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A Novel Thrombolytic and Anticoagulant Serine Protease from Polychaeta, *Diopatra sugokai*

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Ischemic stroke can result from blockage of blood vessels, forming fibrin clots in the body and causing irreparable brain damage. Remedial thrombolytic agents or anticoagulants have been studied; however, because the FDA-approved tissue plasminogen activator has low efficacy and side effects, it is necessary to develop safer and more effective treatment candidates. This study aimed at assessing the fibrinolytic and anticoagulation features of a novel serine protease extracted and purified from *Diopatra sugokai*, a polychaeta that inhabits tidal flats. The purified serine protease was obtained through ammonium sulfate precipitation, affinity chromatography, and ion-exchange chromatography. Its molecular size was identified via SDS-PAGE. To characterize its enzymatic activities, the protease activity at various pH and temperatures, and in the presence of various inhibitors, was measured via azocasein assay. Its fibrinolytic activity and anticoagulant effect were assessed by fibrin zymography, fibrin plate assay, and fibrinogenolytic activity assays. The novel 38 kDa serine protease had strong indirect thrombolytic activity rather than direct activity over broad pH (4–10) and temperature (37°C–70°C) ranges. In addition, the novel serine protease exhibited anticoagulant activity by degrading the α-, β-, and γ-chains of fibrinogen. In addition, it did not produce cytotoxicity in endothelial cells. Therefore, this newly isolated serine protease is worthy of further investigation as a novel alkaline serine protease for thrombolytic therapy against brain ischemia.

Keywords: Ischemic stroke, alkaline serine protease, *Diopatra sugokai*, fibrinolytic activity, bifunctional thrombolytic activity

Introduction

Stroke is a cerebrovascular disease that results in brain damage through neurovascular disorders that produce cerebral blood flow deficiencies associated with neurological defects [1]. Stroke is generally divided into ischemic and hemorrhagic cases. Approximately 85% of stroke cases are caused by ischemia, resulting in cerebral infarction as well as venous thrombosis and embolism [2, 3]. Ischemic stroke is classified as cardiogenic embolism or cerebral infarction in large vessel diseases [4], with the commonest causes being hypertension, diabetes, hyperlipidemia, smoking, and genetic diseases [5]. There are currently two types of stroke treatments: anticoagulant and thrombolytic. Anticoagulants can prevent the occurrence of stroke by reducing the production of fibrin clots [6]. Thrombolytics can dissolve fibrin clots in blocked blood vessels [7] and consist of two types based on the mechanism by which the fibrin clot is degraded. Direct thrombolytic agents convert the fibrin clot into fibrin degradable products. Serine proteases are enzymes that degrade proteins through the cleavage of peptide bonds [8]. Serine proteases for directly dissolving thrombi, such as brinase, plasmin, and trypsin, have been previously studied [9]; however, these treatments have marked disadvantages such as being toxic and unselectively dissolving fibrin clots necessary for maintenance of the blood-brain barrier [10, 11]. Serine proteases that act indirectly, such as tissue plasminogen activator (t-PA), the only FDA-approved thrombolytic agent, cause plasminogen to be activated into plasmin [12]. Indirect thrombolysis is reported to be comparatively safe but to still have negative side effects such as hemorrhage and re-occlusion [13, 14].

Trials to develop t-PA alternatives without side effects have been conducted, but have been unsuccessful to date [13, 15]. Recently, marine organisms were found to have serine proteases that have indirect fibrinolytic activity similar to that of t-PA. In particular, the activity and efficiency of annelid-origin serine proteases have been investigated in several studies [16, 17, 18, 32]. On that basis, screening for bifunctional fibrinolytic enzymes from polychaetas and earthworms was considered worthwhile. *Diopatra sugokai* is affiliated to the Annelida (Polychaeta) as characterized by the Institute of Oceanology, Korean Academy of Sciences. *D. sugokai* is an euryhaline nereid polychaete that occurs significantly in Korea, frequently inhabiting under intertidal zone, rocky-gravel sediments [19]. For these reasons, it is worthy of screening a bifunctional fibrinolytic enzyme from polychaete worms and earthworms. In this study, a novel serine protease was extracted from *D. sugokai* and was confirmed to be a bifunctional alkaline serine protease that had greater indirect fibrinolytic activity than direct activity.

Materials and Methods

Materials

Specimens of *D. sugokai* were collected from the coast of Muan-gun, Korea. A Hitrap Benzamidine affinity fast flow column (AC) and a Hitrap DEAE sepharose ion-exchange column (IEX) were purchased from GE Healthcare Life Sciences (Sweden). Ammonium sulfate, sodium chloride, Coomassie brilliant blue R250, bovine serum albumin (BSA), human fibrinogen, azocasein, plasminogen, hydrocortisone, trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO), basic fibroblast growth factor (bFGF), trypsin-EDTA including phenol red, and Triton X-100 were purchased from Sigma-Aldrich (USA). Acrylamide, sodium dodecyl sulfate (SDS), and ammonium persulfate (APS) were obtained from Bio-Rad (USA). The human brain vascular endothelial cells (hCMEC/D3) were purchased from INSERM (France). Tetramethylethylenediamine (TEMED), Tris, and glycine were procured from Affymetrix (USA). Penicillin-streptomycin, ascorbic acid, hydroxyethylpiperazine ethane sulfonic acid (HEPES), chemically defined lipid concentrates, and fetal bovine serum (FBS) were obtained from Gibco (Korea). Thrombin was obtained from Mybiosource (USA). Human blood plasma was obtained from Chromogenix (USA). Absorbance was measured by using a Sunrise enzyme-linked immunosorbent assay (ELISA) reader (Tecan, Austria). Urokinase plasminogen activator (u-PA) was obtained from PROSPEC (USA) Sodium azide was purchased from Samchun Chemical (Korea). SeeBlue plus2 pre-stained standard and VARIOSKAN LUX were obtained from ThermoFisher (USA). All other reagents and chemicals were of special grade and procured commercially.

Extraction of Serine Protease

D. sugokai was maintained without food for 48 h to empty the intestines, after which 50 g samples were obtained by chopping the worm into small pieces. The samples were incubated for 2 h at 4°C in 100 ml of 20 mM phosphate buffer (Na_2HPO_4, KH_2PO_4 at pH 7.0). Samples were then centrifuged at 8,000 ×*g* for 30 min with ammonium sulfate at a 20% concentration and then incubated for 4 h [14]. The supernatant was then removed and after 20 min an additional centrifugation at 10,000 ×*g* with a 55% concentration of ammonium sulfate solution was performed. After 4 h of incubation at 37°C, the last centrifugation was conducted at 10,000 ×*g* for 15 min. The obtained pellet was dissolved in 20 mM phosphate buffer and stored at −20°C before use.

Purification of Fibrinolytic Enzyme

Crude extracts (1 ml) were loaded onto a Hitrap Benzamidine AC, equilibrated with Tris-HCl including 0.5 M NaCl buffer at pH 7.4. At a flow rate of 1 ml/min, the sample was eluted with 5 ml of 0.5 M glycine buffer at pH 3. Following those affinity purification steps, the serine protease (1 ml) was loaded onto a Hitrap DEAE sepharose ion-exchange column and equilibrated with 20 mM Tris-HCl at pH 8. At a flow rate of 1 ml/min, the sample was eluted with 100 mM~1 M NaCl in Tris-buffer at pH 7.4. After all purification steps, the enzyme activity was measured by using azocasein and a cell cytotoxicity test as described below. For a standard curve, the protein concentration was estimated by using BSA.

Fibrin Plate Assay

Plasminogen-free fibrin plate assay. Fibrinolytic activity was determined by using the fibrin plate method [20]. Fibrin plates were formed by reacting fibrinogen and thrombin. Fibrinogen (1.5% human fibrinogen in 20 mM Tris-HCl buffer, pH 7.4) and thrombin (25 NIH units in 20 mM Tris-HCl buffer, pH 7.4) were allowed to react for 1 h at room temperature. To test the fibrinolytic activity, a small hole was made with a 20 µl pipette tip and 10 µl of crude extraction was treated in the hole. Subsequently, serine protease, u-PA, and the control (PBS) were allowed to react at 37°C for 4 h. The presence of fibrinolytic enzyme activity was then determined by estimating the liquefied area in the fibrin plate.

Plasminogen-rich fibrin plate assay. After preparing the fibrin plate, 5 units of plasminogen was added. Samples were prepared as described above, after which serine protease, u-PA, and the control (PBS) were added and allowed to react at 37°C for 4 h. The amount of fibrinolytic activity was estimated by comparing the dissolved area of the plasminogen-free and plasminogen-rich plates. The dissolved area was measured by using ImageJ software (USA National Institutes of Health).

Effects of Protease Inhibitors and Metal Ions on Enzymatic Activity

The effects of four protease inhibitors (aprotinin, a trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), and ethylenediaminetetraacetic acid (EDTA)) were investigated [27]. The enzyme sample (2 µg) was

preincubated with 50 mM Tris-HCl buffer at pH 7.5 with the addition of the protease inhibitor for 30 min at 37°C. After incubation, assessment of the residual enzyme activity was conducted by using the azocasein assay. The level of inhibition of the inhibitors was determined as a percentage of the control activity level.

Fibrinogenolytic Activity Assay

Fibrinogenolytic activity was determined according to the method of Kim *et al.* [21] with a slight modification. Briefly, 200 µl of 0.5% fibrinogen was incubated with 200 µl of purified serine protease (0, 10, 30, 60, 120, 250, 500, and 1,000 ng) and 25 mM Tris-HCl at pH 7.4 at 37°C for 4 h. The reaction was stopped by the addition of SDS-PAGE sample buffer (1 M urea, 4% SDS, and 4% β-mercaptoethanol). The digested products were analyzed by performing 10% SDS-PAGE.

Azocasein Assay

The azocasein assay was conducted in order to measure protease activity [22]. Briefly, 10 µg/ml of extracted enzyme was mixed with 1 ml of 25 mM Tris-HCl buffer (pH 7.5) and 0.5% azocasein and then reacted at 37°C for 30 min. After stopping the reaction with 10% TCA, the sample underwent 10 min of centrifuging at 10,000 ×*g*. The optical density of the 5 N NaOH-mixed supernatant was measured at 450 nm. After assay completion, the samples were used for pH and temperature characterization.

Effects of pH and Temperature on Fibrinolytic Activity

To determine the effect of temperature on the fibrinolytic activity of the enzyme against azocasein, the crude extract (100 µl of 0.3 µg/ml) was incubated with 500 mM Tris-HCl (pH 7.4) at various temperatures between 4°C and 70°C for 30 min [23]. To determine the effects of pH, the serine protease was examined in various buffers within a pH range of 3–10. Serine protease (100 µl) at 0.3 µg/ml was added to 200 µl of 25 mM glycine-HCl buffer for the pH range of 3–6 or with 25 mM Tris-HCl buffer for the pH 7–10 range. After incubating for 30 min at 3°C, the serine protease activity was assessed by following the same process as that of the azocasein assay.

Determination of Molecular Mass

The molecular mass of the purified serine protease was determined on 10% SDS-PAGE as described by Laemmli [20]. The SDS-PAGE running and stacking gels consisted of 30% acrylamide, 1.5 M Tris-HCl, 0.5 M Tris-HCl, 10% SDS, 10% APS, TEMED, and distilled water [21]. The purified enzyme measurement was carried out by mixing with protein dye and boiling for 3 min at 100°C, followed by electrophoresis. After electrophoresis, the SDS-PAGE gel was stained with Coomassie brilliant blue R-250 and, after 2 h, the gel was destained for 8 h.

Direct and Indirect Fibrin Zymography

Fibrin zymography was performed following the method by Loskutoff *et al.* [25]. Briefly, the fibrin zymography gel was resolved under nonreducing conditions, containing 0.06% fibrinogen, thrombin (1 NIH unit/ml), plasminogen (1 NIH unit/ ml), and 10% SDS-polyacrylamide gel. The purified serine protease and crude enzyme were subjected to fibrin zymography gel electrophoresis. After electrophoresis, the plasminogen-free (direct) and -rich (indirect) gels were washed with 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $CaCl_2$, 1 mM $ZnCl_2$, and 2.5% Triton X-100 for 1 h to remove the SDS. Both gels were then incubated under 5% CO_2 at 37°C for 10 h with a reaction buffer (30 mM Tris-HCl buffer, pH 7.4) that included 200 mM NaCl and 0.02% NaN_3. The crude or purified serine proteases were stained with Coomassie brilliant blue R-250.

Identification of Serine Protease from *Diopatra sugokai* by Protein ID Analysis

To identify the amino acid sequences of *D. sugokai* serine protease, the purified serine protease underwent 10% SDS-PAGE [21]. After electrophoresis, the gel was stained and then destained. Amino acid sequences were analyzed by treating the protease with trypsin and endoprotease and then subjecting the products to Edman degradation using quadrupole time-of-flight mass spectrometry, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

In Vitro Endothelial Cytotoxicity Test

The purified serine protease from *D. sugokai* needed to be tested for cytotoxicity before it could be considered for commercial use in stroke victims. The cell toxicity effects of the purified serine protease on hCMEC/D3 cells were analyzed by treating the cells in EBM-2 cell culture medium containing 1% penicillin-streptomycin, 5% FBS, hydrocortisone (1.4 mM), ascorbic acid (10 µg/ml), 1× chemically defined lipid concentrate, HEPES (10 mM), and bFGF (1 ng/ml). The culture media of the hCMEC/D3 cells was changed after 3 days. For subculturing, collagen type I was coated on a new 75 T-flask. After 1 h, 0.05% trypsin-EDTA including phenol red was treated for 3 min on the 75 T-flask. To perform the MTT assays, 0.05×10^6 hCMEC/D3 cells were seeded on 24-well plates and incubated under 5% CO_2 at 37°C for 1 day. Subsequently, the hCMEC/D3 culture medium was changed to new serum-free medium. The next day, the purified serine protease was treated to the 24-well plates of prepared cells depending on control, and at individual concentrations (50, 100, 200, and 500 ng/ml). Cell viability was assessed by performing MTT assays at 1, 3, and 5 days. The purple color of formazan was detected at 540 nm using VARIOSKAN LUX.

Statistical Analysis

Experimental accuracy was identified by performing statistical analyses. Enzyme activity was assessed by using one-way analysis of variance followed by Tukey's method. All experiments were carried out in triplicate. Statistical significance was decided on the basis of *p*-values (*$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$). All data are presented as the average ± SD values.

Results

Extraction of *D. sugokai* Serine Protease

The *D. sugokai* serine protease was isolated by using ammonium sulfate precipitation. The BCA assay was conducted to quantify the protein concentration of the precipitated samples and the extracted protein concentration was revealed to be 1,915 μg/ml.

Dependence of the Activity of the Extracted *D. sugokai* Serine Protease on Fibrin Plate Plasminogen

To determine whether the extracted enzyme had direct or indirect fibrinolytic activity, the enzymatic samples were allowed to react on plasminogen-free and -rich fibrin plates. As shown in Fig. 1, the serine protease showed stronger indirect activity on the plasminogen-rich plate than on the plasminogen-free plate, although fibrinolytic activities were observed on both fibrin plates. On the basis of this result, it was confirmed that the extracted serine protease had bifunctional activity.

Protease Inhibitor Effects on the Activity of the Extracted *D. sugokai* Serine Protease

The effects of serine protease inhibitors on the fibrinolytic activity of *D. sugokai* serine protease are summarized in Table 1. Enzyme activity was inhibited by 1 mM PMSF, a typical serine protease inhibitor. The enzyme was also inhibited by 1 mM EDTA, a metalloprotease inhibitor, and by aprotinin and a trypsin inhibitor.

Table 1. Effects of various protease inhibitors on the enzyme activity of *Diopatra sugokai* extract.

Inhibitors	Concentration (mM)	Relativie activity (%)
Control	0	100
PMSF	1	42.9 ± 0.008
Trypsin inhibitor	1	11 ± 0.004
EDTA	1	5.6 ± 0.006
Aprotinin	1	-2.3 ± 0.002

Enzyme activities were assessed after incubation with the various protease inhibitors at 37°C and pH 7 for 30 min. Data are presented as the mean ± SD values (n = 3).

Fibrinogenolytic Activity Test

To investigate the ability of *D. sugokai* serine protease to hydrolyze human fibrinogen, the extracted enzyme sample was incubated with human fibrinogen at 37°C and the reactant was evaluated by performing 10% SDS-PAGE. Fig. 2 shows the presence of dose-dependent digestion of fibrinogen after 4 h. The enzyme cleaved all α, β, and γ-chains of fibrinogen.

Effects of Temperature and pH on the Activity of the Extracted *D. sugokai* Serine Protease

Temperature and pH effects on the enzymatic activity were confirmed by azocasein assays. As shown in Fig. 3, the serine protease activity gradually increased with an increase in temperature, especially showing an exponential

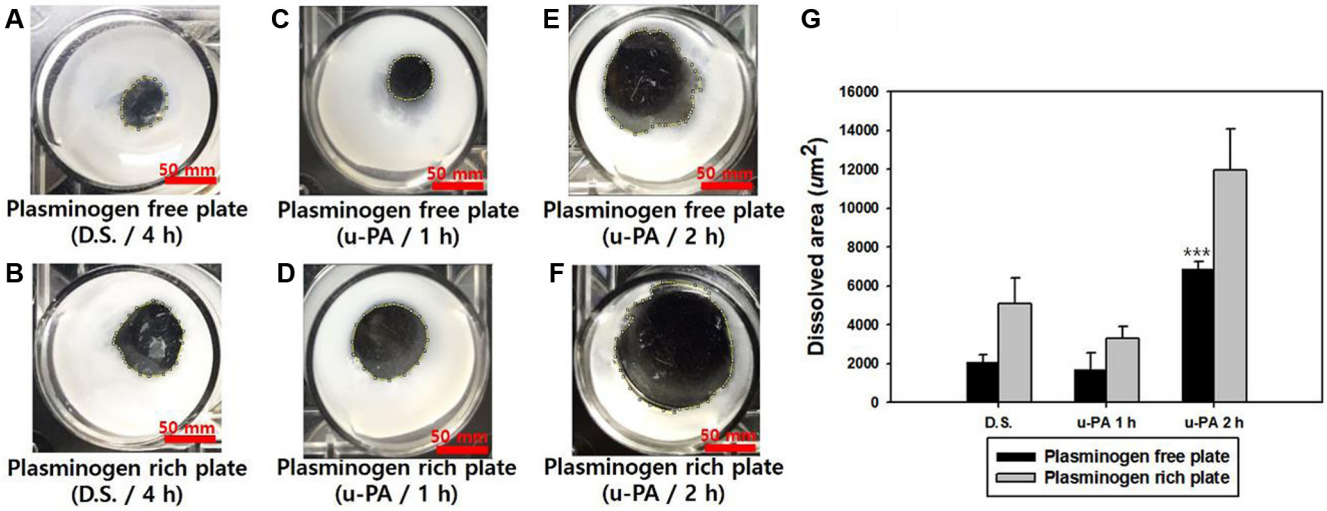

Fig. 1. Dependence of extracted serine protease activity on plasminogen.
(**A**, **B**) Ten microliters of *Diopatra sugokai* extract was treated on plasminogen-free and -rich plates for 4 h. (**C**–**F**) Ten microliters of urokinase plasminogen Diopatra activator (u-PA) was treated on plasminogen-free and -rich plates for 1 or 2 h. (**G**) The dissolved area was quantified by ImageJ software.

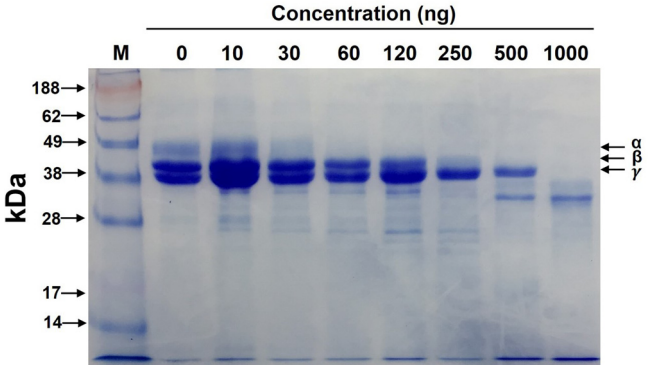

Fig. 2. Fibrinogenolytic activity of *Diopatra sugokai* on human fibrinogen.

Fibrinogenolytic activity of the extracted enzyme was evaluated by 10% SDS-PAGE of 0.5% fibrinogen incubated with *D. sugokai* serine protease (0–1,000 ng) for 4 h. Lane M, molecular mass standards; lane 0, fibrin clot; lanes 10–1,000, fibrin clots incubated with extracted enzyme.

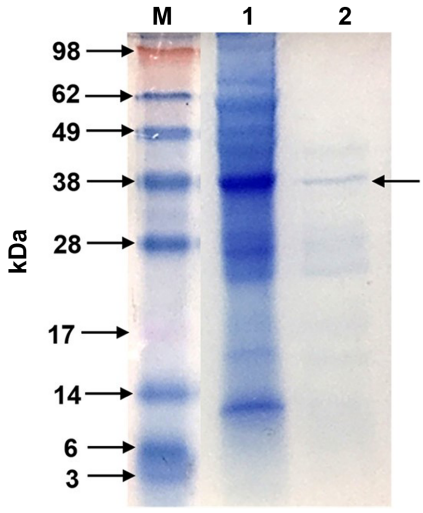

Fig 4. SDS-PAGE of the extracted enzyme.

The extracted and purified *Diopatra sugokai* enzymes (10 μg/20 μl) were separated by 10% SDS-PAGE and the protein bands were stained with Coomassie brilliant blue R-250 and then destained with glacial acetic acid/methanol/distilled water (1:3:6, by volume). Lane M, standard molecular weight markers composed of phosphorylase (98 kDa), BSA (62 kDa), glutamic dehydrogenase (49 kDa), alcohol dehydrogenase (38 kDa), carbonic anhydrase (28 kDa), myoglobin–red (17 kDa), lysozyme (14 kDa), aprotinin (6 kDa), and insulin B chain (3 kDa). Lane 1, crude *D. sugokai* extract. Lane 2, purified *D. sugokai* extract.

increase in the range from 50°C to 70°C. In addition, enzyme activity was measured over a wide range of pH values, similar to that used in previous research [26]. The serine protease activity increased as pH increased, with a maximum at pH 8, implying that the extracted serine protease was an alkaline serine protease.

Purification and Molecular Mass Determination of *D. sugokai* Serine Protease

After purifying the serine protease sample with affinity chromatography and ion-exchange chromatography, a BCA assay was carried out to quantify the purified enzyme. The concentration of purified enzyme was 102 μg/ml. SDS-PAGE of the extracted serine protease was also conducted. Several proteins separated and left some bands with a molecular mass in the range of 62–14 kDa. The purified serine protease was estimated to be 38 kDa (Fig. 4).

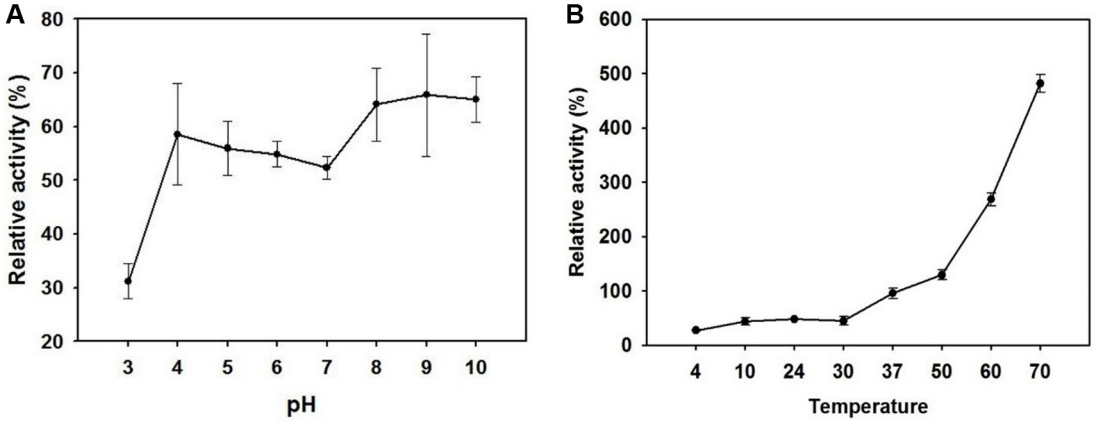

Fig. 3. Effects of pH (**A**) and temperature (**B**) on the fibrinolytic activity of *Diopatra sugokai* serine protease.

The protease activity was measured by azocasein assays at 450 nm. (**A**) The serine protease activity was analyzed by incubating the fibrinolytic enzyme at 37°C for 30 min over a pH range of 3–10. (**B**) The serine protease activity was assessed after incubation at temperatures ranging from 4°C to 70°C.

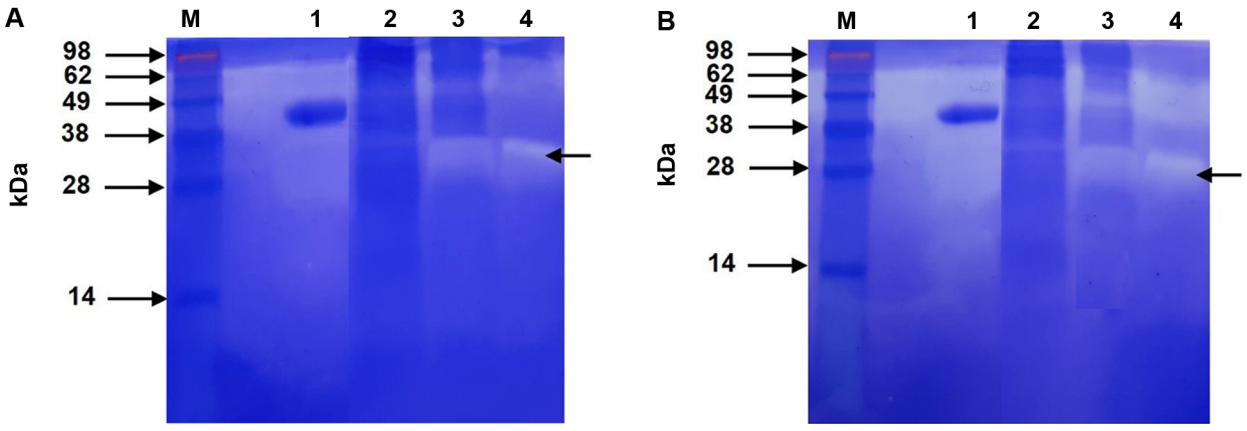

Fig. 5. Results of direct (**A**) and indirect (**B**) fibrin zymography.
Lane M, standard molecular weight marker. Lane 1, urokinase plasminogen activator. Lane 2, crude *Diopatra sugokai* extract. Lane 3, purified *D. sugokai* extract (affinity column). Lane 4, purified *D. sugokai* extract (IEX column).

Direct and Indirect Fibrin Zymography

Fibrin zymography was used to confirm the fibrinolytic activities of the purified enzyme from *D. sugokai* at a specific molecular mass (Fig. 5). Stronger activity was observed on plasminogen-rich gel than on plasminogen-free gel on fibrin zymography. The molecular mass of the active fibrinolytic enzyme was estimated to be 38 kDa. In Fig. 5, lane 1 (control; u-PA) is the range of 90–28 kDa, whereas lanes 2 (crude *D. sugokai*), 3 (purified *D. sugokai*, AC column), and 4 (purified *D. sugokai*, IEX column) show the presence of 62 and 38 kDa proteins. The results, along with those presented in Fig. 4, indicate that a serine protease of 38 kDa was present.

Identification of Serine Protease from *D. sugokai* by Protein Identification Analysis

Seven peptide fragments from *D. sugokai* were identified by using LC-MS (Fig. 6, Table 2) and were compared with the NCBI amino acid database for polychaetes (Table 2). As shown in Table 2, three fragments were confirmed to match the amino acid sequences of fibrinolytic protein from *Sipunculus nudus*.

Cytotoxicity Test

Cell viability over a 5-day period with varying concentrations of *D. sugokai* serine protease was evaluated by performing MTT assays with hCMEC/D3 cells. There were no significant differences in cell viability with respect to serine protease concentration at each day (Fig. 7). The purified serine protease was shown to be nontoxic to hCMEC/D3 cells (Fig. 7).

Discussion

Identification of substitutes for the thrombolytic serine protease t-PA is a goal of current research into the development of drugs for stroke treatment because t-PA has harmful side effects on blood-brain barrier tissues. In general, indirect serine proteases are good candidates for substitution of t-PA or u-PA because they activate the

Table 2. Identification of serine protease from *D. sugokai* by using the Edman method of protein identification.

Species	Time	Precise MW	Precise m/z	Best Sequence	Theoretical MW
SPINU	21.309	945.5461	473.7803	TIVEIETK	945.5383
SPINU	22.45	1513.812	505.6112	VRELEIQLEEER	1513.799
SPINU	26.124	1285.696	643.8552	IALQTELEDVR	1285.688
CAPTE	22.321	1322.693	662.3537	LETTFNTQTR	1322.683
CAPTE	29.007	1203.678	602.8464	DLSSVQTLLTK	1203.671
PERAI	21.363	1433.688	717.8512	AADLAGSDPDYAIR	1433.679
ARECR	21.002	1377.611	689.8128	TCGGCALPDER	1377.602

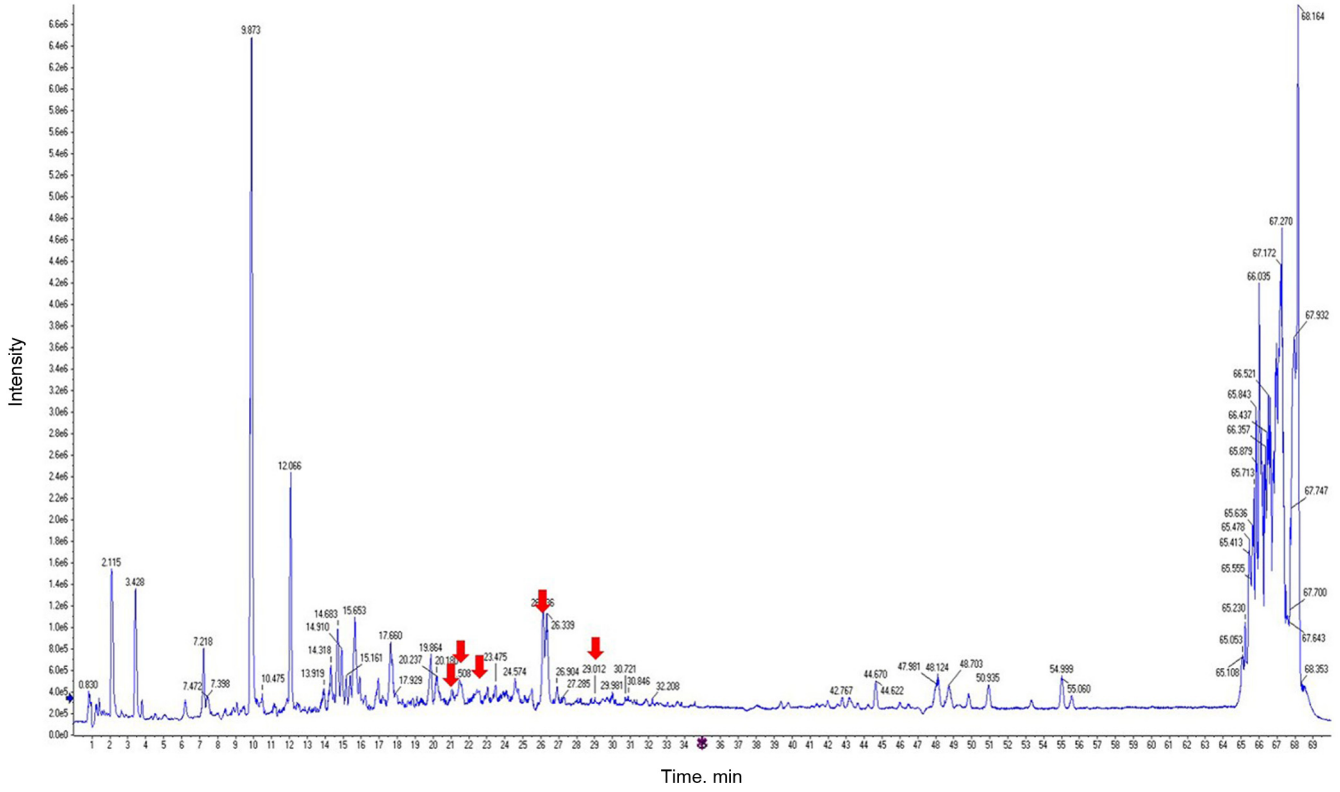

Fig. 6. Identification of *Diopatra sugokai* serine protease by the Edman degradation method of protein identification.
Arrows indicate a measured time and precise molecular weight.

conversion of plasminogen to plasmin, which degrades fibrin clots with fewer side effects than that from using direct nonspecific fibrinolysis [27]. Therefore, this study was conducted to extract, identify, and analyze a serine protease from *D. sugokai* and determine its potential in the treatment of ischemic stroke. The experimental results indicate that the extracted enzyme expressed fibrinolytic activity via both direct and indirect mechanisms (Fig. 1), similar to the mechanisms of u-PA. Following completion of the inhibition tests, the extracted *D. sugokai* sample was confirmed to have several enzymes including serine protease (Table 1). Serine protease has a catalytic triad system containing histidine, serine, and aspartic acid [28]. PMSF, aprotinin, trypsin inhibitor, and EDTA are typical serine protease inhibitors [29, 30] and were observed to lower the *D. sugokai* serine protease activity levels (Table 1). These results showed the extract to be a serine protease with a bidirectional fibrinolytic mechanism. Fibrinogenolytic activity assessment was conducted with human fibrinogen, the last molecule in the coagulant cascade before conversion to fibrin (Fig. 2). The γ-chain, one of three chains conjugating the E-monomer with D-dimer of fibrinogen [31. 32], is the most difficult to be cleaved by enzymes, including plasmin [33]. As shown in Fig. 2, the γ-chain was gradually

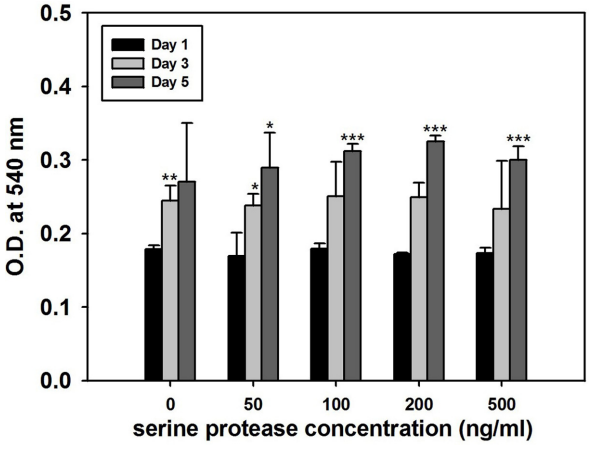

Fig. 7. Cytotoxicity of purified *Diopatra sugokai* serine protease toward hCMEC/D3 cells, assessed by MTT assay.
MTT values were measured at purified serine protease concentrations of 0, 50, 100, 200, and 500 ng/ml. Assay durations were 1, 3, and 5 days and were replicated ($n = 3$). Asterisks indicate the presence and levels of significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

hydrolyzed with an increase in the extracted enzyme and the chain had completely disappeared after treatment with 1,000 ng of *D. sugokai* serine protease. That complete degradation of the γ-chain confirms that the extract has anticoagulant activity [34]. In addition, the serine protease-containing *D. sugokai* sample was characterized over wide ranges of pH and temperature. Under cerebral ischemia conditions, acidosis can result, depending on various factors such as blood glucose level and the reduction of blood flow [35]. Generally, ischemic injury and reduction of glutamate inducing neuronal death occurs in an acidosis environment at pH 6.5–7. Moreover, severe acidosis (under pH 6.4) can exacerbate ischemic injury through denaturation of protein, acid-sensing calcium channels, and ferrous iron release [36]. The serine protease from *D. sugokai* was shown to be active over the pH range of 6.5–7; thus, it could act as a thrombolytic agent in cerebral ischemia under acidosis conditions. In addition, under a cerebral ischemia situation, the infarct volume can be increased by a raised body temperature in humans [37]. Thus, hypothermic therapy, involving the lowering of body temperature to below 35°C, has been performed clinically as it has been reported to be effective in neuroprotection in ischemic stroke [38]. However, hypothermia slows the spontaneous degradation of fibrin clots [39]. Therefore, the presence of thrombolytic activity in serine protease is important under hypothermic conditions in the 34°C–37°C range. As shown in Fig. 3, the *D. sugokai* serine protease had sufficient activity at 34°C–37°C. The purified *D. sugokai* serine protease was confirmed to have a molecular mass of 38 kDa (Figs. 4 and 5) and to have similarity with the fibrinolytic protein from marine *S. nudus* (Table 1 and Fig. 6). This molecular mass is similar to those previously reported for fibrinolytic proteins from other annelids [18, 40, 41]. Finally, the purified *D. sugokai* serine protease was not cytotoxic to endothelial cells even when treated at high concentrations (Fig. 7).

In conclusion, a novel bifunctional serine protease was identified from *D. sugokai*. The serine protease had fibrinolytic and anticoagulant activities. The extracted alkaline serine protease had sufficient activity under broad pH and temperature ranges, and it did not exhibit cytotoxicity in endothelial cells. Considering these results, the enzyme activities and characteristics of *D. sugokai* serine protease warrant further research as an alternative to t-PA treatment of the ischemic brain.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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