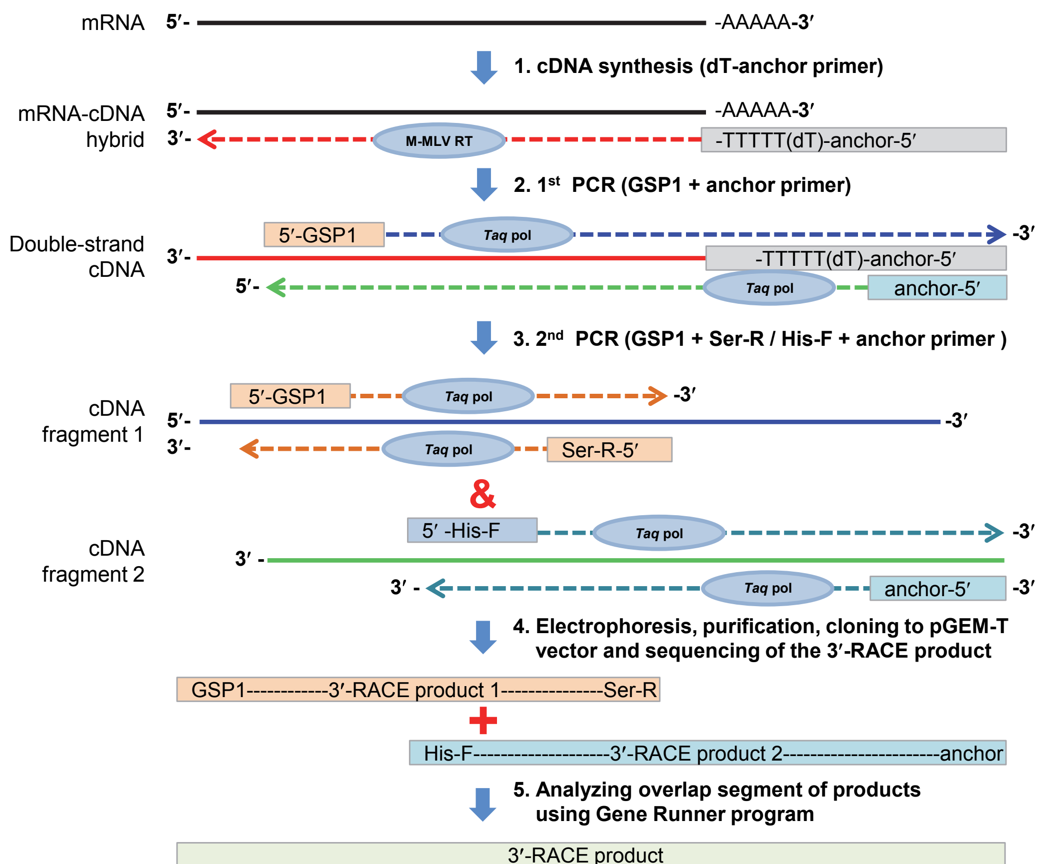

Fig. 32. Outline of 3´-RACE to obtain *CTSP* genes. In the 3´-RACE PCR, (1) Single-strand cDNA was synthesized by reverse transcription using the mRNA and Oligo dT-anchor primer. (2) Double-strand cDNA was amplified by PCR from single-strand cDNA using the Anchor and GSP1 primers. (3) To increase the specificity of the amplified PCR products, 1st PCR products were employed in second round PCR reactions. The reactions were amplified with a pair of GSP1:His-R and Ser-F:Anchor primers. (4) PCR products were separated by agarose gel electrophoresis, and the corresponding bands were recovered and ligated into pGEM-T easy vector. The inserted DNA fragments were analyzed by sequencing. (5) Overall sequence of 3´-RACE product was generated by analyzing overlap segments of products using Gene Runner program.

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C. tentaculata. Binding positions of 3´-RACE PCR primers on the cDNAs are summarized in Fig. 33A. When a pair of PCR primers (GSP1 as forward primer and Anchor primer as reverse primer) were used (Fig. 33A), a specific band could not observed on agarose gel although there was smeared pattern (Fig. 33B). However, the first step PCR seemed to amplify a product that could be served as a template for the second step PCR with a pair of GSP1:Ser-R and His-F:Anchor primers. When the 1st PCR products were used as template, approximately 500 bp DNA was amplified by a pair of primers (GSP1 and Ser-R) (Fig. 33B). Also, a DNA of approximately 700 bp was amplified by a pair of His-F and Anchor primers. Furthermore, the 5´-RACE PCR products for all three CTSPs showed that similar sized band appeared on agarose gel (Fig. 33B). These products were cloned into pGEM-T easy vector and sequenced. Two kinds of cDNA fragments were obtained from 3´-RACE for each CTSP. The 3´-RACE product sequences are shown in Figs. 34A, 35A, and 36A. Overall sequences of 3´-RACE products were reconstructed from the overlap sequences of clones obtained with GSP1:Ser-R and His-F:Anchor primers. The overall nucleotide length of 3´-RACE products of CTSP-1, -2, and -3 were 828, 829, and 820 bp, respectively. The partial nucleotide sequences of three *CTSP* genes were identified and their open reading frames were analyzed using Gene Runner program (http://www.generunner.net). Positions of 3´-RACE PCR primers are shown in Figs. 34A, 35A, and 36A. To amplify the 5´ unknown regions of *CTSP* genes, 5´-RACE PCR primers were designed on the basis of 3´-RACE product sequences. As shown in Figs. 34A, 35A, and 36A, the 5´-RACE PCR primers are underlined. The 5´-RACEs were performed to identify 5´ ends of *CTSP* genes as shown in Fig. 37. In the 5´-RACE PCR, gene specific cDNAs were synthesized by using the each *CTSP* gene specific RT primer (GSP-RT) from mRNA of *C. tentaculata.*

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Fig. 33. Amplification of 3´ ends from *CTSP* genes. (A) Diagrammatic representation showing the cDNA position capable of binding to primers. The cDNA was synthesized by dT-Anchor primer. (B) RACE products amplified by PCR using gene specific and universal primers. The RACE products were separated on 1% agarose gel and stained with ethidium bromide to visualize. The pairs of primers used are indicated at the top of each lane. Lanes G-A, GSP1:Anchor; G-S, GSP1:Ser-R; H-A, His-F:Anchor.

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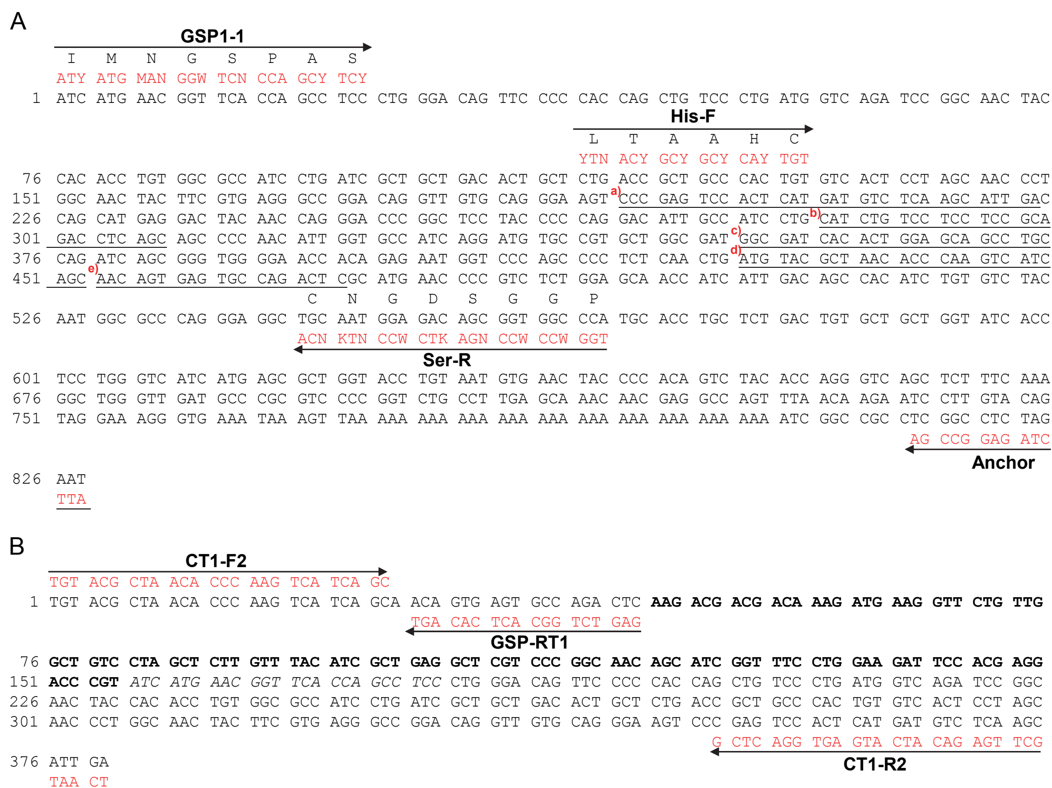

Fig. 34. Nucleotide sequences of 3´- and 5´-RACE products to obtain *CTSP-1* gene. (A) Nucleotide sequence of the 3´-RACE product. Positions of gene specific (GSP1-1) and universal (His-F, Ser-R, and Anchor) primers used in 3´-RACE PCRs were indicated by arrows. Primers for 5´-RACE PCR were underlined. Superscripts a), CT1-R2 primer; b), CT1-R1 primer; c), CT1-F1 primer; d), CT1-F2 primer; e), GSP-RT1 primer. (B) Nucleotide sequence of the 5´-RACE product. Positions of GSP-RT1, CT1-F2 and CT1-R2 primers used in 5´-RACE PCR were indicated by arrows. The bold letters indicate newly identified sequences in 5´-RACE.

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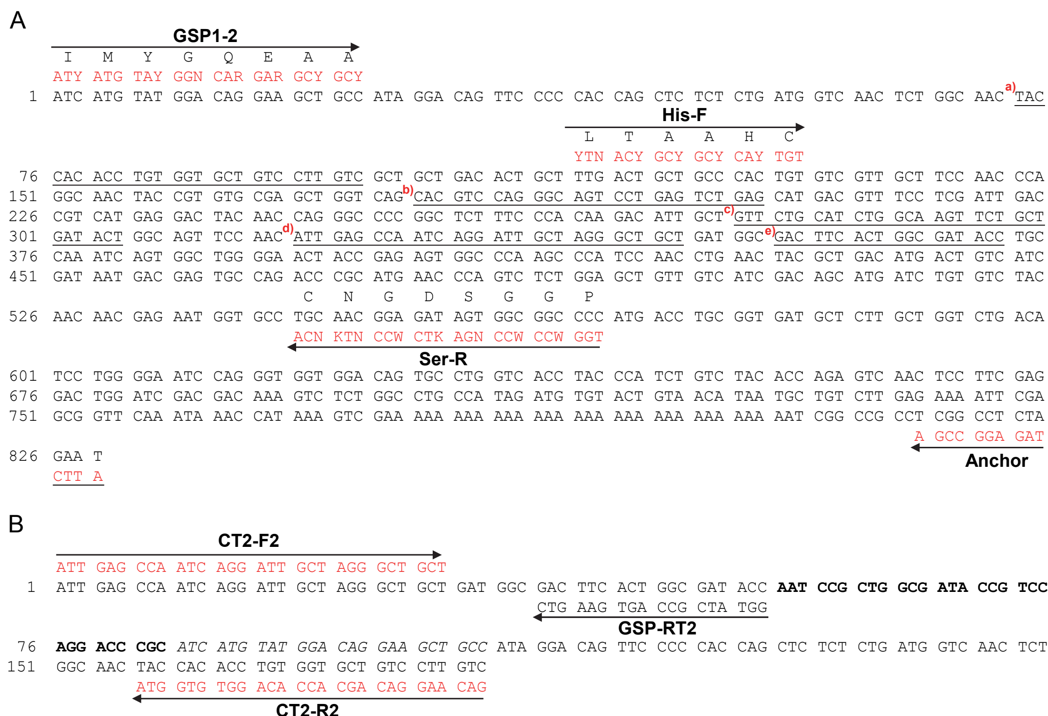

Fig. 35. Nucleotide sequences of 3´- and 5´-RACE products to obtain *CTSP-2* gene. (A) Nucleotide sequence of the 3´-RACE product. Positions of gene specific (GSP1-2) and universal (His-F, Ser-R, and Anchor) primers used in 3´-RACE PCRs were indicated by arrows. Primers for 5´-RACE PCR were underlined. Superscripts a), CT2-R2 primer; b), CT2-R1 primer; c), CT2-F1 primer; d), CT2-F2 primer; e), GSP-RT2 primer. (B) Nucleotide sequence of the 5´-RACE product. Positions of GSP-RT2, CT2-F2 and CT2-R2 primers used in 5´-RACE PCR were indicated by arrows. The bold letters indicate newly identified sequences in 5´-RACE.

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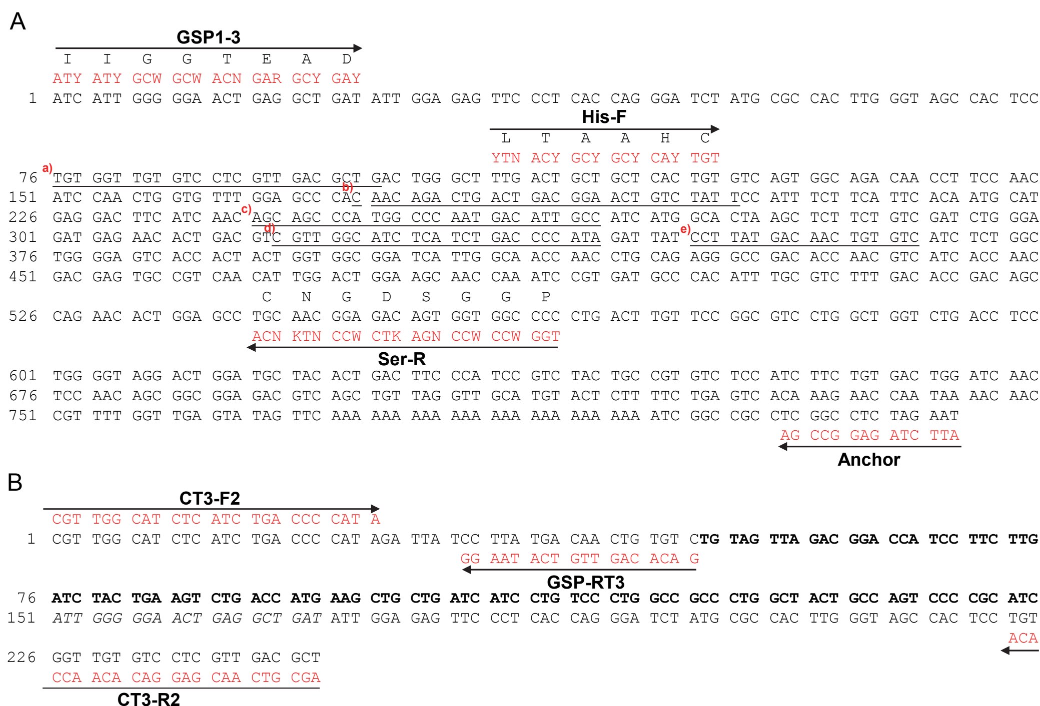

Fig. 36. Nucleotide sequences of 3´- and 5´-RACE products to obtain *CTSP-3* gene. (A) Nucleotide sequence of the 3´-RACE product. Positions of gene specific (GSP1-3) and universal (His-F, Ser-R, and Anchor) primers used in 3´-RACE PCRs were indicated by arrows. Primers for 5´-RACE PCR were underlined. Superscripts a), CT3-R2 primer; b), CT3-R1 primer; c), CT3-F1 primer; d), CT3-F2 primer; e), GSP-RT3 primer. (B) Nucleotide sequence of the 5´-RACE product. Positions of GSP-RT3, CT3-F2 and CT3-R2 primers used in 5´-RACE PCR were indicated by arrows. The bold letters indicates newly identified sequences in 5´-RACE.

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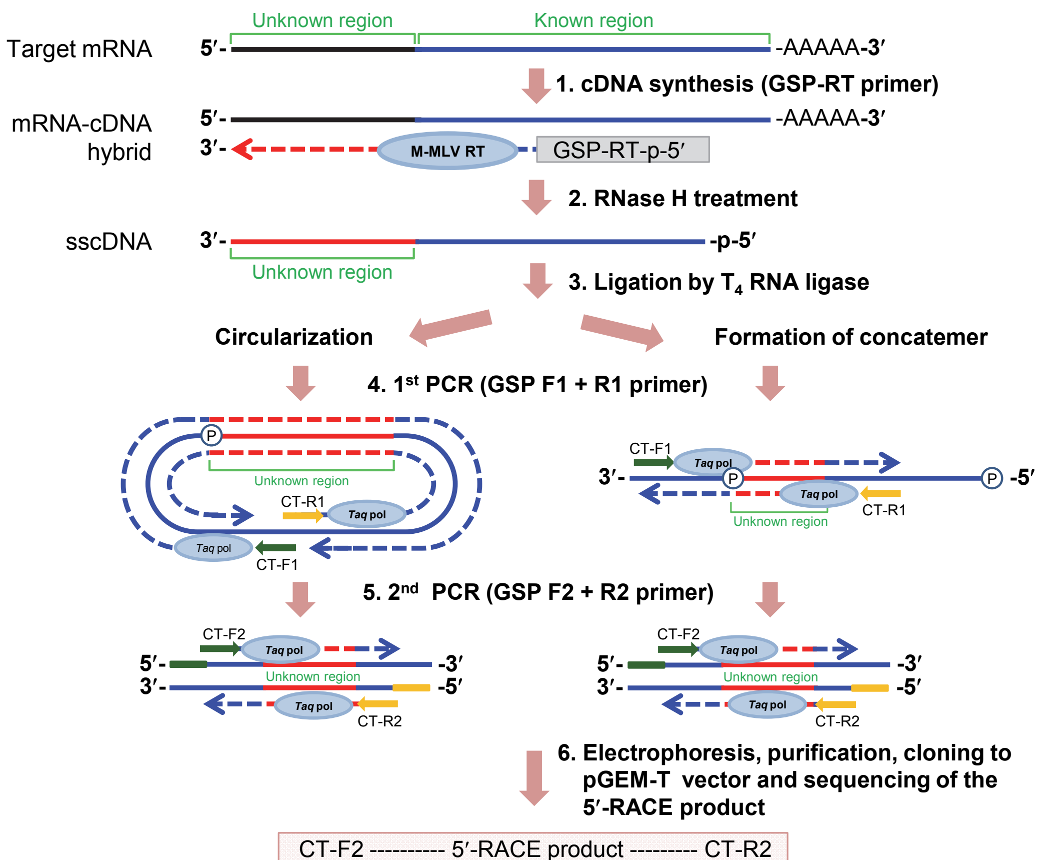

Fig. 37. Outline of 5´-RACE to obtain *CTSP* genes. In the 5´-RACE PCR, (1) single-strand cDNA was synthesized by reverse transcription using the mRNA and 5´-phosphorylated GSP-RT primer. (2) To remove the mRNA, RNaseH was added to reaction and incubated. (3) Single-strand cDNAs were concatemerized by T4 RNA ligase. (4) Double-strand cDNAs were amplified by PCR from single-strand concatemer cDNA using the CT-F1 and CT-R1 primers. (5) To increase the specificity of the amplified PCR products, 1st PCR products were employed in second round PCR reactions. The reactions were amplified with CT-F2 and CT-R2 primers. (6) PCR products were separated by agarose gel electrophoresis, and the corresponding bands were recovered and ligated into pGEM-T easy vector. The inserted DNA fragments were analyzed by sequencing.

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The synthesized single-strand cDNAs were treated with RNase H to remove mRNA template. After removing the mRNA, single-strand cDNAs were concatemerized by T_4 RNA ligase. In the first step PCR, the cDNA concatemers were used as a template for a pair of CT-F1 and CT-R1 primers. Binding positions of 5´-RACE PCR primers on the cDNAs are summarized in Fig 38A. On the agarose gel electrophoresis, the 1st PCR product size of CTSP-1, -2, and -3 were 400, 300, and 400 bp, respectively (Fig. 38B). To increase the specificity of the amplified PCR products, 1st PCR products were employed in second round PCR reactions. When the 1st PCR products were used as template, approximately 200-300 bp DNAs were amplified by a pair of CT-F2 and CT-R2 primers (Fig. 38B). These products were cloned into pGEM-T easy vector and sequenced. As shown in Figs. 36B, 37B, and 38B, the nucleotide sequencing results showed that the 5´-RACE products of CTSP-1, -2, and -3 were composed of 380, 183, and 246 bp, respectively. However, newly obtained sequences of 5´-RACE products were 113, 33, and 110 bp, respectively. Finally, full length CTSP genes were generated by analyzing contiguous segments of RACE PCR products using Gene Runner program. For further confirmation of full length *CTSP* genes, each *CTSP* gene specific reverse primers (Table 3) were designed on the basis of sequence analysis of 3´-RACE PCR products. Another gene specific forward primers (Table 3) were designed on the basis of sequence analysis of 5´-RACE PCR products. To amplify the full length cDNA of *CTSP* genes, CTSP-F and CTSP-R primers were employed in a RT-PCR with total cDNA of *C. tentaculata.*

III-2-2. Characterization of *CTSP* genes

Using the CTSP-F and CTSP-R primers, the full length coding

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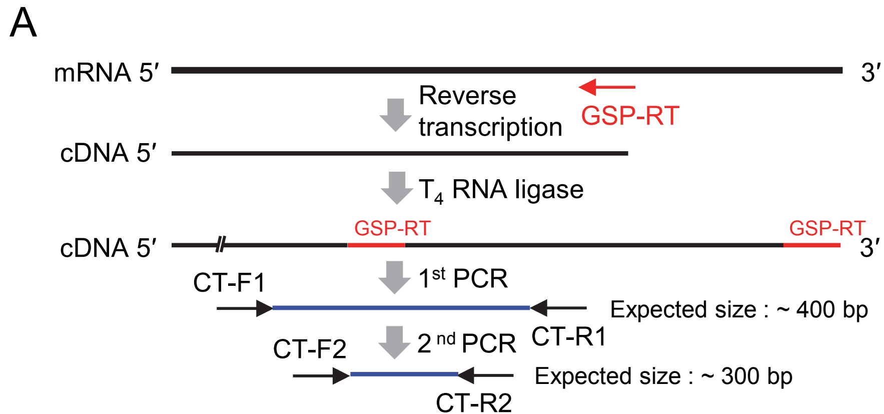

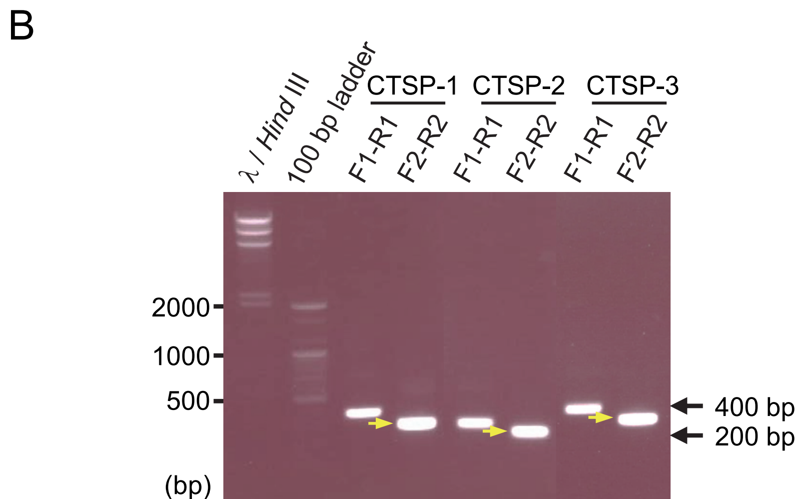

Fig. 38. Amplification of 5´ ends from *CTSP* genes. (A) Diagrammatic representation showing the cDNA position capable of binding to primers. The cDNAs were synthesized by GSP-RT primers and then ligated each other with T_4 RNA ligase. (B) RACE products amplified by PCRs using gene specific primers. The resulting RACE products were separated on 1% agarose gel and stained with ethidium bromide to visualize. The pair of primers used are indicated at the top of each lane. Lanes F1-R1, CT-F1:CT-R1; F2-R2, CT-F2:CT-R2.

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regions of CTSP-1, -2, and -3 were cloned into pJET1.2/blunt vector. The resulting constructs were named pJET-*CTSP-1*, pJET-*CTSP-2*, and pJET-*CTSP-3*. The final nucleotide sequences of three genes, *CTSP-1, -2*, and *-3* encoding the fibrinolytic enzymes were 884 bp, 805 bp, and 869 bp, respectively (Figs. 39-41). Nucleotide sequence analysis showed that the sequences of *CTSP-1*, *-2*, and *-3* genes had open reading frames composed of 804, 738, and 756 bp, each capable of encoding 267, 246, and 252 amino acids, respectively (Figs. 39-41). As shown in Fig. 42, the deduced amino acid sequences of CTSP enzymes were compared with each other. Amino acid identity between CTSP-1 and -2, CTSP-1 and -3, and CTSP-2 and -3 were found to be 73.3%, 39.9%, and 44.2%, respectively. In the sequence comparison also showed that there was a putative catalytic triad composed of His, Asp, and Ser residues within consensus motif, that are frequently found in trypsin-like serine protease family (Brenner, 1988). The deduced amino acid sequences of CTSP enzymes were compared to other annelid serine proteases in the NCBI protein databases using the BLAST tool: as shown in Table 8. CTSP-1 showed homology with other serine protease, especially 44.07% with the fibrinolytic enzyme from *Lumbricus bimastus* (accession number: AAD05563.1) and 41.53% with chymotrypsinogen from *Arenicola marina* (accession number: CAA64472.1). CTSP-2 showed 42.3% and 41.7% homology with the fibrinolytic enzymes from *Lumbricus bimastus* (accession number: AAD05563.1) and lumbrokinase from *Eisenia fetida* (accession number: ABW04905.1), respectively. CTSP-3 showed homology with other serine proteases, expecially 43.78% and 42.92% with the serine proteinase from *Enchytraeus japonensis* (accession number: BAL43180.1) and fibrinolytic enzyme from *Lumbricus bimastus* (accession number: AAD05563.1), respectively. Comparison of enzymes having a relatively high sequence similarity to CTSP enzymes are shown in Fig. 43,

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```
  1 AAGACGACGACAAAGATGAAGGTTCTGTTGGCTGTCCTAGCTCTTGTTTACATCGCTGAG
                    M  K  V  L  L  A  V  L  A  L  V  Y  I  A  E   15
 61 GCTCGTCCCGGCAACAGCATCGGTTTCCTGGAAGATTCCACGAGGACCCGTATCATGAAC
     A  R  P  G  N  S  I  G  F  L  E  D  S  T  R  T  R  I  M  N   35
121 GGTTCACCAGCCTCCCTGGGACAGTTCCCCCACCAGCTGTCCCTGATGGTCAGATCCGGC
     G  S  P  A  S  L  G  Q  F  P  H  Q  L  S  L  M  V  R  S  G   55
181 AACTACCACACCTGTGGCGCCATCCTGATCGCTGCTGACACTGCTCTGACCGCTGCCCAC
     N  Y  H  T  C  G  A  I  L  I  A  A  D  T  A  L  T  A  A  H   75
241 TGTGTCACTCCTAGCAACCCTGGCAACTACTTCGTGAGGGCCGGACAGGTTGTGCAGGGA
     C  V  T  P  S  N  P  G  N  Y  F  V  R  A  G  Q  V  V  Q  G   95
301 AGTCCCGAGTCCACTCATGATGTCTCAAGCATTGACCAGCATGAGGACTACAACCAGGGA
     S  P  E  S  T  H  D  V  S  S  I  D  Q  H  E  D  Y  N  Q  G  115
361 CCCGGCTCCTACCCCCAGGACATTGCCATCCTGCATCTGTCCTCCTCCGCAGACCTCAGC
     P  G  S  Y  P  Q  D  I  A  I  L  H  L  S  S  S  A  D  L  S  135
421 AGCCCCAACATTGGTGCCATCAGGATGTGCCGTGCTGGCGATGGCGATCACACTGGAGCA
     S  P  N  I  G  A  I  R  M  C  R  A  G  D  G  D  H  T  G  A  155
481 GCCTGCCAGATCAGCGGGTGGGGAACCACAGAGAATGGTCCCAGCCCCTCTCAACTGATG
     A  C  Q  I  S  G  W  G  T  T  E  N  G  P  S  P  S  Q  L  M  175
541 TACGCTAACACCCAAGTCATCAGCAACAGTGAGTGCCAGACTCGCATGAACCCCGTCTCT
     Y  A  N  T  Q  V  I  S  N  S  E  C  Q  T  R  M  N  P  V  S  195
601 GGAGCAACCATCATTGACAGCCACATCTGTGTCTACAATGGCGCCCAGGGAGGCTGCAGT
     G  A  T  I  I  D  S  H  I  C  V  Y  N  G  A  Q  G  G  C  S  215
661 GGAGACAGCGGTGGCCCATGCACCTGCTCTGACTGTGCTGCTGGTATCACCTCCTGGGTC
     G  D  S  G  G  P  C  T  C  S  D  C  A  A  G  I  T  S  W  V  235
721 ATCATGAGCGCTGGTACCTGTAATGTGAACTACCCCACAGTCTACACCAGGGTCAGCTCT
     I  M  S  A  G  T  C  N  V  N  Y  P  T  V  Y  T  R  V  S  S  255
781 TTCAAAGGCTGGGTTGATGCCCGCGTCCCCGGTCTGCCTTGAGCAAACAACGAGGCCAGT
     F  K  G  W  V  D  A  R  V  P  G  L  P  *                    268
841 TTAACAAGAATCCTTGTACAGTAGGAAAGGGTG
```

Fig. 39. The nucleotide and deduced amino acid sequences of *CTSP-1* gene from *C. tentaculata*. The underlined nucleotide sequences indicate the primer binding sites used for pJET1.2/blunt vector cloning. Amino acids shown by bold characters indicate the N-terminal amino acid sequence of purified CTSP-1 enzyme.

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1 AATCCGCTGGCGATACCGTCCAGGACCCGCATCATGTATGGACAGGAAGCTGCCATAGGA
N P L A I P S R T R **I M Y G Q E A A** I G 20
61 CAGTTCCCCCACCAGCTCTCTCTGATGGTCAACTCTGGCAACTACCACACCTGTGGTGCT
Q F P H Q L S L M V N S G N Y H T C G A 40
121 GTCCTTGTCGCTGCTGACACTGCTTTGACTGCTGCCCACTGCGTCGTTGCTTCCAACCCA
V L V A A D T A L T A A H C V V A S N P 60
181 GGCAACTACCGTGTGCGAGCTGGTCAGCACGTCCAGGGCAGTCCTGAGTCTGAGCATGAC
G N Y R V R A G Q H V Q G S P E S E H D 80
241 GTTTCCTCGATTGACCGTCATGAGGACTACAACCAGGGCCCCGGCTCTTTCCCACAAGAC
V S S I D R H E D Y N Q G P G S F P Q D 100
301 ATTGCTGTTCTGCATCTGGCAAGTTCTGCTGATACTGGCAGTTCCAACATTGAGCCAATC
I A V L H L A S S A D T G S S N I E P I 120
361 AGGATTGCTAGGGCTGCTGATGGCGACTTCACTGGCGATACCTGCCAAATCAGTGGCTGG
R I A R A A D G D F T G D T C Q I S G W 140
421 GGAACTACCGAGAGTGGCCCAAGCCCATCCAACCTGAACTACGCTGACATGACTGTCATC
G T T E S G P S P S N L N Y A D M T V I 160
481 GATAATGACGAGTGCCAGACCCGCATGAACCCAGTCTCTGGAGCTGTTGTCATCGACAGC
D N D E C Q T R M N P V S G A V V I D S 180
541 ATGATCTGTGTCTACAACAACGAGAATGGTGCCTGCAGCGGAGATAGTGGCGGCCCCATG
M I C V Y N N E N G A C S G D S G G P M 200
601 ACCTGCGGTGATGCTCTTGCTGGTCTGACATCCTGGGGAATCCAGGGTGGTGGACAGTGC
T C G D A L A G L T S W G I Q G G G Q C 220
661 CTGGTCACCTACCCATCTGTCTACACCAGAGTCAACTCCTTCGAGGACTGGATCGACGAC
L V T Y P S V Y T R V N S F E D W I D D 240
721 AAAGTCTCTGGCCTGCCATAGATGTGTACTGTAACATAATGCTGTCTTGAGAAAATTCGA
K V S G L P * 246
781 GCGGTTCAAATAAACCATAAAGTCG

Fig. 40. The nucleotide and deduced amino acid sequences of *CTSP-2* gene from *C. tentaculata*. The underlined nucleotide sequences indicate the primer binding sites used for pJET1.2/blunt vector cloning. Amino acids shown by bold characters indicate the N-terminal amino acid sequence of purified CTSP-2 enzyme.

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```
  1 TAGTTAGACGGACCATCCTTCTTGATCTACTGAAGTCTGACCATGAAGCTGCTGATCATC
                                                M  K  L  L  I  I    6
 61 CTGTCCCTGGCCGCCCTGGCTACTGCCAGTCCCCGCATCATTGGGGAACTGAGGCTGAT
     L  S  L  A  A  L  A  T  A  S  P  R  I  I  G  G  T  E  A  D   26
121 ATTGGAGAGTTCCCTCACCAGGGATCTATGCGCCACTTGGGTAGCCACTCCTGTGGTTGT
     I  G  E  F  P  H  Q  G  S  M  R  H  L  G  S  H  S  C  G  C   46
181 GTCCTCGTTGACGCTGACTGGGCTTTGACTGCTGCTCACTGTGTCAGTGGCAGACAACCT
     V  L  V  D  A  D  W  A  L  T  A  A  H  C  V  S  G  R  Q  P   66
241 TCCAACATCCAACTGGTGTTTGGAGCCCACAACAGACTGACTGACGGAACTGTCTATTCC
     S  N  I  Q  L  V  F  G  A  H  N  R  L  T  D  G  T  V  Y  S   86
301 ATTTCTTCATTCACAATGCATGAGGACTTCATCAACAGCAGCCCATGGCCCAATGACATT
     I  S  S  F  T  M  H  E  D  F  I  N  S  S  P  W  P  N  D  I  106
361 GCCATCATGGCACTAAGCTCTTCTGTCGATCTGGGAGATGAGAACACTGACGTCGTTGGC
     A  I  M  A  L  S  S  S  V  D  L  G  D  E  N  T  D  V  V  G  126
421 ATCTCATCTGACCCCATAGATTATCCTTATGACAACTGTGTCATCTCTGGCTGGGGAGTC
     I  S  S  D  P  I  D  Y  P  Y  D  N  C  V  I  S  G  W  G  V  146
481 ACCACTACTGGTGGCGGATCATTGGCAACCAACCTGCAGAGGGCCGACACCAACGTCATC
     T  T  T  G  G  G  S  L  A  T  N  L  Q  R  A  D  T  N  V  I  166
541 ACCAACGACGAGTGCCGTCAACATTGGACTGGAAGCAACCAAATCCGTGATGCCCACATT
     T  N  D  E  C  R  Q  H  W  T  G  S  N  Q  I  R  D  A  H  I  186
601 TGCGTCTTTGACACCGACAGCCAGAACACTGGAGCCTGCAACGGAGACAGTGGTGGCCCC
     C  V  F  D  T  D  S  Q  N  T  G  A  C  N  G  D  S  G  G  P  206
661 CTGACTTGTTCCGGCGTCCTGGCTGGTCTGACCTCCTGGGGTAGGACTGGATGCTACACT
     L  T  C  S  G  V  L  A  G  L  T  S  W  G  R  T  G  C  Y  T  226
721 GACTTCCCATCCGTCTACTGCCGTGTCTCCATCTTCTGTGACTGGATCAACTCCAACAGC
     D  F  P  S  V  Y  C  R  V  S  I  F  C  D  W  I  N  S  N  S  246
781 GGCGGAGACGTCAGCTGTTAGGTTGCATGTACTCTTTTCTGAGTCACAAAGAACCAATAA
     G  G  D  V  S  C  *                                          252
841 AACAACCGTTTTGGTTGAGTATAGTTC
```

Fig. 41. The nucleotide and deduced amino acid sequences of *CTSP-3* gene from *C. tentaculata*. The underlined nucleotide sequences indicate the primer binding sites used for pJET1.2/blunt vector cloning. Amino acids shown by bold characters indicate the N-terminal amino acid sequence of purified CTSP-3 enzyme.

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```
                        N-terminal propeptide
CTSP-1  MKVLLAVLALVYIAEARPGNSIGFLEDSTRTRIMNGSPASLGQFPHQLSLMVRSGNYHTC  60
CTSP-2  ----------------------NPLAIPSRTRIMYGQEAAIGQFPHQLSLMVNSGNYHTC  39
CTSP-3  -MKLLIILSLAALATASP-------------RIIGGTEADIGEFPHQGS-MRHLG-SHSC  44
           ** :*:*. :* * *             **: *  * :*:**** * * . *  *:*

CTSP-1  GAILIAADTALTAAHCVTPSNPGNYFVRAGQVVQGSPESTHDVSSIDQHEDYNQGPGSYP 120
CTSP-2  GAVLVAADTALTAAHCVVASNPGNYRVRAGQHVQGSPESEHDVSSIDRHEDYNQGPGSFP  99
CTSP-3  GCVLVDADWALTAAHCVSGRQPSNIQLVFGAHNRLTDGTVYSISSFTMHEDFIN-SSPWP 103
        *.:*: ** ********   :*.*  :  *   : :  : :.:**:  ***: : ...:*

CTSP-1  QDIAILHLSSSADLSSPNIGAIRMCRAGDGDHTGAACQISGWGTTENGPS--PSQLMYAN 178
CTSP-2  QDIAVLHLASSADTGSSNIEPIRIARAADGDFTGDTCQISGWGTTESGPS--PSNLNYAD 157
CTSP-3  NDIAIMALSSSVDLGDENTDVVGIS-SDPIDYPYDNCVISGWGVTTTGGGSLATNLQRAD 162
        :***:: *:**.* .. *   : :. :   *..   * *****.* .* .  .::*  *:

CTSP-1  TQVISNSECQTRMNPVSGATIIDSHICVYNGAQ---GGCSGDSGGPCTCSDCAAGITSWV 235
CTSP-2  MTVIDNDECQTRMNPVSGAVVIDSMICVYNNEN---GACSGDSGGPMTCGDALAGLTSWG 214
CTSP-3  TNVITNDECRQHWTGSN--QIRDAHICVFDTDSQNTGACNGDSGGPLTCSGVLAGLTSWG 220
          ** *.**: : .  .   : *: ***::  .   *.*.****** **..  **:***

CTSP-1  IMSAGTCNVNYPTVYTRVSSFKGWVDARVPG-LP- 268
CTSP-2  IQGGGQCLVTYPSVYTRVNSFEDWIDDKVSG-LP- 247
CTSP-3  RTG---CYTDFPSVYCRVSIFCDWINSNSGGDVSC 252
          .   * . :*:** **. * .*:: .  * :.
```

Fig. 42. Alignment of amino acid sequences between three CTSP enzymes from *C. tentaculata*. Identical residues in all three sequences are asterisked. Three amino acid residues shown by boxes indicate the members of a putative catalytic triad consensus motives in the active enzyme, which are typically found in trypsin-like serine protease.

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Table 8. Classification and homology between annelid proteases.

Phylum	Class / Order	Species	Function or Character	Database No.	Identity (%)		
					CTSP-1	CTSP-2	CTSP-3
Annelida	Polycaeta	*Cirriformia tentaculata*	CTSP-1	This study	100	73.31	39.91
		Cirriformia tentaculata	CTSP-2	This study	73.31	100	44.21
		Cirriformia tentaculata	CTSP-3	This study	39.91	44.21	100
		Arenicola cristata	Fibrinolytic enzyme	AGS48987.1	30.08	34.75	34.33
		Arenicola marina	Trypsin-like protease	CAA72624.1	24.58	30.51	29.18
		Arenicola marina	Chymotrypsinogen	CAA64472.1	41.53	40.25	39.48
		Capitella teleta	Hypothetical chymotrypsin-like serine protease	BAK20402.1	38.98	36.86	42.06
		Perinereis aibuhitensis	Fibrinolytic protease	ACL12061.1	31.36	33.9	30.9
		Periserrula leucophryna	Trypsin-like serine protease	AAP51250	17.37	18.64	21.03
	Clitellata	*Enchytraeus japonensis*	Serine proteinase	BAL43180.1	36.02	37.71	43.78
		Enchytraeus japonensis	Fibrinolytic enzyme	BAL43186.1	40.68	39.83	42.92
		Eisenia fetida	Fibrinolytic protease 1	ABD76397.1	40.68	41.53	39.91
		Eisenia fetida	Lumbrokinase	ABW04905.1	40	41.7	40.34
		Eisenia fetida	Lumbrokinase	ABW04906.1	39.41	41.53	39.91
		Eisenia fetida	Fibrinolytic protease 0	ABG68022.1	38.22	40.89	38.67
		Helobdella robusta	Hypothetical trypsin-like serine protease	ESO12707.1	35.17	34.75	32.62
		Helobdella robusta	Hypothetical trypsin-like serine protease	ESO00207.1	35.59	36.86	38.63
		Lumbricus bimastus	Fibrinolytic enzyme	AAD05563.1	44.07	42.37	42.92
		Lumbricus bimastus	Lmbrokinase	AAL28118.1	31.36	30.51	33.91
		Lumbricus rubellus	Fibrinolytic enzyme	BAB40767.1	29.24	30.93	30.04
		Lumbricus rubellus	Chymotrysin	CAA11132.1	23.73	29.24	30.04
Echiura	Echiuroinea	*Urechis unicinctus*	Fibrinolytic enzyme	ADL28819.1	44.92	46.19	40.77

```
CTSP-1      MKVLLAVLALVYIAEARPGNSIGFLEDSTRTR-IMNGSPASLGQFPHQLSLMVRSGN-YH 58
CTSP-2      ----------------NP------LAIPSRTR-IMYGQEAAIGQFPHQLSLMVNSGN-YH 36
CTSP-3      -MKLLIILS--------------LAALATASPRIIGGTEADIGEFPHQGSMRHLG---SH 42
Am-CT       -MKLSLIILAVALCEARPRDVDLTARASVAAPFIINGSPADISNFPYQCSLRYAG---SH 56
Lb-FE       ---------------------------------VIGGTNASPGEFPWQLSQQRQSGSWSH 27
Ef-LK       ----------------------------------IGGTDASPGEFPWQLSQTRGG---SH 23
Ej-SP       -MKFFVVCL--------------LLPALVAGGRIVGGGDADNGEWPWQLSLRNLG---SH 42
Ej-FE       MLPLLFLIATAGLIEARP-SAMLKPSFGFSMMGVVGGTDASPGEFPWQLSQQRLGGSWSH 59
                                                  : *  *  .::* * *    .    *

CTSP-1      TCGAILIAADTALTAAHCVTPSNPGNYFVRAGQVVQGS-PES--THDVSSIDQHEDYNQG 115
CTSP-2      TCGAVLVAADTALTAAHCVVASNPGNYRVRAGQHVQGS-PES--EHDVSSIDRHEDYNQG 93
CTSP-3      SCGCVLVDADWALTAAHCVSGRQPSNIQLVFGAHNRLTDGTVYS---ISSFTMHEDFINS 99
Am-CT       TCGCSVLNAGVVLTAAHCVDGRVATAFSVLAGSTDRT--VSG--DIDASGFTMNSAYDGN 112
Lb-FE       SCGASLLSSTSALSASHCVDGVLPNNIRVIAGLWQQSD-TSGTQTANVDSYTMHENYGAG 86
Ef-LK       SCGASLLNALNGLSASHCVDGAAPGTITVIAGLHDRSG-TPGSQEVDITGYTMHENYNQG 82
Ej-SP       SCGASLIRPNWAVCAAHCV-GSSPSAYTIIAGTNQRSCPGSNCEERRANSATRHEDFQNI 101
Ej-FE       SCGASLIGATRALSAAHCVDGASASILRVIAGLHQRSN-TAGTQTSNVASYTMHESYNQG 118
            :**. :: .   : *:***    .    :  *   :             .   :. :

CTSP-1      P-GSYPQDIAILHLSSSADLSSPNIGAIRMCRAGDGDHTGAACQISGWG--TTENGPSPS 172
CTSP-2      P-GSFPQDIAVLHLASSADTGSSNIEPIRIARAADGDFTGDTCQISGWG--TTESGPSPS 150
CTSP-3      --SPWPNDIAIMALSSSVDLGDENTDVVGISSD-PIDYPYDNCVISGWGVTTTGGGSLAT 156
Am-CT       A-GGFPNDIATVALTSNLNLGDPNIAAASLPPNNNDQFVGSQCTITGWGR-TGTSNILPA 170
Lb-FE       T-ASYSNDIAILHLATSISLGG-NIQAAVLPANNNNDYAGTTCVISGWGR-TDGTNNLPD 143
Ef-LK       T-NTYANDIAILHFASAINIGG-NVQAALLPANNNNDYNGLTCVISGWGR-TGSSNVLPD 139
Ej-SP       GLLGFPNDISIIHWVDAIAESSGSIQYVPLAT--TADQVGRNCYITGWGR-LYGNGPIPE 158
Ej-FE       S-ATFANDVAILNLATAITTGG-NIAFATL-AQGSNDFAGTTCVISGWGR-TSASNALPD 174
                :.:*:: :        .. .     :      :     * *:***      .  .

CTSP-1      QLMYANTQVISNSECQTRMNPVSGATIIDSHICVYN---GAQGGCSGDSGGPCTCSD--- 226
CTSP-2      NLNYADMTVIDNDECQTRMNPVSGAVVIDSMICVYN---NENGACSGDSGGPMTCGD--- 204
CTSP-3      NLQRADTNVITNDECRQHWTGSN--QIRDAHICVFDTDSQNTGACNGDSGGPLTCSG--- 211
Am-CT       TLQQVTMPIISNAECASRMSSVSGANVNDGHICIYN---GDSGSCNGDSGGPMNCQG--- 224
Lb-FE       ILQKSSIPVITTAQCTAAMVGVGGANIWDNHICVQDPA-GNTGACNGDSGGPLNCPDGG- 201
Ef-LK       TLQKASIEVIGTTQCQSLMGSIG--NIWDNHICLYDNA-NNVGSCNGDSGGPLNCPDGG- 195
Ej-SP       NLQEAHIDLLTTAECSSMWS-PT--PVTDSQVCVFDKATQARGACNGDSGGPLVCELSSG 215
Ej-FE       TLQKASIPVISGTQCQSLVAGIG--TIWDGHICLYDSA-GNIGSCNGDSGGPLNCPSGGS 231
             *      ::   :*           :  *  :*: :      *.*.******  *

CTSP-1      --CAAGITSWVIMS-AGTCNVNYPTVYTRVSSFKGWVDARVPGLP-- 268
CTSP-2      --ALAGLTSWGIQG-GGQCLVTYPSVYTRVNSFEDWIDDKVSGLP-- 246
CTSP-3      --VLAGLTSWGRTG----CYTDFPSVYCRVSIFCDWINSNSGGDVSC 252
Am-CT       --YVAGVTSWGISSALGNCMVSYPSVYTRTSYFLSWIANNS------ 263
Lb-FE       -TRVVGVTSWVVSSGLGTCLPDYPSVYTRVSAYLGWIGDNSR----- 242
Ef-LK       -TRVAGVTSWGVSSGAGNCLQTYPSVYTRTSAYLSWIANNS------ 235
Ej-SP       SWELVGATSWGRSG----CSTDYPSVYTRVSAFNSWILNQIGE---- 254
Ej-FE       -TVVAGVTSWGISS-LGVCRQDYPSVYTRVSYYYTWINSHL------ 270
                .* ***   .    *   :*:** *.. :  *:  .
```

Fig. 43. Amino acid sequence alignment of serine proteases from different species. The boxes indicate the conserved regions of the putative catalytic triads of serine proteases. CTSPs, serine protease from *C. tentaculata* (this study); Am-CT, chymotrypsinogen from *A. marina*; Lb-FE, fibrinolytic enzyme from *L. bimastus*; Ef-LK, lumbrokinase from *E. fetida*; Ej-SP, serine proteinase from *E. japonensis*; Ej-FE, fibrinolytic enzyme from *E. japonensis.*

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sequence alignment of the CTSP enzymes and high homology other annelid fibrinolytic enzymes showed significant sequence similarity at the catalytic triad consensus motif.

III-2-3. Analysis of three dimensional structures of CTSP enzymes

The structural models of three CTSP enzymes were built based on the high resolution crystal structure of earthworm fibrinolytic enzyme (2.3 angstrom; PDB code 1m9u.1.A) using homology modeling technique implemented in the software SWISS-MODEL (Figs. 44-47). The high degree of sequence identity (>43%) was found between these proteases with respect to their template 1m9u.1.A. The homology models of CTSP enzymes showed that there monomeric proteases contain thirteen β-sheet structures. In addition, α-helix structures in the CTSP-1, -2, and -3 enzymes were found to be 4, 3, and 3, respectively (Figs. 44A, 45A, and 46A). In the 3D model structure of CTSP-1 enzyme, six of the eleven cysteines seemed to form three disulfide bonds: Cys28-Cys44, Cys113-Cys195, and Cys155-Cys173, whereas Cys125, Cys182, Cys190, Cys192, and Cys210 are free (Fig. 44). In the 3D model structure of CTSP-2 enzyme, six of the eight cysteines formed three disulfide bonds: Cys28-Cys44, Cys155-Cys173, and Cys182-Cys210., whereas Cys125, and Cys192 are free (Fig. 45). In the CTSP-1 and -2 enzymes, the disulfide bond Cys28-Cys44 connected the N-terminus of β2-sheet and the α1-helix including a catalytic residue His43, whereas disulfide bond Cys155-Cys173 connects the α2-helix and β10-sheet. His43, Asp90, and Ser186 residues were constituted the active site where the breakage of peptide bonds in substrates might occur (Figs. 44 and 45). In the 3D model structure of CTSP-3 enzyme, four of the twelve cysteines formed two disulfide bonds: Cys26-Cys42 and Cys153-Cys169, whereas

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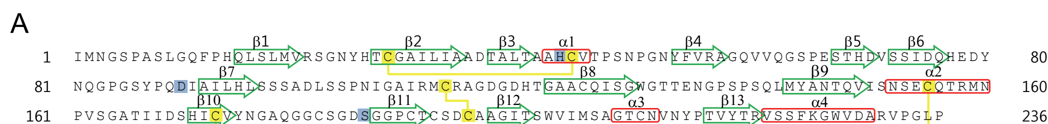
A



B

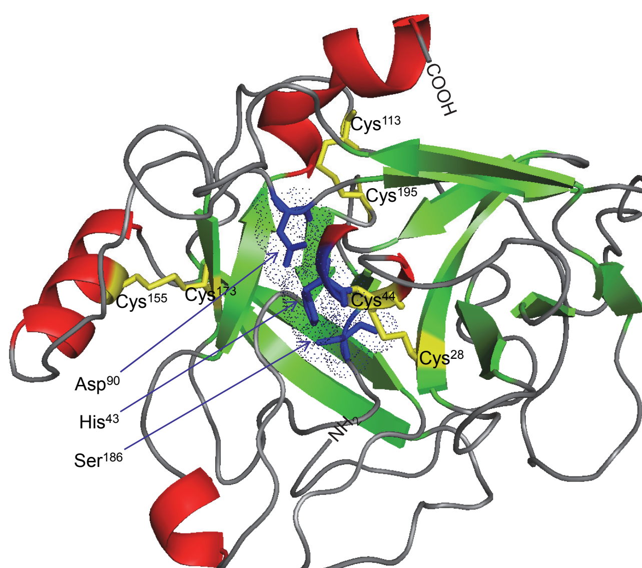


Fig. 44. The secondary and three dimensional structures of CTSP-1. (A) The secondary structure. Helices, sheets, and disulfide bonds are indicated with red column, green arrow, and yellow lines, respectively. (B) The three dimensional structure. α-helices and β-sheets are indicated in red and green patches. Turns and loops are indicated in silver lines. Active site residues (His^{43}, Asp^{90}, and Ser^{186}) are indicated as blue sticks. Disulfide bonds are indicated as yellow sticks.

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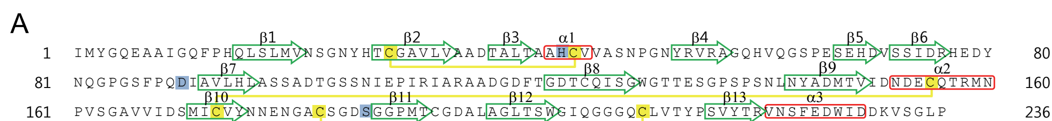
A



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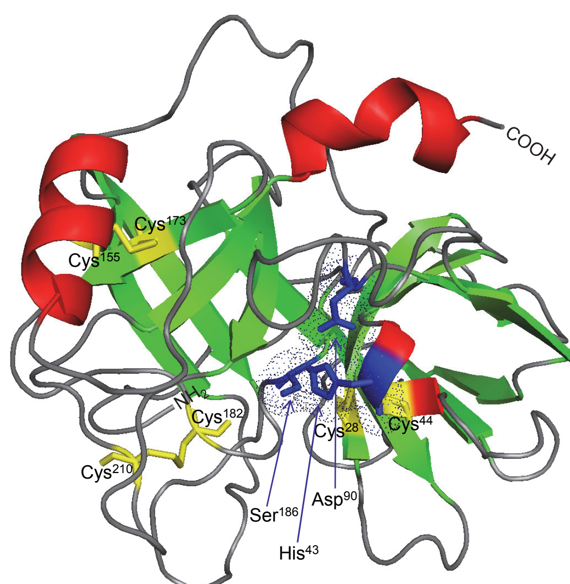

Fig. 45. The secondary and three dimensional structures of CTSP-2. (A) The secondary structure. Helices, sheets, and disulfide bonds are indicated with red column, green arrow, and yellow lines, respectively. (B) The three dimensional structure. α-helices and β-sheets are indicated in red and green patches. Turns and loops are indicated in silver lines. Active site residues (His[43], Asp[90], and Ser[186]) are indicated as blue sticks. Disulfide bonds are indicated as yellow sticks.

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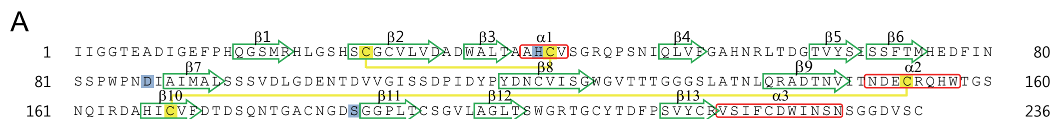
A



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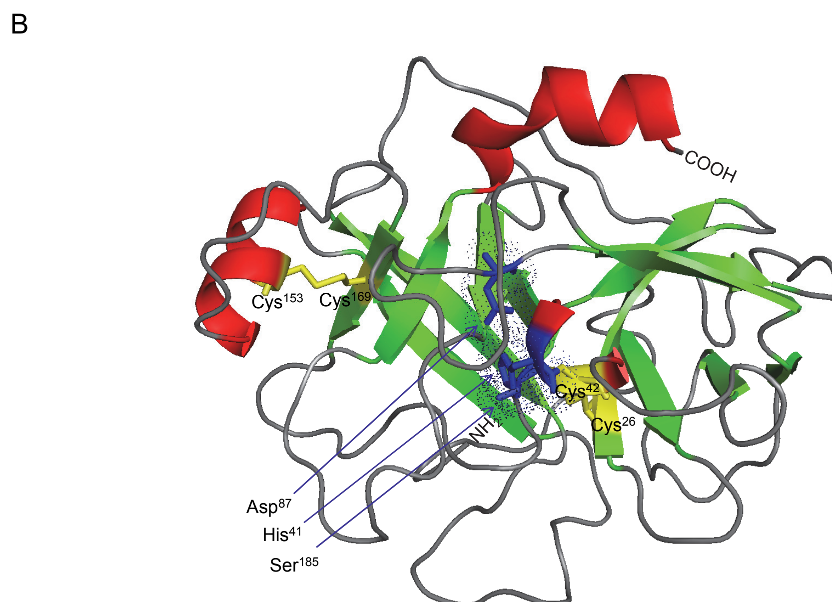

Fig. 46. The secondary and three dimensional structures of CTSP-3. (A) The secondary structure. Helices, sheets, and disulfide bonds are indicated with red column, green arrow, and yellow lines, respectively. (B) The three dimensional structure. α-helices and β-sheets are indicated in red and green patches. Turns and loops are indicated in silver lines. Active site residues (His^{41}, Asp^{87}, and Ser^{185}) are indicated as blue sticks. Disulfide bonds are indicated as yellow sticks.

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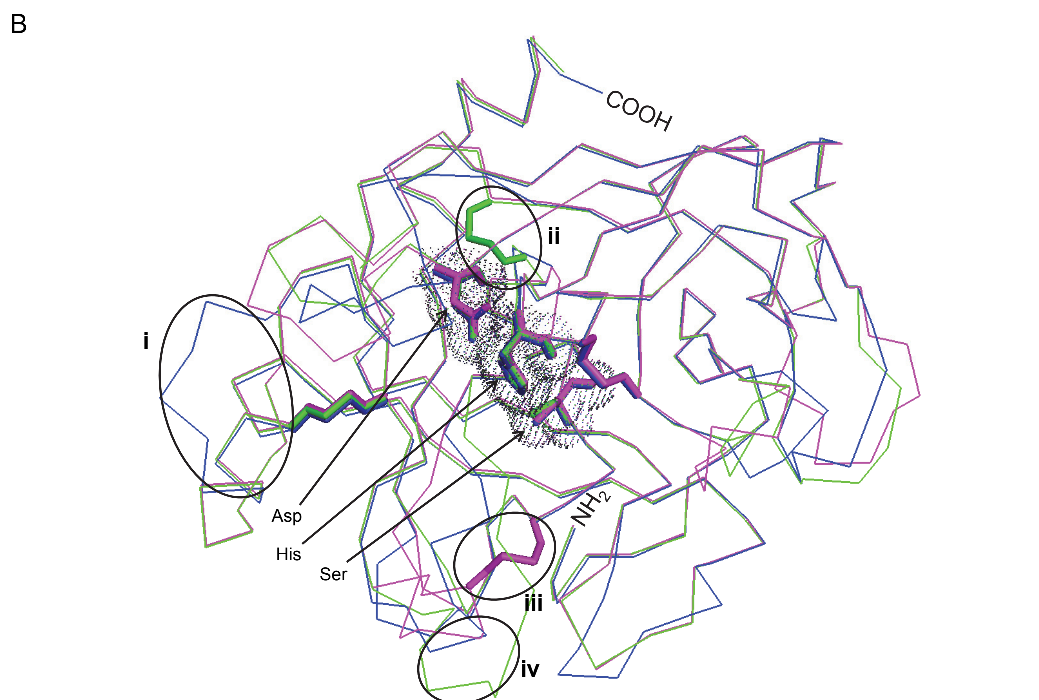

Fig. 47. Comparative models of CTSP enzymes. (A) Multiple sequence alignment and secondary structures of CTSP enzymes. Helices and sheets are indicated with red column and green arrow, respectively. (B) Backbone superposition of the three CTSP enzymes. Backbones of CTSP-1, -2, and -3 enzymes are colored as green, magenta, and blue, respectively; and the structural regions exhibiting relatively large conformational difference are labeled: (i) the loop region (residues 158-163) of CTSP-3; (ii) disulfide bond (Cys^{113}-Cys^{195}) in CTSP-1; (iii) disulfide bond (Cys^{182}-Cys^{210}) in CTSP-2; and (iv) the α-helix (residues 208-211) of CTSP-1. The residues of catalytic triad (Asp, His, and Ser) and disulfide bonds are rendered as stick models.

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Cys121, Cys181, Cys191, Cys206, Cys215, Cys221, and Cys234 are free (Fig. 44). The disulfide bond Cys26-Cys42 connected the N-terminus of β2-sheet and the α1-helix including a catalytic residue His41, whereas disulfide bond Cys153-Cys169 connected the α2-helix and β10-sheet. His^{41}, Asp^{87}, and Ser^{185} residues were constituted the active site where the breakage of peptide bonds in substrates might occur (Fig. 46). The catalytic triad residues (Asp, His, and Ser) are completely conserved among the CTSP enzymes as shown in the multiple sequence alignment and comparative model (Fig. 47). Despite the similarity between these structural models, relatively large conformational differences in local structures could be found upon the superposition of structural models (Fig. 47B). Close inspection of Fig. 47B revealed four regions exhibiting backbone conformational differences: i) the surface-exposed loop comprising residues 158-163 with a high percentage of polar residues located between α2-helix and β10-sheet of CTSP-3; ii) disulfide bond (Cys^{113}-Cys^{195}) in CTSP-1 enzyme; iii) disulfide bond (Cys^{182}-Cys^{210}) in CTSP-2 enzyme; iv) the surface-exposed α3-helix region (residues 208-211) located between β12- and β13-sheet of CTSP-1 enzyme. In addition, the little differences were observed merely in surface-exposed loop comprising a high percentage of polar residues.

III-2-4. Construction of recombinant expression vector pPICZαA-*CTSP-3*

The cloning of mature *CTSP-3* gene was achieved with PCR. The PCR product was digested with *Xho* I and *Xba* I and then ligated into *Xho* I/*Xba* I-cut pPICZαA vector to make the construct shown in Fig. 48A.

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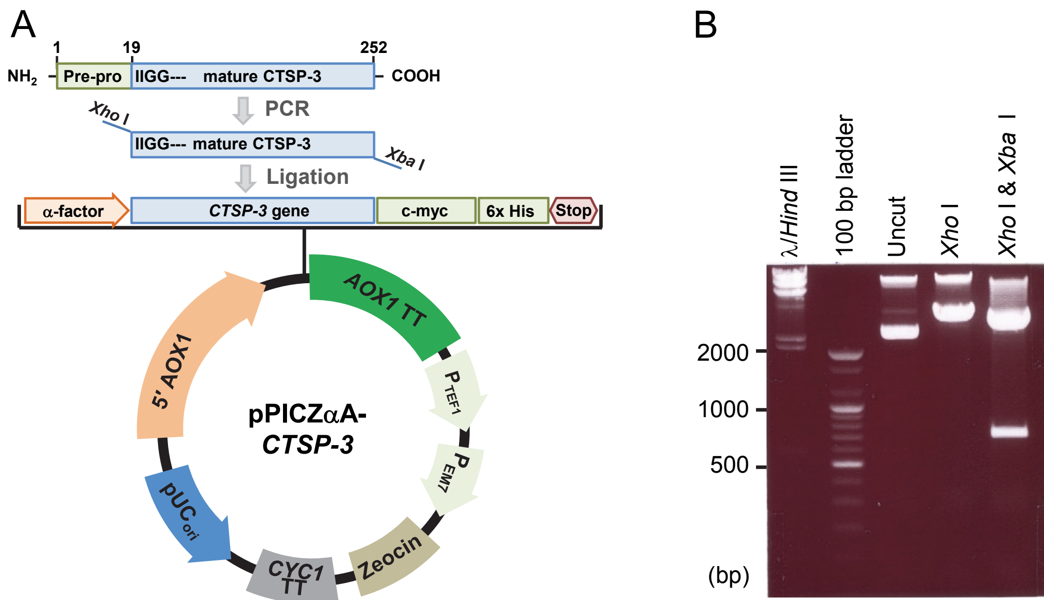

Fig. 48. Construction of a recombinant plasmid pPICZαA-*CTSP-3*. (A) Schematic diagram of the recombinant plasmid pPICZαA-*CTSP-3*. The gene was amplified by PCR from the pJET-*CTSP-3* construct and cloned into pPICZαA vector doubly digested with *Xho* I and *Xba* I restriction enzymes. (B) Agarose gel electrophoresis of recombinant plasmid pPICZαA-*CTSP-3* and its digests of *Xho* I and *Xba* I restriction enzymes. The recombinant plasmid harbors 705 bp insert DNA containing *CTSP-3* gene.

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The resulting 4.3 kb recombinant plasmid named pPICZαA-*CTSP-3* harbored *CTSP-3* gene. A 705 bp *Xho* I and *Xba* I fragment of the *CTSP-3* gene obtained by restriction enzyme digestion was cloned into recombinant vector pPICZαA-*CTSP-3* (Fig. 48). The nucleotide sequence of cloned *CTSP-3* gene was determined from which amino acid sequence was also deduced (Fig. 49). As shown in Fig. 49, pPICZαA-*CTSP-3* had one open reading frame composed of α-factor secretion signal peptide, CTSP-3 enzyme, C-terminal c-myc epitope, and poly-histidine tag. The pPICZαA-*CTSP-3* expression vector also had the zeocin resistance gene, bacterial pUC origin of replication for maintenance and propagation in *E. coli,* and *AOX 1* transcriptional regulatory element for recombinant protein expression in *P. pastoris*.

III-2-5. Expression of recombinant CTSP-3 enzyme in the *P. pastoris* expression system

The linearized plasmid pPICZαA-*CTSP-3* was transformed into *P. pastoris* X-33 and the expression level of CTSP-3 enzyme was analyzed. Moreover, several parameters were tested including media composition and time of induction. Recombinant CTSP-3 enzyme (named yrCTSP-3) was expressed in *P. pastoris* as an N-terminal fusion protein with α-factor (Fig. 49). This signal sequence is known as *Saccharomyces cerevisiae* mating ormone and it is responsible for the secretion of fusion protein across the cell membrane into the culture media. Therefore, the expressed yrCTSP-3 enzyme could be isolated from the culture media. Proteins from culture supernatant were subjected to TCA precipitation and analyzed by SDS-PAGE and Western blotting. As shown in Fig. 50, the time course for expression of yrCTSP-3 enzyme in *P. pastoris* X-33 was analyzed by immunoblotting

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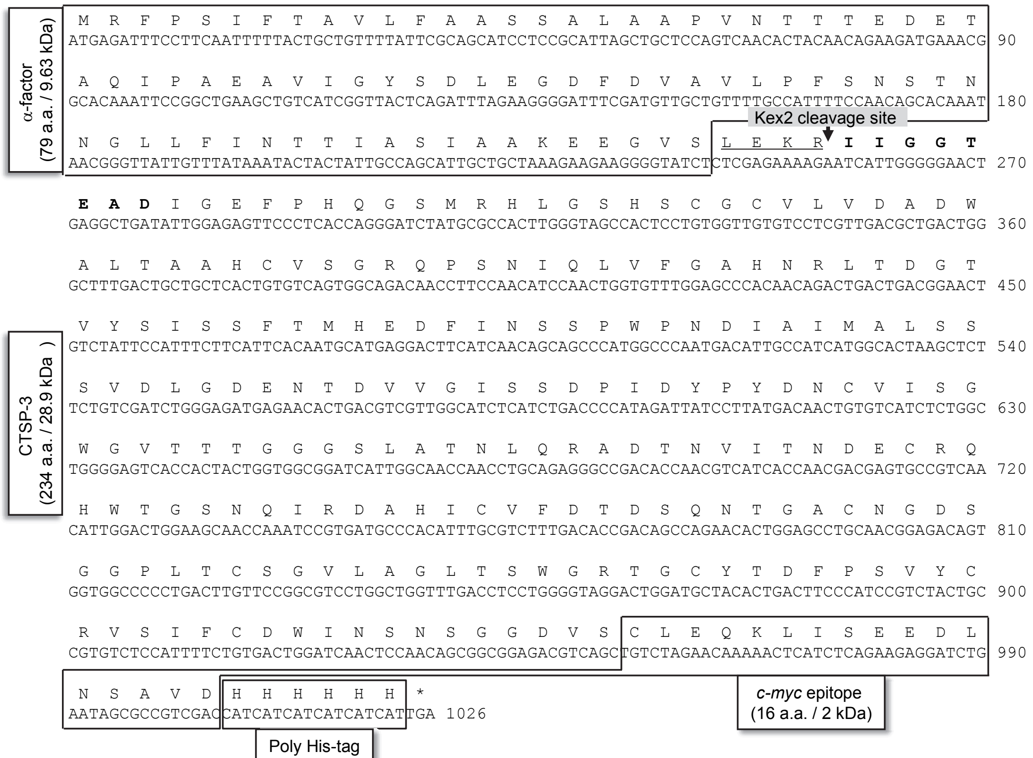

Fig. 49. The nucleotide sequence of pPICZaA-*CTSP-3* harboring *CTSP-3* gene and its deduced amino acid sequence. The N-terminal sequence of mature CTSP-3 enzyme was IIGGTEAD. The α-factor, *c-myc* epitope, and poly His-tag sequences are in boxes. The underlined residues are Kex2 protease cleavage site.

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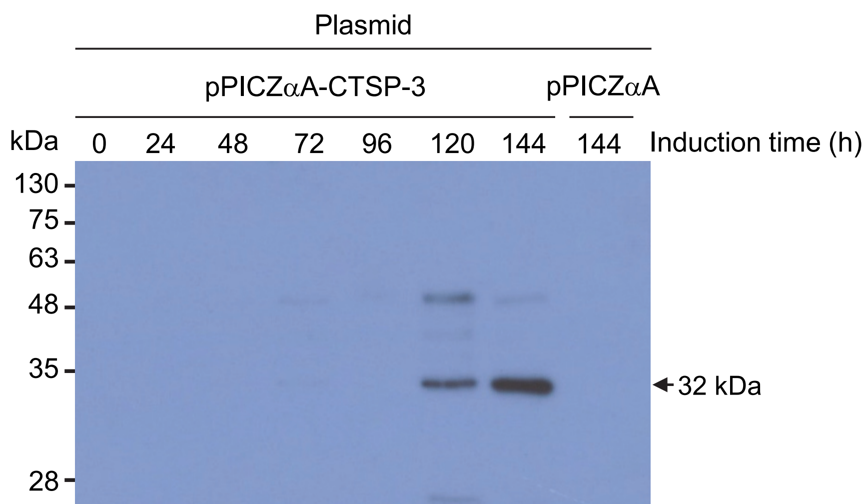

Fig. 50. Expression of recombinant CTSP-3 enzyme in *P. pastoris* X-33. The cells harboring pPICZαA-*CTSP-3* were induced by the addition of 0.5% methanol for the indicated time periods. Cells were sampled at different time points and centrifuged at 10,000 x*g* for 5 min and the 1 ml of supernatants were concentrated by TCA. The expressed proteins were subjected onto 12% SDS-PAGE and Western blotting was performed with anti-His tag antibody.

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with anti-His tag antibody. The secretion of yrCTSP-3 enzyme was detectable on 72 h and reached the maximal level after 144 h of induction. Immunoblot analyses of expressed yrCTSP-3 enzyme was revealed a major protein band with an molecular mass of approximately 32 kDa (Fig. 50). The estimate molecular weight of the fusion protein was approximately 41 kDa (α-fctor, 9.3 kDa; CTSP-3, 28.4 kDa; C-terminal tag, 2.5 kDa). The result indicates that the 9.3 kDa of α-factor signal sequence have been removed from N-terminus of the secreted yrCTSP-3 enzyme.

III-2-6. Isolation and purification of recombinant CTSP-3 enzyme

The secreted yrCTSP-3 enzyme in culture supernatant was initially precipitated using 70% $(NH_4)_2SO_4$, and then purified by one step of chromatography. The precipitated proteins were applied onto a HiTrap chelating affinity column (Fig. 51). The proteins bound on the column were eluted by a linear imidazole gradient of 0-0.3 M and the proteolytic activity of each fraction was assayed with S-2586 as a substrate. As shown Fig. 51, one peak from elution volumes 4-8 ml showed protease activity and the proteins were pooled as an active fraction. The proteins pooled were concentrated by ultrafiltration using an YM 10 membrane. Purified yrCTSP-3 enzyme appeared as a single band on an SDS-polyacrylamide gel stained with Coomassie brilliant blue and the molecular mass of purified yrCTSP-3 enzyme was estimated to be 32 kDa (Fig. 52A). However, as shown in Fig. 52B, western blot analysis of the eluted proteins showed several bands. These results suggest that major protein band correctly reflects the molecular mass of yrCTSP-3 enzyme and other smaller minor protein bands indicate degradation products of recombinant enzyme. The purification result of CTSP-3 enzyme is summarized in Table 9. The specific activity of purified

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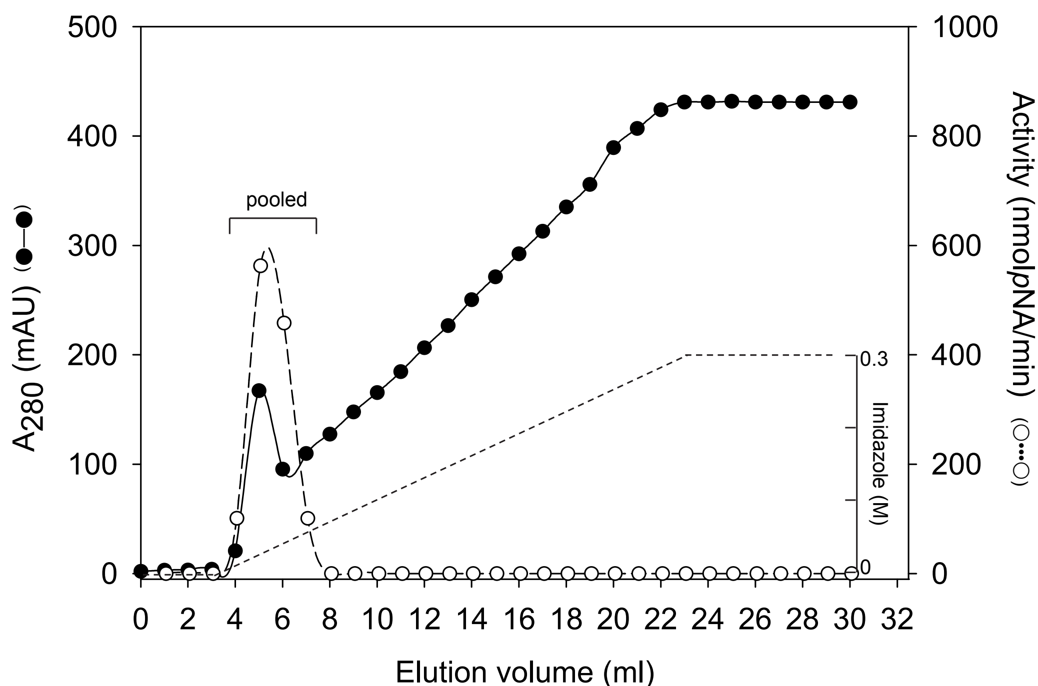

Fig. 51. Purification of recombinant CTSP-3 enzymes from *P. pastoris* X-33. Proteins concentrated with 0-70% ammonium sulfate were loaded onto an histidine affinity HiTrap chelating column and the proteins bound were eluted by a imidazole linear gradient of 0-0.3 M. Active fractions were pooled from elution volumes 4-8 ml as indicated. Protein elution was monitored by measuring the absorbance at 280 nm (●). The protease activity (○) was assayed with S-2586 as a substrate.

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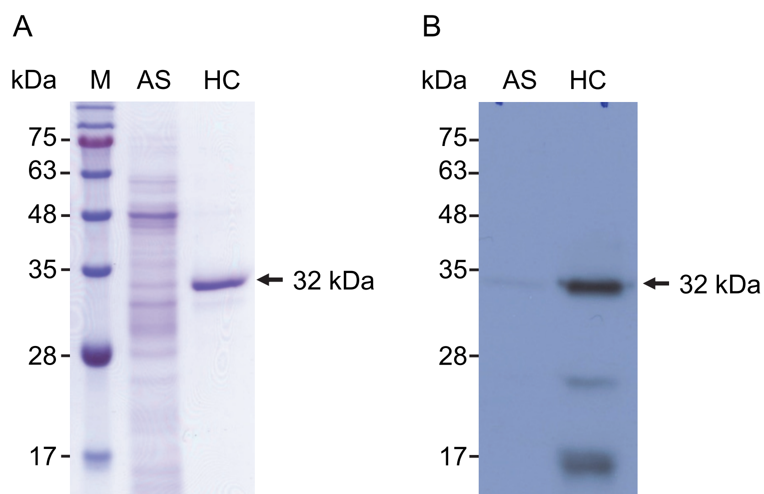

Fig. 52. SDS-PAGE and western blot analysis of the recombinant CTSP-3 from *P. pastoris* X-33. (A) Proteins collected from each purification step were electrophoresed on a 12% SDS polyacrylamide gel and stained with Coomassie brilliant blue to visualize. (B) Detection of C-terminal His-tag of recombinant CTSP-3 enzyme by Western blot. M, molecular mass marker; AS, ammonium sulfate precipitation (0 to 70%); HC, HiTrap chelating column chromatography.

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yrCTSP-3 enzyme was estimated to be 4.953 U/mg, and 0.04 mg of enzymes could be obtained from 11.85 mg of total cultured media proteins (Table 9).

III-2-7. Determination of the specific and fibrinolytic activity of purified recombinant CTSP-3 enzyme

The chromogenic substrate (S-2586) was used for the analysis of enzyme kinetics. As shown in Table 10, K_M value of yrCTSP-3 enzyme was estimated to be 0.225 mM. K_{cat} value of the same enzyme was found to be 7.133 sec^{-1}. In particular, K_{cat}/K_M value of yrCTSP-3 (Table 10) was 6.8-fold higher than that of native CTSP-3 (Table 6) when S-2586 was used as a substrate. These results suggest that yrCTSP-3 enzyme is more active than native CTSP-3 enzyme purified from *C. tentaculata*, as it has the highest turnover number and catalytic efficiency. The fibrinolytic activity of yrCTSP-3 enzyme was observed by fibrin plate assay (Fig. 53). When plasmin (0.04 U) and CTSP-3 enzymes (each 2 μg) were applied into the wells made in the fibrin plate and incubated for 6 h at 37°C, halo zones appeared clearly. In this assay, the diameter of halo zones formed by the treatments with plasmin, native CTSP-3, yrCTSP-3 enzymes were 2.2, 2.6, and 2.8 cm, respectively. Therefore, the adjusted plasmin units of native CTSP-3 and yrCTSP-3 enzymes were equivalent to 0.047 and 0.05 units, respectively. These results suggest that yrCTSP-3 enzyme shows similar fibrinolytic activity in the native CTSP-3 enzyme.

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Table 9. Summary of the purification of recombinant CTSP-3 enzyme from *P. pastoris* X-33.

Purification step	Total protein (mg)	Total activity (U)[a]	Specific activitya(U/mg)	Yield[b] (%)
Culture supernatant	11.85	ND[c]	ND[c]	ND[c]
70% $(NH_4)_2SO_4$	8.61	0.2359	0.027	100
HiTrap chelating	0.04	0.2006	4.953	85.03

[a] One unit (U) was defined as the amount of protease that catalyzes the release of 1 μmol of pNA/min when the chromogenic substrate (S-2586) was used as a substrate.

[b] The total activity of the 70% $(NH_4)_2SO_4$ was assigned the value of 100%.

[c] ND, not detectable.

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Table 10. Kinetic parameters of recombinant CTSP-3 enzyme.

Enzyme	K_M (mM)[a]	K_{cat} (sec^{-1})[a]	K_{cat}/K_M ($mM^{-1}sec^{-1}$)
yrCTSP-3	0.225 ± 0.0157	7.133 ± 1.460	31.489

[a] Kinetic constants were determined at pH 8.5 using S-2586 as a substrate. Data are shown as means ± SD (n = 4).

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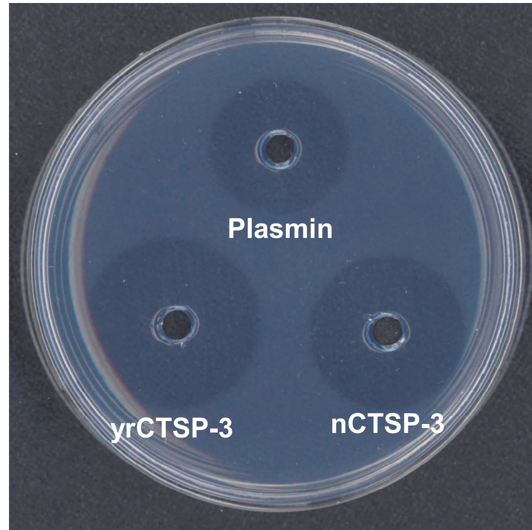

Fig. 53. Fibrinolytic activity of recombinant CTSP-3 enzyme. PBS, plasmin (0.04 U), and CTSP-3 enzymes (2 μg) were inoculated into the holes in the fibrin plate and incubated for 6 h at 37°C as indicated. yrCTSP-3, recombinant CTSP-3; nCTSP-3, native CTSP-3.

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IV. DISCUSSION

Blood homeostasis is achieved by maintaining a balance between the formation and degradation of fibrin. Abnormal thrombotic occlusions encompass a variety of cardiovascular diseases, such as myocardial infarction and acute stroke. Development of effective thrombolytic agents is progressing. However, drawbacks in the typical therapeutic agent are also being increasingly reported. (Mine *et al*., 2005; Toombs 2001). Plasminogen activators are commonly used for thrombolytic therapeutic agents, such as urokinase and streptokinase, and new classes of directly acting fibrinolytic enzymes have become available from earthworms (Hu *et al*., 2004; Nakajima *et al*., 2003). Nevertheless, few fibrinolytic enzymes have been reported from polychaete worms, such as *P. leucophryna* (Joo *et al*., 2001; Koo *et al*., 2010), *Neanthes japonica* (Wang *et al*., 2011), and *P. aibuhitensis* (Li *et al*., 2006).

This study describes purification and characterization of fibrinolytic enzymes, named CTSP-1, -2, and -3 from polychaete *C. tentaculata*. The final yields of CTSP-1, -2, and -3 were approximately 0.5%, 0.7%, and 1.9%, respectively (Table 4). Purified CTSP-1, -2, and -3 were found to be 28.8, 30.9, and 28.4 kDa, respectively (Fig. 13). These molecular weights are similar to other fibrinolytic enzymes such as 28 kDa protease from *Bacillus amyloliquefaciens* CB1 (Heo *et al*., 2013), 24.6 to 33.0 kDa lumbrokinase iso-enzymes from *Lumbricus rubellus* (Cho *et al*., 2004), 27.5 to 34.5 kDa protease from *Perionyx excavates* (Phan *et al*., 2011), and 28.6 to 33.5 kDa protease from *N. japonica* (Wang *et al*., 2011). The comparison of amino acid sequences revealed the presence in N-termini of all CTSP enzymes of the typical hepta-peptide (I-X-X-G-X-X-A) conserved in serine proteases from several annelid species, which include PLFP from *P. leucophryna* (Koo *et al*.,

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2010), fibrinolytic isozymes A and B from *L. rubellus* (Nakajima *et al*., 1993), and EFE-3 from *E. foetida* (Wang *et al*., 2003). These results suggest that the three CTSP iso-enzymes are not products of proteolytic cleavage during purification.

The results of substrate specificity analysis suggest that CTSP-1 and -2 hydrolyze the peptide bond at the side of an arginine, whereas CTSP-3 cleaves at the carboxyl side of tyrosine residue in their peptide substrates (Figs. 17-19). Furthermore, CTSP-1 and -2 efficiently hydrolyzed the substrate for urokinase (S-2444; pyro-Glu-Gly-Arg-*p*NA), while CTSP-3 efficiently hydrolyzed the substrate for chymotrypsin (S-2586; MeO-suc-Arg-Pro-Tyr-*p*NA) (Table 5). These results suggest that CTSP-1 and -2 might be potent urokinase-like proteases and probably exhibit fibrinolytic activity, whereas CTSP-3 might be a potent chymotrypsin-like protease.

In examination of the effects of various protease inhibitors on the proteolytic activities, three CTSP enzymes were found to be completely inhibited by DFP and PMSF. In addition, two enzymes CTSP-1 and -2 were strongly inhibited by TLCK and aprotinin, whereas CTSP-3 was diminished by TPCK (Table 7). The trypsin-like serine proteases are effectively inhibited by inhibitors such as TLCK and aprotinin, but not by TPCK. Chymotrypsin-like proteases, on the other hand, are inhibited by TPCK, but not by TLCK and aprotinin (Brabcová *et al*., 2012). Therefore, the results obtained with the inhibitors demonstrate that CTSP-1 and -2 enzymes are trypsin-like serine proteases, and CTSP-3 is a chymotrypsin-like serine protease (Dodia *et al*., 2008). The temperature-dependent experiments imply that the CTSP enzymes are stable up to 50°C (Gohel and Singh, 2012). It is generally conceded that the optimum pH and temperature for thermostable alkaline enzymes are between pH 9 and 11 and from 50-70℃, respectively (Fig. 20 and Fig. 21) (Jayakumar *et al*., 2012; Purohit and Singh, 2013; Simkhada *et al*., 2010).

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On this criterion, the purified CTSP enzymes can be categorized into thermostable alkaline serine proteases (Purohit and Singh, 2013; Thumar and Singh, 2009), although CTSP-3 showed its optimum activity at 50℃ under pH 8.5 (Fig. 20 and Fig. 21).

The fibrinogenolytic activity assay of CTSP enzymes (Figs. 23A, 24A, and 25A) showed that the CTSP enzymes were very active fibrinogenolytic enzymes, in that they can cleave even the γ chain of fibrinogen efficiently unlike other fibrinogenolytic enzymes (Swenson and Markland, 2005; Xiao *et al*., 2007). The γ-chains of fibrinogen form a triple-stranded α-helical coiled coil structure, which is often extremely resistant cleaving by most of fibrinogenolytic enzymes. Therefore, their abilities of efficiently cleaving the γ-chains of fibrinogen would have a great advantage for removing fibrin clots *in vivo*. In addition, the fibrinolytic activity assay showed that CTSP enzymes have stronger fibrinolytic activities than plasmin when they cleave the cross-linked fibrin. Most fibrinogenolytic enzymes cleave preferentially either the Aα or the Bβ chains of fibrinogen (Assakura *et al*., 1994; Swenson and Markland, 2005). For instance, α-fibrinogenases that are typical metalloproteases can hydrolyze only the Aα chain of fibrinogen, but not cleave the Bβ and γ chains (Huang *et al*., 1995; Xiao *et al*., 2007). In addition, α-fibrinogenases favorably hydrolyze the Bβ chain, with a delayed cleavage of the Aα and γ chains (Swenson and Markland, 2005). It is also known that most of fibrin(ogen)olytic serine proteases have a tendency to cleave the Bβ chain of fibrinogen, with a decrease in fibrinolytic activity towards the Aα chain (Markland, 1998). For example, fibrinogenolytic enzymes from *L. rubellus* can efficiently hydrolyze the Aα and Bβ chains, but not cleave the γ chain (Cho *et al*., 2004). Even plasmin does not efficiently cleave the γ chain, while it actively degrades the Aα and Bβ chains of fibrinogen (Siritapetawee *et al*., 2012). Thereby, CTSP enzymes are

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somehow unique and strong proteases, since they can efficiently cleave all chains of fibrinogen within 20 min at the enzymes versus fibrinogen mass ratio of 1:20 (Figs. 23A, 24A, and 25A). However, several enzymes including NJF from *N. japonica* (Deng *et al*., 2010), N-V from *Neanthes virens* (Sars) (Zhang *et al*., 2007), and CSP from *Cordyceps sinensis* (Li *et al*., 2007) exhibit similar activities in cleaving the chains of fibrinogen with much lower hydrolyzing activities. The cross-linked α-α and γ-γ chains formed from fibrin polymers by FXIIIa are resistant to plasmin degradation because of their stable and insoluble structures (Mosesson, 1992; Mosesson, 2005). However, CTSP enzymes can actively digest the fibrin polymers as judged by turbidity assay (Fig. 26) and also cleave the α-α and the γ-γ chains of the cross-linked fibrins formed in the presence of FXIIIa, as shown by fibrin plate assay (Figs. 23B, 24B, and 25B) and cleavage assay on SDS-polyacrylamide (Fig. 27).

Human blood plasma contains a large amount of proteins (normally 60-85 mg per ml) (Anderson and Anderson, 1977). These include albumin (~55%), globulin (~38%), fibrinogen (~7%), and (pro)enzymes and protease inhibitors (less than 1% each) (Adkins *et al*., 2002; Anderson and Anderson, 1977). The plasma also holds inorganic materials such as Na^+, Cl^-, K^+, and Mg^{2+}. Therefore, it is generally assumed that the fibrinolytic activities of CTSP enzymes can be greatly affected by the components of blood plasma. According to Jensen (Jensen and Sottrup-Jensen, 1986), α_2-macroglobulin is present at high concentration (approximately 2.0 μM) in blood plasma and inhibited almost all four classes protease; cysteine, serine, aspartate, and metallo (Galliano *et al*., 2006). Furthermore, to study the effect of α_2-macroglobulin on earthworm fibrinolytic enzyme III-1 (EFE-III-1) from *L. rubellus,* the activity of EFE-III-1 is decreased to 65% when incubated with a α_2-macroglobulin (Wu *et al*., 2002). Interestingly, proteolytic cleavage assay of

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CTSP enzymes to various plasma proteins showed that α_2-macroglobulin was cleaved by CTSP-1, -2, and -3 enzymes within 20 min (Figs. 14-16). However, effects of serum albumin and blood plasma on the enzyme activities showed that the protease activities of plasmin and CTSP enzymes can be greatly reduced by plasma proteins (Fig. 28). It has been shown that plasma contains anti-protease components, not only α_2-macroglobulin, but also anti-thrombin III, α_2-anti-plasmin, and act as endogenous inhibitors (Adkins *et al*., 2002). However, even with the decreased proteolytic activities, the three CTSP enzymes could actively cleave the fibrin clot in plasma as shown by turbidimetric lysis assay (Fig. 29). In other words, CTSP enzymes directly hydrolyze the fibrinogen, fibrin, and other plasma proteins *in vitro.* On the other hand, CTSP enzyme's proteolytic activities were greatly decreased in the plasma milieu, while fibrinolytic activity was retained. These characteristic of CTSP enzymes give an important advantage in pharmaceutical application. The results of this study suggest that CTSP enzymes could decrease risk of induction of hemorrhage on the treatment of thrombus. Therefore, it is necessary to further investigate the inhibition mechanism of CTSP enzymes by the plasma proteins and the molecular mechanism for its thrombolytic effect *in vivo*.

In addition, this study also investigate the isolation of *CTSP* genes from *C. tentaculata* and the construction of recombinant CTSP-3 enzyme expression systems. The successful expression and purification of recombinant CTSP-3 enzyme would provide stable enzymes for further studies. The *C. tentaculata* produces three alkaline fibrinolytic serine proteases. The identification of *CTSP* genes was achieved with 3´-RACE using three gene specific degenerate primers (GSP1-1, -2, and -3) and two universal degenerate primers (His-F and Ser-R) derived from the amino acid sequencing results of CTSP enzymes. In the nucleotide sequence analysis, 3´-RACE PCR products

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were found to be 828, 829, and 820 bp cDNA fragments, respectively (see Figs. 36A, 37A, and 38A, respectively). To obtain the complete *CTSP* gene of the cDNAs, the primers for 5´-RACE PCR were designed based on the nucleotide sequences that show a difference between the three 3´-RACE PCR products. Consequently, 111, 33, and 101 bp cDNA fragments were obtained by nucleotide sequence analysis of 5´-RACE PCR products, (see Figs. 36B, 37B, and 38B, respectively). The complete cDNA nucleotide sequences confirm by 5´ and 3´ sequencing primer were designed based on the end of 3´ and 5´-RACE products, respectively. Nucleotide sequence analysis showed that the CTSP-1, -2, and -3 contained 804, 738, and 756 bp open reading frame (ORF) encoding 267, 246, and 252 amino acids, respectively (Figs. 39-41). The deduced amino acid sequences of CTSP enzymes contained N-terminal pro-peptides of 32, 10, and 18 amino acids, respectively, as was predicted by the N-terminal sequencing results of purified CTSP enzymes. However, the identified *CTSP-2* gene did not have a start codon (ATG) and initial methionine (Met) (Fig. 40). Deduced amino acid sequence comparison showed that N-terminus sequence of the CTSP-2 enzyme was shorter than those of CTSP-1 and -3 enzymes (Fig. 42). These results indicate that the identified *CTSP-2* gene is a partial sequence. After removing the pro-peptide, the mature CTSP-1, -2, and -3 enzymes were composed of 236, 236, and 234 amino acids with a predicted molecular weight of 24.43 (pI 5.24), 24.70 (pI 4.28), and 24.78 kDa (pI 4.33), respectively, as calculated by the ProtParam tool (http://web.expasy.org/protparam/). The amino acid sequence comparison showed that there were putative active-site serine and histidine residues within consensus motives, [DNSTAGC]-[GSTAPIMVQH]-x(2)-G-[DE]-S-G-[GS]-[SAPHV]-[LIVMFYWH]-[LIVMFYSTANQH] and [LIVM]-[ST]-A-[STAG]-H-C that are frequently found in the trypsin-like serine protease family (Brenner, 1988).

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Furthermore, the deduced amino acid sequences of CTSP enzymes showed 39-73% similarity. In addition, they showed less than 45% homology compared to those of serine proteases from annelid at the level of amino acid sequence. These results demonstrate that CTSP enzymes are novel fibrinolytic serine proteases.

Expression of recombinant CTSP enzymes had been tried in bacteria. The CTSP enzymes were expressed in *E. coli,* however, the enzyme products were not secreted and remained as inclusion bodies in cytoplasm (data not shown). Therefore, in this study *P. pastoris* was used for expression of CTSP-3 enzyme. Recombinant protein production in yeast has many advantages, such as ease of handling, rapid growth, and high efficiency transformation (Hong *et al*., 2007). In addition, protein expression in *P. pastoris* has been a subject of much research, and many proteins have been successfully produced (Daly and Hearn, 2005; Macauley-Patrick *et al*., 2005; Cregg *et al*., 2009). A 705 bp of mature CTSP-3 enzyme DNA fragment was amplified using the pJET-*CTSP-3* construct as a template. The fragment was ligated into yeast expression vector pPICZαA and fused with *Saccharomyces cerevisiae* α-factor secretion signal peptide, which directs the secretion of expressed CTSP-3 enzyme from the cell. The C-terminal of expressed recombinant CTSP-3 contained c-myc peptide and 6x His tags. Recombinant vector pPICZαA-*CTSP-3* harboring *CTSP-3* gene was successfully expressed in *P. pastoris* X-33 (Fig. 50).

The western blot analysis data following methanol-induced expression suggest that the α-factor sequence efficiently secreted the recombinant CTSP-3 enzyme into the culture media and that signal peptide was processed by KEX2 protease in the *P. pastoris* secretory pathway (Cereghino and Cregg, 2000), and expressed enzyme containing a His tag at the C-terminus. The purification process involves two steps, ammonium sulfate

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precipitation and His tag affinity chromatography. The catalytic efficiency of the purified recombinant CTSP-3 enzyme (named yrCTSP-3) against the chromogenic substrate S-2586 turned out to be six-fold higher than that of CTSP-3 enzyme purified from *C. tentaculata* (Table 6 and 10). Furthermore, the fibrinolytic activity of yrCTSP-3 enzyme was observed by fibrin plate assay (Fig. 53). However, purification result indicate that expression levels of yrCTSP-3 enzyme in *P. pastoris* to be low. This may be due to codon usage difference in the *C. tentaculata* (Outchkourov *et al*., 2002), low copy number of the gene (Vasileva *et al*., 2001), and not-established expression conditions, such as media pH, induction temperature, methanol concentration, and expression scale. In this regard, it is worth mentioning that *P. pastoris* can increase protein production 30-fold by scale up from flask to fermentator (Cregg *et al*., 2000; Cereghino and Cregg, 2000; Clare *et al*., 1991). Moreover, differences in codon usage preference among organisms lead to a variety of problems concerning heterologous gene expression; however, these can be overcome by site-directed mutagenesis and gene synthesis (Bennetzen and Hall, 1982; Ikemura, 1985).

The rare codon analysis was performed using the OptimumGene™ algorithm (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis) to examine the number of highest- and lowest-usage codons in the various organisms (Table 11). As a result, Codon Adaptation Index (CAI) (Sharp and Li, 1987) of CTSP-3 was found to be 0.69 in the *P. pastoris*. The lower CAI (<0.8) suggested that CTSP-3 will be expressed in low level. Rare codons were analysed more concretely using a Graphical codon usage analyser (http://gcua.schoedl.de/index.html) (Fig. 54). As shown in Fig.54, the relative codon adaptiveness was plotted to usage of each codon in the *P. Pastoris*. Relative adaptiveness of codon usage with a value of 100 indicates that the codons are highly used for a given amino acid. Conversely, a value of less

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Table 11. Comparison of the codon usage of *CTSP* genes in various host organisms.

Host		*E. Coli*		*P. pastoris*		Human		Mouse		Insect	
Parameter / Enzyme	GC (%)[a]	CAI[b]	<30 CFD (%)[c]	CAI[b]	<30 CFD (%)[c]	CAI[b]	<30 CFD (%)[c]	CAI[b]	<30 CFD (%)[c]	CAI[b]	<30 CFD (%)[c]
CTSP-1	58.4	0.69	6	0.65	1	0.82	0	0.84	0	0.88	1
CTSP-2	55.9	0.68	5	0.68	3	0.78	0	0.80	0	0.86	1
CTSP-3	54.3	0.67	5	0.69	1	0.80	0	0.82	0	0.86	1

[a] Average GC content. The ideal percentage range of GC content is between 30% to 70%
[b] Codon adaptation index. Possibility of high protein expression level is correlated to the value of CAI. A CAI of 1.0 is considered to be ideal while a CAI of >0.8 is rated as good for expression in the desired expression organism.
[c] Codon frequency distribution. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. Codons with values lower than 30 are likely to hamper the expression efficiency.

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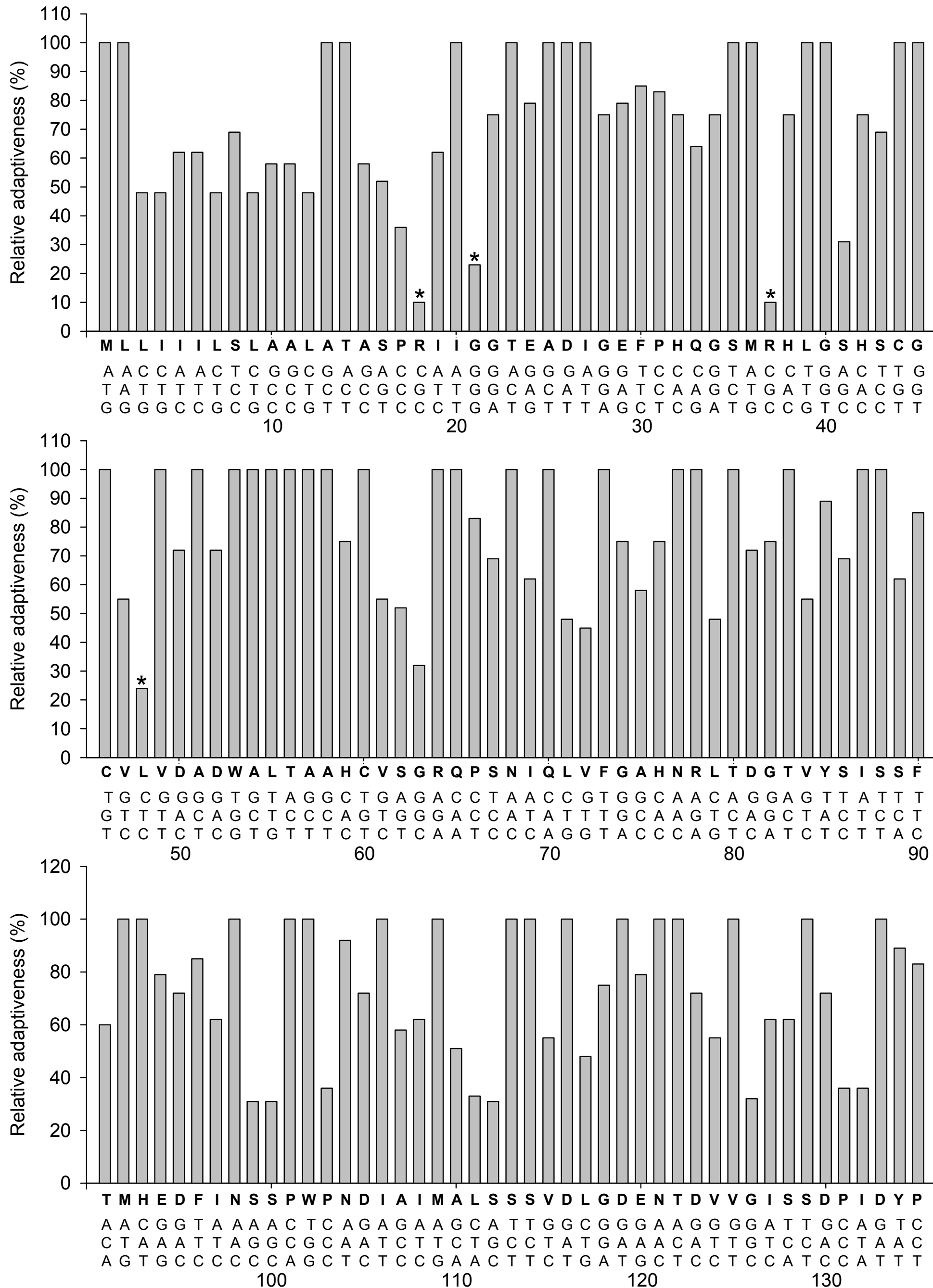

Fig. 54. The distribution of relative codon adaptiveness of each codon in the *P. pastoris.* Asterisks represent rare codon (CFD<30) in the *P. pastoris*.

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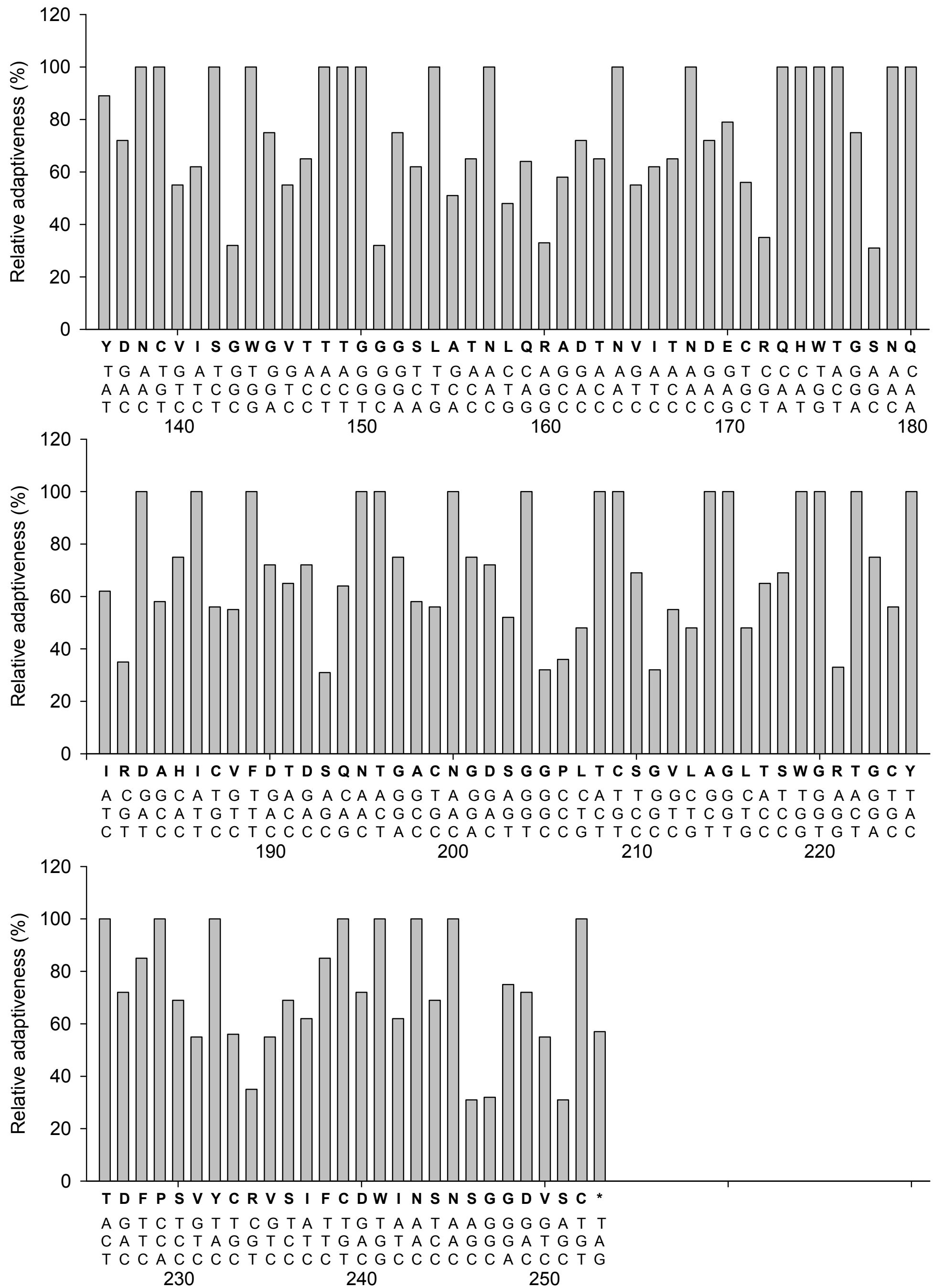

Fig. 54. Continued.

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than 30 indicates few used codons, which is likely to negatively affect the expression efficiency. Very low-frequency codons in the *P. pastoris* as CGC, GGG, and CTC were 10%, 23%, and 24%, respectively. This result suggested that the *CTSP-3* gene contain a significantly small number of rare codons that may reduce translational efficiency of the gene. Therefore, the expression levels of CTSP-3 enzyme in *P. pastoris* can be improved through a codon harmonization approach, which will enable efficient mass production.

In summary, fibrinolytic enzymes, named CTSP-1, -2, and -3 were purified from *C. tentaculata,* as they migrated as single bands with molecular masses of approximately 28.8, 30.9, and 28.4 kDa, which were composed of 236, 236, and 234 amino acids, respectively. CTSP-1 and -2 enzymes are trypsin-like alkaline serine proteases and hydrolyze the peptide bond at the side of arginine. On the other hands, the CTSP-3 enzyme is a chymotrypsin-like alkaline serine protease and cleaves at the carboxyl side of tyrosine residue in the peptide. Furthermore, CTSP enzymes are very active fibrino(geno)lytic enzymes, in that they can cleave the all chain (Aα, Bβ, and γ) of fibrinogen. In addition, they can actively digest the fibrin polymers and cross-linked fibrin (α-α and γ-γ chains). The activities of CTSP enzymes could be greatly reduced in plasma milieu because of endogenous inhibitors. However, even with their decreased proteolytic activities, the CTSP enzymes could cleave actively the fibrin clot in human plasma. This is a very important characteristic and suggests that CTSP enzymes may minimize the potential for hemorrhage on the treatment of thrombus. The deduced amino acid sequences of CTSP enzymes showed that there was 39 to 73% similarity between them. In addition, they showed less than 45% homology compared to those of serine proteases from annelid at the level of amino acid sequence. These results indicate that CTSP enzymes are novel fibrinolytic serine proteases. Using a yeast *P. pastoris* expression system,

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yrCTSP-3 enzyme could be successfully produced as a soluble and active enzyme, exhibiting a similar fibrinolytic activity and enzymatic properties to its native enzyme.

Taken together, the results obtained by the present study demonstrate that the three CTSP enzymes have potential as becoming therapeutic agents for thrombus dissolution. However, the optimization of growth conditions and yrCTSP-3 enzyme production in *P. pastoris* requires further study. In addition, the molecular mechanism for its thrombolytic effect *in vivo,* and inhibition mechanism of CTSP enzymes by the plasma proteins require further investigation.

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V. 적 요

갯지렁이 유래 세린계열 단백질분해효소의 혈전분해 특성 및 발현

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생명과학과

혈액의 응고와 항상성 유지에는 다양한 종류의 단백질분해효소들이 관여한다. 혈액응고 반응은 FXa, FIXa 및 트롬빈과 같은 다양한 세린계열 단백질분해효소에 의해 유도되며, 이들은 전구체 형태로 혈액 내에 존재한다. FXa에 의해 잘려 프로트롬빈으로부터 활성화된 트롬빈은 피브리노겐을 가수분해하여 피브린을 만든다. 이렇게 형성된 피브린은 혈소판 및 혈구세포들과 응집하여 혈전을 형성한다. 그러나 이 혈전은 또 다른 세린계열 단백질분해효소인 플라스민에 의해 분해되어 제거된다. 따라서 혈액의 항상성은 응고계 및 분해계의 균형으로 유지될 수 있다. 그러나 혈액의 항상성이 파괴되어 순환계 내에 혈전이 축적되면 심근경색 및 뇌졸증과 같은 치명적인 혈관 폐쇄성 질환들이 유발된다. 현재 임상에서는 혈전의 제거 및 치료에 uPA(urokinase-type plasminogen activator) 또는 tPA(tissue-type plasminogen activator)와 같은 플라스미노겐을 플라스민으로 활성화시키는 간접작용 효소를 사용하고 있다. 그러나 이들 효소의 사용은 과도한 출혈 및 알레르기의 발생 등과 같은 부작용을 유발할 뿐만 아니라 치료비용도 매우 크다는 단점을 지니고 있다. 따라서 다양한 생물소재로부터 선택적이고

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직접적으로 혈전을 분해할 수 있는 효소를 개발하는 연구는 매우 중요한 의미를 지닌다. 본 연구에서는 국내 서·남해안에 서식하는 다모류중 하나인 명주실타래갯지렁이(*Cirriformia tentaculata*)로부터 CTSP(*C. tentaculata* serine protease)-1, -2 및 -3로 명명한 3종의 알칼리성 세린계열 단백질분해효소를 정제하여 효소활성 등을 포함한 효소의 생화학적 특성을 규명하였으며, 분자생물학wjr 기법을 이용하여 이들을 암호화하는 유전자들을 클로닝하여 그 구조를 분석하였다. 또한 효모세포에서 CTSP-3 효소를 발현시키는 유전자 발현 연구도 수행하였다. 효소의 분리 및 정제와 관련한 연구의 주요 결과는 다음과 같다. (1) 정제한 CTSP-1, -2 및 -3의 분자량은 각각 28.8, 30.9, 28.4 kDa이었으며, 최적 pH와 온도범위는 8.5~9.0 및 50~60℃였다. (2) 3종의 CTSP는 모두, PMSF(phenylmethylsulfonyl fluoride) 및 DFP(diisopropyl fluorophosphate) 와 같은 세린계열 단백질분해효소 저해제에 의해서 효소 활성이 완전히 억제되었으나, 1,10-PT(1,10-phenanthroline) 및 bestatin과 같은 금속성단백질분해효소 저해제에 의해서는 거의 영향을 받지 않았다. 이러한 결과는 이 3종의 CTSP 효소는 모두, 열에 안정한 알칼리성 세린계열 단백질분해효소임을 시사하는 것이다. (3) 발색성 펩티드 기질을 이용한 기질 절단자리 분석을 통하여 CTSP-1과 CTSP-2는 아르기닌 잔기의 카르복시 말단을, CTSP-3는 타이로신 잔기의 카르복시 말단을 절단함을 확인하였다. (4) 아미노말단 서열분석 결과, CTSP-1은 ‘Ile-Met-Asn-Gly-Ser-Pro-Ala-Ala’, CTSP-2는 ‘Ile-Met-Tyr-Gly-Gln-Glu-Ala-Ala’, CTSP-3는 Ile-Ile-Gly-Gly-Thr-Glu-Ala-Asp’서열을 지님을 확인하였다. 또한 이들 단백질분해효소는 아미노 말단에 ‘I-X-X-G-X-X-A’의 공통서열을 가지고 있음도 확인하였다. (5) 이 3종의 CTSP 효소들은 피브리노겐의 Aα, Bβ, γ 사슬을 모두 20분 안에 절단하였고, 교차연결 피브린도 잘 분해한다는 사실을 확인하였다. 또한 이 효소들은 혈장에서 그 효소활성이 억제되었지만 혈장 내의 교차연결 피브린 섬유도 효과적으로 분해할 수 있는 효소능을 발휘하였다. 이와 같은 결과는 CTSP들이 피브린의 Aα 또는 Bβ만을 가수분해할 수 있는 여타의 혈전분해 효소들과는

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달리 혈전분해에 매우 효과적인 효소임을 시사하는 것이다. 본 연구는 또한 CTSP-1, -2 및 -3의 cDNA 유전자를 RACE(Rapid amplification of cDNA end)-PCR(polymerase chain reaction) 방법을 이용하여 분리한 후, 발현벡터에 클로닝하여 재조합단백질을 얻는데도 성공하였다. 이로부터 얻은 연구결과는 다음과 같다. (1) 염기서열을 분석한 결과, CTSP-1, -2, 및 -3 cDNA 유전자는 각각 804 bp, 738 bp, 756 bp로 이루어져 있으며, 각각 268, 246, 252개의 아미노산을 암호화할 수 있는 열린읽기틀(open reading frame; ORF)을 지니고 있었다. (2) ORF로부터 유추한 아미노산 서열을 비교분석한 결과, CTSP-1과 -2, CTSP-1과 -3 및 CTSP-2와 -3 사이에는 각각 73.3%, 39.9% 및 44.2%의 상동성을 지니고 있었다. 또한 이 효소들은 모두, 세린계열 단백질분해효소에 진화적으로 보존되어 있는 '세린-아스파르트산-히스티딘'으로 이루어진 촉매 아미노산 잔기 삼조(catalytic triad)를 가지고 있음도 확인하였다. (3) 효모세포에서 재조합 CTSP-3 효소를 발현시키기 위해 *CTSP-3* cDNA 유전자를 pPICZaA 벡터(메탄올에 의해 단백질 발현이 유도되며, 효모의 α-인자 신호서열을 가지고 있어 발현된 재조합단백질을 세포 밖으로 배출할 수 있음)에 클로닝하여 재조합 플라스미드 pPICZaA-*CTSP-3*를 재조하였다. 이 재조합 플라스미드를 효모의 일종인 *Pichia pastoris* X-33 세포에 형질전환시킨 후, 메탄올을 처리하여 재조합단백질의 발현을 유도한 결과, 32 kDa 크기를 지닌 CTSP-3 재조합효소(yrCTSP-3로 명명)가 배양액에 발현되었으며, 이를 Western blotting과 피브린 평판법을 통하여 확인하였다. (4) 형질전환 효모세포의 배양액에 함유되어 있는 총 11.85 mg의 단백질을 0-70% ammonium sulfate 침전으로 얻은 후, His-tag 친화성 크로마토그래피를 수행한 결과, 85.03%의 수율로 약 40 μg의 yrCTSP-3 효소를 순수분리 및 정제할 수 있었다. (5) CTSP-3의 발색기질인 S-2586을 사용하여 yrCTSP-3의 K_M, K_{cat}, and K_{cat}/K_M값을 측정한 결과, 각각 0.225 mM, 7.13 sec^{-1}, and 31.49 $mM^{-1}sec^{-1}$임을 확인하였다. (6) 피브린 평판법을 통하여 yrCTSP-3의 피브린분해 활성을 측정한 결과, 이 재조합효소가 명주실타래갯지렁이의 생체에서 분리한 CTSP-3에 상응하는 활성을 지님을 확인하였다. 이상의 결과는 생체 CTSP-3

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효소와 동일한 활성과 역가를 지닌 재조합 CTSP-3 효소를 효모세포에서 발현시켜 얻을 수 있음을 의미한다. 결론적으로, 본 연구에서 얻은 결과들은 생체 적용성 연구가 추가적으로 수행된다면 CTSP 효소들을 직접성 혈전분해제로 개발할 수 있는 가능성을 보여준다.

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감사의 글

어느덧 실험실 생활을 시작한지 10년째에 접어들었습니다. 정신없이 지나가버린 시간과 과연 내가 최선을 다했나 하는 생각에 아쉬움이 남지만, 많은 분들의 격려와 도움을 통해 학위논문을 완성하게 되어 감사의 뜻을 전하고자 합니다.

우선 9년이 넘는 긴 시간동안 저를 지도해주시고 부족한 제자 가르치시느라 스트레스 받으시고 또 고생해주신 이정섭교수님께 깊은 감사와 더불어 죄송하다는 말씀을 드리고 싶습니다. 그리고 갯지렁이 연구를 할 수 있도록 도와주시고 심사를 맡아주신 윤성명 교수님, 바쁘신 와중에 흔쾌히 심사를 맡아주시고 또 멀리까지 오셔 많은 조언해주신 이원태 교수님, 박종군 교수님, 그동안 많은 가르침을 주시고 심사까지 맡아주신 전홍성 교수님과 생명과학과 김성준 교수님, 정현숙 교수님, 박윤경 교수님께도 감사드립니다.

실험실 생활을 시작하면서부터 계속 함께한 정은누나와 좋은 말씀 많이 해주신 태원이형과 홍석이형, 더운 여름이나 추운 겨울 할 것 없이 갯지렁이 채집을 해주셔 실험을 할 수 있게 도와주신 현기형과 종국이 에게도 감사의 뜻을 전하고 싶습니다. 그밖에 다 언급하기 힘들 정도로 도움을 주신 많은 분들과 친구들 그리고 실험실 후배들에게도 감사 뜻을 전합니다.

마지막으로 아끼고 사랑하는 우리 가족들.. 힘들게 일 하시면서도 막내아들 공부를 위해 지원을 아끼시지 않는 아빠, 엄마, 항상 나이 먹은 동생 챙기느라 고생이 많은 형과 누나.. 가족들에게 부담주면서 까지 내 욕심만 챙기고 있는 것 같아 죄송스럽고 또 너무 감사합니다.

논문을 준비하면서 다시 한 번 제 자신의 부족함을 느끼고 반성할 수 있는 계기가 되었습니다. 아쉬웠던 점들을 마음속 깊이 세기고 이를 바탕으로 부족함을 채우고 더 나은 모습으로 성장하기 위해 노력하겠습니다. 그동안 지켜봐주신 모든 분들께 다시 한 번 감사의 뜻을 전하며, 앞으로도 많은 관심 부탁드립니다.

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저작물 이용 허락서

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논문제목	한글 : 갯지렁이 유래 세린계열 단백질분해효소의 혈전분해 특성 및 유전자 발현				
	영문 : Biochemical and molecular characterization of fibrinolytic serine proteases from a marine polychaete *Cirriformia tentaculata*				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 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 편집과 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 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의(0) 반대( )

2014 년 08 월 25 일

저작자: 박 종 우 　(인)

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