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Identification of a new serine protease from polychaeta, *Marphysa sanguinea*, for its thrombolytic and anticoagulant activity

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Abstract−A serine protease was purified from *Marphysa sanguinea* through ammonium sulfate followed by ion exchange chromatography, and its N-terminal amino sequence was identified to be IVGGSEATPYQFPFQ. Fibrinolytic activity was depended on both direct fibrinolysis and indirect plasminogen-mediated cascade and had a consistent activity irrespective of pH. The serine protease could be confirmed to degrade α-, β-, and γ-chains of human fibrinogen through fibrinogenolytic assay and did not express significant cytotoxicity to endothelial cells. These imply the enzyme has anticoagulant as well as thrombolytic activity, not significantly impairing endothelial cells comprising brain blood brain barrier (BBB) tissue. Conclusively, the new serine protease is worthy of being a candidate to substitute tissue-Plasminogen Activator (t-PA) for acute ischemic reperfusion injury of brain.

Keywords: Serine Protease, *Marphysa sanguinea*, Brain Ischemia, Thrombolysis, Anticoagulant

INTRODUCTION

Circulatory diseases, including ischemic stroke, myocardial infarction, deep-vein thrombosis, pulmonary embolism, are known to mainly occur by blocking blood flow by thrombus in the blood vessel [1]. Among them acute ischemic stroke, more than 80% of the strokes and second leading cause of death in the world, need a quick reperfusion of the blood flow which is blocked by fibrin clots [2,3]. To date, recombinant tissue-type plasminogen activator (rt-PA) is the only thrombolytic agent commercially approved by the FDA. However, in clinical practice, a short time window, potential neurotoxicity, and hemorrhage are causes of tissue-type plasminogen activator (t-PA) failure [4-6], along with its short half-life [7-9] and low efficacy [10]. Accordingly, many candidates have been developed with higher fibrin specificity and lower risk, but all of them were destined to fail, including the recent clinical trials of desmoteplase, which had been regarded as a promising thrombolytics [11,12].

The most important characteristics for t-PA alternatives are strong activity and low side effects. Fibrin clots can be directly degraded by serine proteases, but which causes non-specific damage to brain tissue. Therefore, t-PA-like enzymes with indirect fibrinolysis are regarded as promising, where serine protease activates plasminogen to plasmin and which specifically degrades fibrin clots [13,14]. Nevertheless, even t-PA degrades non-specifically NMDA receptor of blood brain barrier (BBB) which is thick blood vessel that protects the brain, resulting in hemorrhagic side effect [15-17]. After t-PA is treated, BBB is collapsed by matrix metallo protease-9 (MMP-9) and secondary damages are induced. Or, the endogenously released t-PA from the damaged parenchymal cells post ischemia have been known to have cytotoxic effects within cerebral tissue [18-20]. For this reason, a candidate for t-PA alternative is confined to those with indirect fibrinolysis activity and no cytotoxicity to cells comprising BBB.

Recently, serine proteases have been reported that have a potential thrombolytic agent from marine polychaeta worms [21,22]. In this research, a new fibrinolytic serine protease was isolated from *Marphysa sanguinea*, characterized by its enzymatic functions, and assessed for its cytotoxicity to cells comprising BBB.

MATERIALS AND METHODS

1. Purification of Serine Protease

Following ammonium sulfate precipitation method [21], the crude protein complex was harvested. Briefly, the *M. sanguinea* tissue dissected and auto-lysed in 100 mL of 20 mM phosphate (Na_2HPO_4, KH_2PO_4 and pH 7.0) (Sigma Aldrich, USA) for 2 hr at 4 °C was centrifuged at 8,000 g for 30 min, followed by saturation of the supernatant by ammonium sulfate (Sigma Aldrich, USA) to 20% and then incubating for 4 hr. The supernatant post centrifugation at 10,000 g for 20 min was adjusted to 55% ammonium sulfate and incubated for 4 hr, after which the pellet post centrifugation at 10,000 g for 15 min was solubilized in 5 mL of 20 mM phosphate buffer and stored at −20 °C. Serial chromatography was performed using ion exchange columns (GE healthcare, 1 mL), equilibrated with Tris-HCl and eluted with NaCl buffer (pH 7.4).

2. Identification of Serine Protease

Gel electrophoresis was performed to identify the molecular weight of the isolated serine protease. SDS-PAGE gel constitutes 30% acrylamide, 10% SDS, 10% APS, TEMED, 1.5 M Tris-HCl (0.5 M Tris-

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HCl) and distilled water, and sample is loaded on 12% acrylamide gels with loading buffer. Finally, purified protein was stained with Coomassie Blue R 250 (MERCK, USA) to identify protein bands.

For identifying N-terminal amino sequence of *M. sanguinea* serine protease, the serine protease electrophoresed by SDS-PAGE was transferred to PVDF membrane (Millipore, USA). 15 residue amino sequence identified by Edman degradation method was further compared with other protease amino sequences by NCBI blast.

To ascertain whether the purified protein is serine protease, three protease inhibitors (Aprotinin, trypsin inhibitor, PMSF) were tested and examined for their effect on the change of enzyme activity. A mixture of the protein sample (2 μg) and each protease inhibitor (1 mM) was incubated in 50 mM Tris-HCl buffer, pH 7.5 for 30 min at 37 °C. For the interaction assay with metal ions Na^+, K^+, Fe^{3+}, Ca^{2+}, a mixture of the protein sample with 1 mM each metal ion was prepared by incubation in 50 mM Tris-HCl, pH 7.5 for 30 min at 37 °C. Azocasein assay was performed to measure enzyme activity, and the inhibition level of enzyme was expressed as a percentage of its activity relative to that of non-inhibited one.

3. Measurement of Activity of Serine Protease

Fibrin plate was made by mixing 300 NIH thrombin (20 mM Tris-HCl buffer, pH 7.4) (MyBioSource, USA) with 7 mL fibrinogen (1% human fibrinogen (20 mM Tris-HCl buffer, pH 7.4)) (Sigma Aldrich, USA) and 7 ml agarose (Invitrogen, USA). After reacting serine protease on the plate, fibrinolytic activity was measured by the dissolved area of the plate. Serine protease (20 μg/mL) and urinary-Plasminogen Activator (u-PA) (2 μg/mL) were added to compare the fibrinolytic activity. Additionally, plasminogen-rich plate was performed by mixing with samples and plasminogen, and protease activity was measured by azocasein assay. 30 μg/mL of purified enzyme was reacted with 0.2 mL 25 mM tris-HCl buffer (pH 7.5) with 0.5% azocasein (Sigma Aldrich, USA) at 37 °C for 30 min, after which the solution was mixed with 10% TCA 0.1 ml to stop the reaction and then centrifuged at 10,000 g for 10 min. Optical density of the supernatant with NaOH was measured at 450 nm. To determine the optimal temperature and pH for the fibrinolytic enzyme, azocasein assays were performed in each condition. For temperature, the purified sample was evaluated at 4, 10, 25, 30 37, 50, 60 and 70 °C. The reactions were conducted at pH 7.0 for 1 h. The optimal pH of serine protease depends on range of pH 3.0-10.0. The solutions at pH 3-6 were made with glycine-HCl, and the solutions at pH 7-10 were made with Tris-HCl. The reactions were conducted in incubator at 37 °C for 1 h.

4. Plasminogenolytic Assay

Plasminogenolytic assay was performed by SDS-PAGE. The mixed solution was prepared with 10 μl (1 mg/ml) plasminogen and 10 μg *M. sanguinea* serine protease, and then was reacted in 37 °C for 30 min. After 30 min, the reaction was stopped by SDS-PAGE buffer (1 M urea, 4% SDS, and 4% β-mercaptoethanol), and then the reactants were electrophoresed by 10% SDS-PAGE.

5. Fibrinogenolytic Assay

A modified Matsubara method [23] was used to examine fibrinogenolytic activity. After incubating 200 μl (1 μg) serine protease with 200 μl of 0.5% fibrinogen in 25 mM Tris-HCl, pH 7.4 at 37 °C for each 0, 1, 2, 4, 8 and 12 h, the reaction was stopped by SDS-PAGE buffer (1 M urea, 4% SDS, and 4% β-mercaptoethanol), after which the reactants were electrophoresed by 10% SDS-PAGE.

6. *In Vitro* Cytotoxicity Test

Human brain vascular endothelial cells (hCMEC/D3) (INSERM, France) were cultured in Endothelial Basal Medium-2 (EBM-2) (Lonza, USA) supplemented with 5% Fetal Bovine Serum, 1% Penicillin-Streptomycin, 1.4 μM hydrocortisone (Sigma Aldrich, USA), 5 μg/ml, chemically defined lipid concentrate 1X (Gibco, USA), 10 mM HEPES (Gibco, USA), 1 ng/ml bFGF (Sigma Aldrich, USA). Culture media was changed every three days. For the subculture, 0.05% trypsin-EDTA was treated for 3 min and reloaded to a new t-flask coated with collagen type I (Advanced biomatrix, USA). For experiment, 0.05×10^6 cells were seeded and cultured on 24-well plate under 5% CO_2 at 37 °C for two days, after which the old culture media was exchanged with serum free media and cultured for another one day. At day three, serine protease was treated onto the prepared cells according to the concentration (10 μg). In one, three and five days after treatment of serine protease, MTT assays were performed following the manufacturer's guide. The purple color formazan could be detected in 540 nm using VARIOSKAN LUX (Thermo scientific, USA).

7. Statistical Analysis

Enzyme activity, turbidity, and cytotoxicity assays underwent one-way analysis of variance (ANOVA). All experiments were performed in triplicate and statistical significance was considered with p-values (*<0.05, **<0.01, or ***<0.001). All data are expressed as the average values ± the standard deviation (S.D.).

RESULTS

1. Characterization of the Purified Serine Protease

Referencing the previous research [24], the saturation level of ammonium sulfate was adjusted to selectively precipitate serine prote-

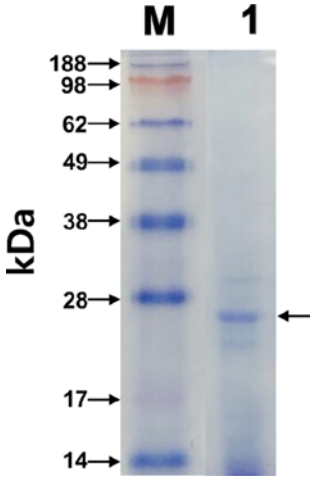

Fig. 1. Determination of purity by SDS-PAGE. SDS-PAGE of the purified serine protease extracted *M. sanguinea*. Lane M is standard protein size marker and lane 1 is sample extracted enzyme. The protein standard marker in lane M consists of myosin (188 kDa), phosphorylase (98 kDa), BSA (62 kDa), glutamic dehydrogenase (49 kDa), alcohol dehydrogenase (38 kDa), carbonic anhydrase (28 kDa), myoglobin-red (17 kDa), and lysozyme (14 kDa).

Table 1. Summary of total and specific activities of serine protease produced by *M. sanguinea*

Purification steps	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Ammonium sulfate	5.02	3325.2	662.4	100
Ion-exchange column	0.32	310.8	971.3	9.35

Table 2. Effects of protease inhibitor and metal ions on purified *M. sanguinea* serine protease

Inhibitors	Concentration (mM)	Relative activity (%)
Control	1	100
PMSF	1	33.1±0.08
Trypsin inhibitor	1	3.8±0.04
EDTA	1	108.8±0.18
Aprotinin	1	4.8±0.04
Fe^{3+}	1	105.9±0.18
Ca^{2+}	1	22.6±0.07
Na^{+}	1	95.1±0.17
K^{+}	1	97±0.17

ase, after which ion exchange chromatography was performed to purify the serine protease. As seen in SDS-PAGE result (Fig. 1), the molecular weight of the purified serine protease was estimated to be 27 kDa. Table 1 summarizes the total and specific activities of the protease. As purification steps proceeded, the specific activity increased to the last 971 units/mg, about 1.5 fold higher than that of the crude extract. Several protease inhibitors were used to confirm whether one of the protease isolated from *M. sanguinea* included serine protease. The *M. sanguinea* protease was inhibited by all serine protease inhibitors including PMSF, trypsin inhibitor, and aprotinin, but not by metalloprotease inhibitor EDTA. Additionally, the purified serine protease was rarely inhibited by metal ions except to Ca^{2+}.

2. Determination of N-terminal Amino Sequence of *M. sanguinea* Serine Protease

The *M. sanguinea* amino sequence was IVGGSEATPYQFPFQ, and compared with other serine proteases by NCBI blast. As shown in Table 3, the serine protease of *Ixodes scapularis* had 73.3% as highest identity compared with *M. sanguinea* serine protease, while the next identity sequence was 66.7% of *Pseudoalteromonas rubra*, *Culex quinquefasciatus*, *Helobdella robusta*.

3. The Dependency of Activity of the Purified Serine Protease on Plasminogen

On the agarose gel to pertaining thrombin and fibrinogen, thrombin cleaves two Aα and two Bβ of fibrinogen to generate fibrin monomers, which spontaneously aggregate to fibrin polymers [25]. Plasminogen-free plate is for investigating whether *M. sanguinea* serine protease directly cleaves fibrin polymer, and plasminogen-rich one is for whether the enzyme activate plasminogen to plasmin which cleaves fibrin polymer. The *M. sanguinea* serine protease displayed activity on both plasminogen-free and plasminogen-

Table 3. Determination of amino sequence of *M. sanguinea* serine protease by Edman degradation method

Enzyme	Amino sequence	Identity	Accession number
Original amino sequence	IVGGSEATPYQFPFQ		
Ixodes scapularis	IVGGSDATPLEFPWQ	73.3	Sequence ID: XP_002411301.1
Pseudoalteromonas rubra	IVGGGEATPFAVPFM	66.7	Sequence ID: WP_010380284.1
Culex quinquefasciatus	IVGGDEAAPHEFPYQ	66.7	Sequence ID: XP_001842458.1
Helobdella robusta	VIGGSEATPNEFPSQ	66.7	Sequence ID: XP_009018205.1

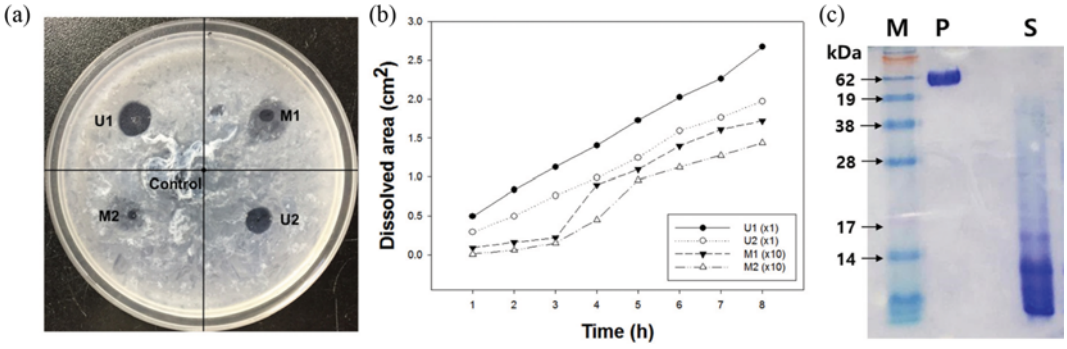

Fig. 2. Confirmation of direct and indirect fibrinolytic activity of *M. sanguinea* serine protease. (a) Fibrin plate assay to confirm the fibrinolytic activity of *M. sanguinea* serine protease and u-PA on 90 π petri-dish after 2 hr treatment. M1 is plasminogen-rich *M. sanguinea* serine protease and M2 is plasminogen-free *M. sanguinea* serine protease. U1 is plasminogen-rich u-PA and U2 is plasminogen-free u-PA. (b) Dissolved area over the time. (c) The plasminogen was dissolved in distilled water. The plasminogen was hydrolyzed to small molecules. (M is protein ladder, P is plasminogen, and S is mixed sample with plasminogen and serine protease purified from *M. sanguinea*).

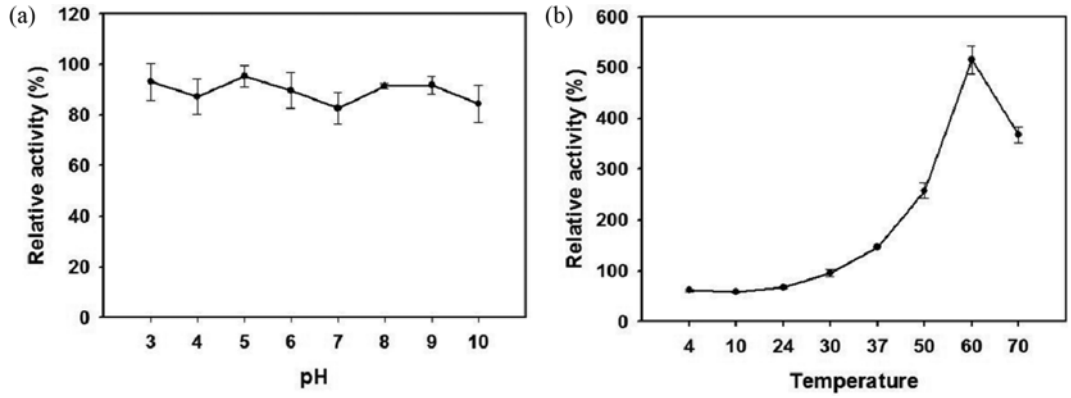

Fig. 3. Characterization of serine protease about pH and temperature. Relative activity was measured by azocasein assay (relative activity= (protease OD value−control OD value)/control OD value). (a) Fibrinolytic enzyme activity of *M. sanguinea* serine protease according to pH was measured by the azocasein assay. (b) Fibrinolytic enzyme activity according to temperature was confirmed at pH 7.5 by the azocasein assay.

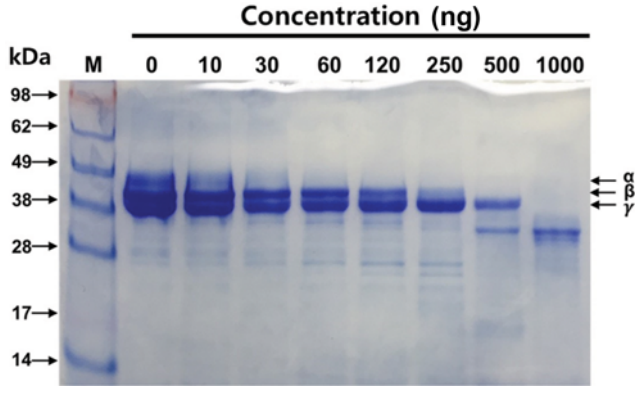

Fig. 4. Analysis of fibrinogenolytic activity of *M. sanguinea* serine protease. The fibrinogen was dissolved in Tris-HCl buffer at pH 7.5. E monomer and D dimer of fibrinogen was linked by α, β, γ chain. The *M. sanguinea* serine protease hydrolyzed α, β, γ chains.

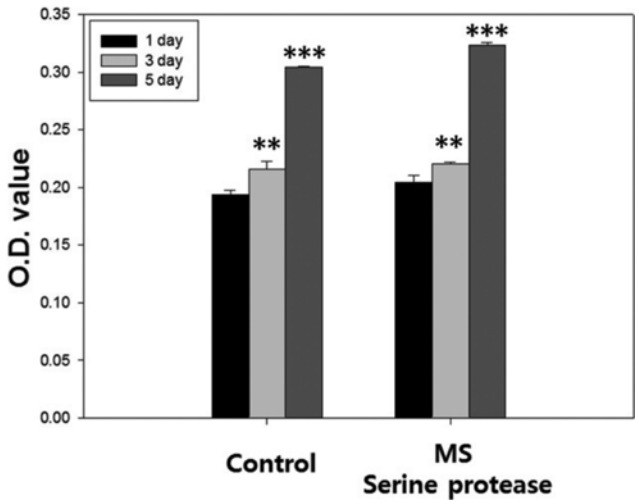

Fig. 5. Cytotoxicity test of serine protease on endothelial cells. Cytotoxicity of non-treated- and treated serine protease was measured by MTT assay on endothelial cells. Serine protease was treated with 10 μg/ml. The p-values of three days and five days of control and *M. sanguinea* serine protease were compared with one day.

rich fibrin plates, which means the purified enzyme had both direct and indirect thrombolysis mechanism. Indirect activity was stronger than direct one by 2.8-fold in 2 h after treatment (Fig. 2(a)). However, both direct and indirect activities of *M. sanguinea* serine protease were much weaker than those of u-PA (Fig. 2(b)).

4. Plasminogenolytic Activity of *M. sanguinea* Serine Protease

Indirect activity of *M. sanguinea* serine protease was conducted by SDS-PAGE. As shown in Fig. 2(c), the band of plasminogen disappeared in the serine protease, leaving broad range of bands in low molecular weights. The serine protease had great indirect activity in 10 μg.

5. Temperature and pH Effects on the Purified Serine Protease

Azocasein assay was used to measure the optimal temperature and pH of *M. sanguinea* serine protease. The enzyme activity was maintained high over wide range of pH 3-pH 10 (Fig. 3(a)). In addition, the enzyme activity increased with temperature up to 60 °C but dropped over 70 °C (Fig. 3(b)).

6. Fibrinogenolytic Activity of *M. sanguinea* Serine Protease

M. sanguinea serine protease was tested onto human fibrinogen and the post-reactant was electrophoresed. As seen in Fig. 4, α-, β-, and γ-chain of fibrinogen began to disappear as concentration of *M. sanguinea* serine protease increased, and completely was degraded from 120, 500, 1,000 ng/ml of the enzyme, respectively. The degraded peptides were explicitly observed between 28 and 38 kDa.

7. Cytotoxicity of *M. sanguinea* Serine Protease on Endothelial Cells

Viability of endothelial cells showed no statistical differences between 10 μg/ml *M. sanguinea* serine protease-treated and non-treated samples (Fig. 5). However, cells were revealed to be damaged under *M. sanguinea* serine protease above 10 μg/ml (data not shown).

DISCUSSION

Our research objective was to isolate a novel thrombolytic ser-

ine protease targeted to human brain ischemic reperfusion (IR). The purified protease by ammonium sulfate/ion exchange was identified by enzyme inhibition assays. PMSF is a well-known typical serine protease inhibitor and the others are those to trypsin-like serine protease [29]. The three different serine protease inhibitors all lowered the activity of the purified enzyme, which implies the enzyme is a serine protease. Considering that N-terminal sequence of *M. sanguinea* IVGGSEATPYQFPFQ had the highest similarity 73.3% with *Ixodes scapularis*, it can be told a novel serine protease.

Ions also could influence enzyme activity, and thus a candidate of serine protease should be tested of its activity in human blood stream including several ions [30]. In this sense, the purified enzyme could be expected to preserve its activity in blood since metal ions except Ca^{2+} rarely influenced the enzyme activity. The t-PA and u-PA have plasminogen-dependent fibrinolytic activity (indirect) stronger than direct fibrinolysis (direct), of which phenomena has been regarded to be favorable to safety of BBB during reperfusion of blood vessel post IR [31]. The indirect fibrinolytic mechanism appeared in the *M. sanguinea* serine protease, even though the activity level was much weaker than that of the control u-PA. The only thrombolytic agent that has been commercially approved by US FDA is t-PA, but u-PA is a drug that is commonly used in many countries and its fibrinolytic activity is comparable to t-PA [10,26]. Some reports said u-PA has fibrinolytic activity stronger than t-PA [27], and thus many studies used u-PA as a control sample [21,28]. The degradation of plasminogen by *M. sanguinea* serine protease confirmed the indirect mechanism of fibrinolysis (Fig. 2(c)). Also, high activity of the enzyme at acidic condition can be said to be adequate for human IR, since the injected t-PA substitute is needed to maintain its activity at acidic environment around post IR brain tissue [32].

Another significant side effect of t-PA is reocclusion in the blood vessel [33], and thus anticoagulants should be administered to prohibit fibrins from clotting after t-PA treatment [34]. *M. sanguinea* serine protease revealed capability to degrade α-, β-, γ-chains of human fibrinogen. Especially, the enzyme efficiently cleaves γ-chain that is resistant to enzyme due to the triple-stranded coil structure [35]. The chain cleavage results confirm that the purified protease is not a α-fibrinogenase like metalloprotease but a β-fibrinogenase since α-fibrinogenase can cleave only Aα [36,37]. Many fibrinogenolytic enzymes including plasmin likely hydrolyze Aα and Bβ but rarely γ chains [38]. Therefore, the *M. sanguinea* serine protease is unique due to the capability of hydrolyzing γ chains and is anticipated to prohibit fibrinogen from being transformed to fibrin clot *in vivo*. All the results imply that *M. sanguinea* serine protease has anticoagulant activity in addition to thrombolysis, both of which are important to permit blood flow stably through the vessel. Finally, no cytotoxicity to endothelial cells up to 10 μg/ml strengthens *M. sanguinea* serine protease as a candidate to reperfuse the IR blood vessel.

CONCLUSION

Many efforts have lasted decades to increase the time window and to minimize side effects like hemorrhage and reocclusion of t-PA. This work contains purification and identification of functions of a novel serine protease from *M. sanguinea* with N-terminal sequence of IVGGSEATPYQFPFQ. A consecutive purification through ammonium sulfate precipitation and ion exchange chromatography revealed a serine protease that has fibrinolysis activity through both direct and indirect mechanisms. The serine protease also degraded α-, β-, and γ-chains of human fibrinogen, but did not explicitly show cytotoxicity to endothelial cells. In addition, over a wide range of pH, enzyme activity was maintained. All these results make it possible to anticipate that the enzyme would be working as a thrombolytic and anticoagulant agent in acidic post IR environment of brain, although in-depth researches on safety and efficacy should be further performed *in vitro* and *in vivo*.

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