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Magnesium Enhanced Fibrinolytic Activity of Protease from *Schizophyllum commune*

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ABSTRACT: Prevention and therapy of thrombotic diseases have attracted much attention in developed countries during recent years. Investigators have been looking for cheaper and safer thrombolytic agents for therapy of thrombotic diseases. Recently, we have discovered a fibrinolytic protease from *Schizophyllum commune*. In this study, the protease was proven to degrade blood clot effectively. Seven divalent metal ions were used to test the selectiveness on enhancing protease activity. The treated rat blood was traced by thromboelastography to assess the viscoelastic properties of whole blood. As the result, fibrinolytic activity of the protease was enhanced remarkably by Mg^{2+} in reducing the strength of blood clot and showed the innovative anti-thrombotic effects. This is the first study of anti-thrombotic effects from fungal-derived fibrinolytic protease using thromboelastography and delineates the efficacy of magnesium supplementation in enhancement of thrombolytic activity from *S. commune* fibrinolytic protease.

KEY WORDS: Fibrinolytic protease, *Schizophyllum commune*, Magnesium, Anti-thrombotic, Thromboelastography.

INTRODUCTION

Myocardial infarction, ischemic stroke and many serious cardiovascular diseases are caused by abnormal clot, or thrombus, which interferes with blood flow, oxygen and nutrient transportation in arteries or veins. In hemostasis, clot is important in wound healing by termination of bleeding and must be efficiently dissolved via fibrinolysis, induced by plasmin. The imbalance between clotting and fibrinolysis results in harmful thrombus or hemorrhage observed in humans (Norris, 2003). Intravenous administration of thrombolytic agents has become widely accepted therapy in clinical setting for thrombotic diseases (de Bono, 1995). Current clinical thrombolytic agents are plasminogen activators that convert the proenzyme plasminogen to the active enzyme plasmin, which degrades fibrin (Collen and Lijen, 2005). Recently, new thrombolytic agents are developed for fibrin-specific property, acting on the surface of thrombus that avoids excessive induction of systemic fibrinolytic system. These agents can reduce bleeding tendency induced by tissue type plasminogen activator (t-PA), single-chain urokinase-type PA (scu-PA) and staphylokinase (Ueshima and Matsuo, 2006). Because of the limited efficacy and the risk for internal hemorrhage with orally administrated thrombolytic agents, investigators are looking for cheaper and safer agents to resolve this problem (Peng et al., 2005).

Although carpophores and mushroom-derived compounds have been used clinically as therapeutic agents, their anti-thrombotic effects have not been thoroughly investigated (Sullivan et al., 2006). *Schizophyllum commune* is a commonly found mushroom that is known for its fibrinolytic activity with currently unclear mechanism. In research, a cheese-like food which exhibited thrombosis prevention activity was produced by *S. commune* derived enzymes (Okamura-Matsui et al., 2001). Usage of herbal medicine such as *S. commune* is popular amongst patients whom suffer from thrombotic diseases. The therapeutic effects on haemostasis of herbal medicine have been suggested as platelet inhibition, antithrombotic enzymes production, and thrombin-fibrinogen binding inhibition (Doljak et al., 2001; Kwok et al., 2005). The purpose of present study is to prove the anti-thrombotic functions of *S. commune* and to examine them via metal ions test through thromboelastography.

MATERIALS AND METHODS

Submerged fungi culture

Schizophyllum commune was obtained from Bioresource Collection and Research Center (BCRC, R.O.C.). It was cultured via submersion in YM broth (composed of enzymatic digestive gelatin, malt extract, dextrose and yeast extract) purchased from Acumedia, Neogen Corporation (Lansing, MI). Submerged cultures were maintained with constant stirring for 7 days at 28°C in a 15 L bottle.

Experimental animal and blood sampling

Animal studies and experimental procedures were performed in accordance with the protocol approved by the Nation Taiwan University Animal and Use Committee. Four-weeks-old male Wistar rats were acquired from Laboratory Animal Center at College of Medicine (National Taiwan University, NTU) and maintained in standard housing condition. Rats are anaesthetized by i.p. pentobarbital sodium salt (50 mg/kg) (Siegfried CMS, Zofingen, Switzerland) and blood was withdrawn by cardiac puncture and stored in a BD Vacutainer® (Becton, Dickinson and Company) without anticoagulant, and another with 0.1 M sodium citrate for citrated whole blood.

Purification of fibrinolytic protease

Fibrinolytic protease of *Schizophyllum commune* was extracted from culture broth and isolated by sequential use of hydrophobic, ion-exchange, and gel-filtration chromatography. Purified protease characterization and fibrinolytic activity identification were investigated and published (Lu et al., 2010).

Determination of protein concentration

Protein concentration of purified protease from *S. commune* was determined by Bio-Rad Protein Assay Kit I with bovine serum albumin as the standard.

Clot degradation test

Rat blood was withdrawn without anticoagulant. A column piece of clot from BD Vacutainer® tube was cut into disks in 0.5 cm height. Clot disk was placed on Petri dish, followed by dropping 0.2 μg and 0.5 μg *S. commune* fibrinolytic protease on clot disks and incubated in 20°C for 8 hrs to observe the clot degradation effects. Twenty microliter 50 mM phosphate buffer was dropped on clot disk as the control treatment and another clot disk as the blank without treatment.

Assay of protease activity

Protease activity was determined by measuring the release of acid-soluble peptides from azocasein that was used as the substrate for human plasmin (Hummel et al., 1965). In short, eluted protein fraction (100 μL) was mixed with 100 μL 0.5% azocasein solution and incubated at 37°C for 20 min, followed by the addition of ice-cold 10% (W/V) trichloroacetic acid to stop reaction and subsequently, by centrifugation for 15 min at 10,000×g at 4°C. The supernatant was mixed with an equal volume of 0.5 N NaOH, and the contained fibrinolytic activity was measured by the absorbance at 440nm. The blank absorbance was measured using boiled protein fraction and had followed the same procedures described above.

Effect of divalent metal ion on protease activity

The effects of divalent metal ions were investigated using $MgCl_2$, $CoCl_2$, $CaCl_2$, $HgCl_2$, $C_4H_6O_4Zn \cdot 2H_2O$, $CuSO_4$, $Pb(NO_3)_2$ or PBS (control). Three replicates of 90 μL protease solution were mixed with 10 μL 10 mM divalent metal ion solution for 1 hr at 37°C. After incubation, the residual protease activity was measured using azocasein assay. The effects of metal ions on protease activity were expressed as percentage of absorbance relative to the control (at 100%).

Thromboelastography (TEG) technique

TEG analysis was used to measure the dynamics of blood clot formation. A rod is suspended in a cup of blood by torsion wire. As the clot was formed, fibers composed of platelets and fibrins increased the torque on the rod by increasing viscosity. The rod's movement is restricted, and the change in the shear elasticity of the blood as a function of time is thus plotted by TEG® Analytical Software. The following measurements were obtained: R value (reaction time - the time blood is placed in the TEG until initial fibrin formation), K value (coagulation time - the time to clot formation), α value (angle - a measurement of the velocity of clot formation) and MA value (maximum amplitude - maximum strength of the developed clot) (Chandler, 1995; Landskroner et al., 2005).

Thromboelastography (TEG) analysis of fibrinolytic protease coexist to Mg^{2+}

Fresh blood was withdrawn from Wistar rat and citrated with 10% 0.1 M sodium citrate, then stored at 4°C for up to 150 min for TEG analysis. Proteases or magnesium solution were prepared in 20 μL with 50 mM phosphate buffer and loaded in cup of TEG system. Before insertion of the rod to the cup, citrated blood (300 μL) and 20 μL 0.2 M $CaCl_2$ were then added and mixed for recalcification to initiate thromboelastography. Results of TEG values were expressed relative to the control sample (at 100%).

Statistical analysis

Statistical analysis was carried out using SAS 9.1 statistical software on computer. Statistical significance was determined using ANOVA, followed by a Fisher's least significance difference test. A difference was considered statistically significant at $p < 0.05$.

RESULTS

Clot degradation test

After 8 hrs of incubation, jelly-like blood clot disks were dissolved by 0.2 μg and 0.5 μg treatments of *S. commune* fibrinolytic protease, while no visible effect was observed in the control and blank treatments (Fig. 1).

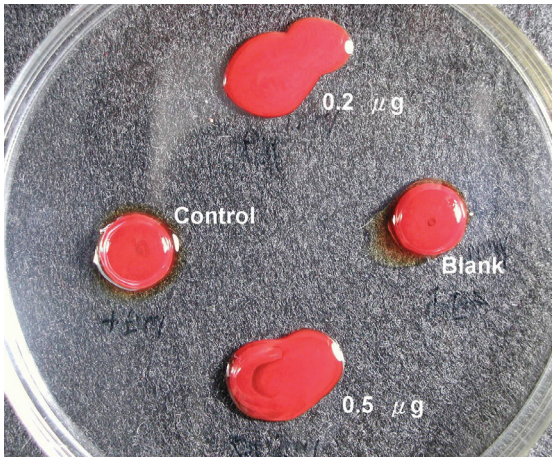

Fig. 1. Clot degradation by *Schizophyllum commune* fibrinolytic protease. 0.2 μg and 0.5 μg: blood clot disks were treated with 0.2 μg and 0.5 μg *S. commune* fibrinolytic protease respectively; Control: clot disk treated with PBS in 20 μL; Blank: blank blood clot disk.

Although the degradation ratio was unable to be determined in this test model, it was apparent that the protease was capable of degrading blood clot.

Effects of divalent metal ions on protease activity

The effect of divalent metal ions on protease activity using azocasein assay after incubation with Mg^{2+}, Co^{2+}, Ca^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+} or Pb^{2+} is summarized in Table 1. Protease activities were found to be enhanced by the addition of Mg^{2+}, but were inhibited by the addition of Hg^{2+}, Cu^{2+}, Co^{2+} or Zn^{2+}. The presence of both Ca^{2+} and Pb^{2+} showed slight effects in the protease activity (Table 1).

Thromboelastography analysis of fibrinolytic protease and Mg^{2+}

In this study, 0.6 μg *S. commune* fibrinolytic protease caused significant changes in MA value but not R, K and α value in citrated blood. Addition of 2.5 mM Mg^{2+} to citrated blood did not affect the blood coagulation status (Fig. 2). Result of magnesium ion supplementation was corresponded to an intravenous infusion experiment without interfering in coagulation (Ravn et al., 2001). With the presence of magnesium ions, 0.6 μg *S. commune* fibrinolytic protease treatment demonstrated significant decrease in MA value, and the reduction was similar to 1.2 μg fibrinolytic protease used (Fig. 2D).

DISCUSSION

Mushroom-derived proteases with fibrin degradation activity were discovered and investigated during recent years (Choi and Sa, 2001; Doljak et al., 2001; Kim and Kim, 2001; Lee et al., 2005; Li et al., 2007; Park et al., 2007; Kim et al., 2008). This activity toward fibrin substrates was reported to digest fibrin clot directly and be used in thrombolytic therapy (Omura et al., 2005; Peng et al., 2005).

Fibrinolytic metalloendopeptidases from edible mushrooms, *Armillaria mellea* (AMMEP) and *Tricholoma saponaceum* (TSMEP) have been investigated with divalent metal ion (zinc) in structure, and the metal core was shown to maintain the fibrinolytic activity in action (Nonaka et al., 1995; Kim and Kim, 2001). This study demonstrated that the activity of *S. commune* fibrinolytic protease was enhanced by Mg^{2+} but no other divalent metal ions. This phenomenon suggests the peculiarity of this protease to other mushroom fibrinolytic metalloendopeptidases and indicates that magnesium is a cofactor for this protease to execute substrate degradation.

The procedure of thromboelastography (TEG) was developed in 1948 (Hartert, 1948). It has been applied to assay viscoelastic properties of whole blood during clinical cardiac and transplant operations. Moreover, it provides the important interactions between cellular and plasmatic coagulation factors which are absent in test of prothrombin time (PT) and activated partial thromboplastin time (APTT) (Landskroner et al., 2005). Blood from living organisms in different states of hemostasis can be monitored by TEG tracings (Mallett and Cox, 1992). In this study, *S. commune* fibrinolytic protease reduced the clot amplitude without inhibition of blood coagulation initiation, clotting process and clotting speed (reflected on R, K and α values). The protease

Table 1. Effects of divalent metal ion on protease activity of *Schizophyllum commune* fibrinolytic protease.

Ions	Chemical reagent	Concentration	Residual activity (100%)[a]
Mg^{2+}	$MgCl_2$	10 mM	386 ± 27
Co^{2+}	$CoCl_2$	10 mM	50 ± 7
Ca^{2+}	$CaCl_2$	10 mM	104 ± 6
Hg^{2+}	$HgCl_2$	10 mM	22 ± 6
Zn^{2+}	$C_4H_6O_4Zn \cdot 2H_2O$	10 mM	77 ± 9
Cu^{2+}	$CuSO_4$	10 mM	29 ± 2
Pb^{2+}	$Pb(NO_3)_2$	10 mM	96 ± 5
Control	PBS buffer	50 mM	100

[a] Protease activity to azocasein was expressed as percentage of absorbance relative to the control (at 100%).

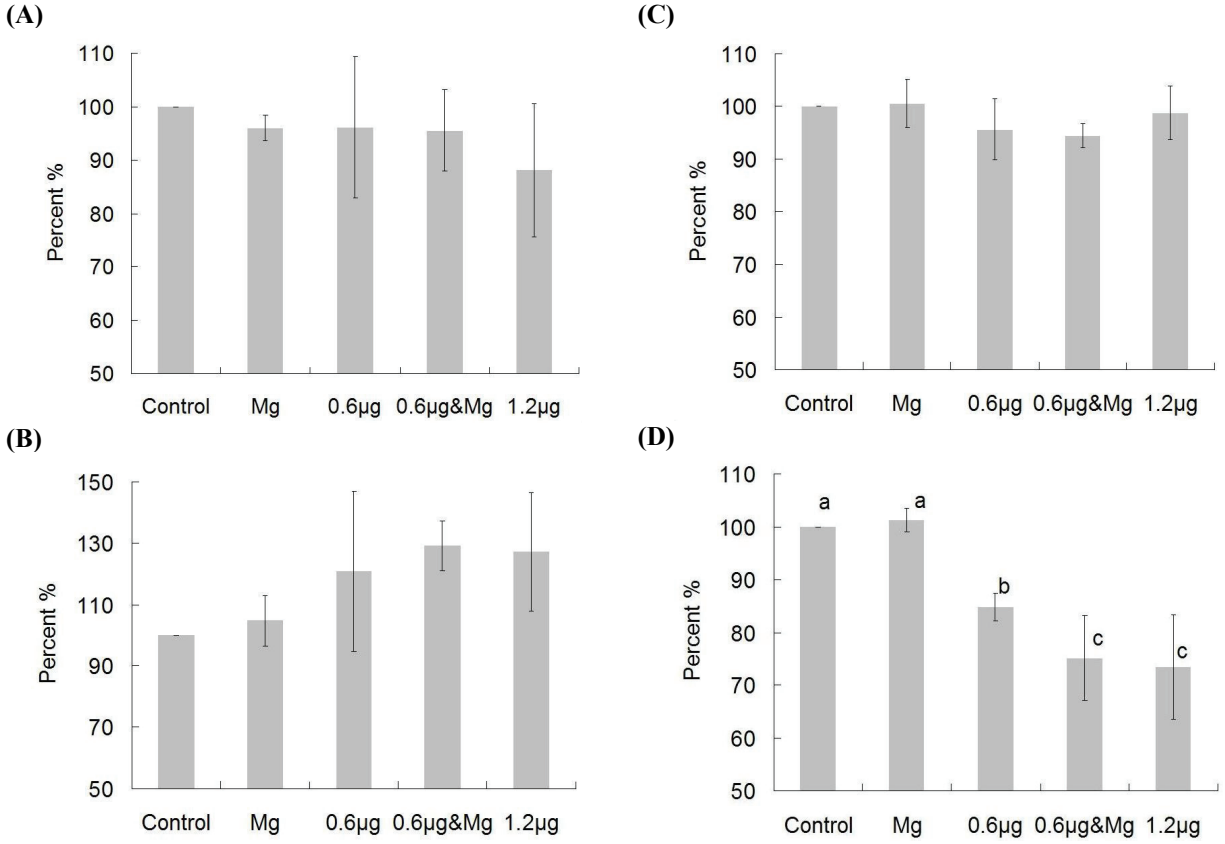

Fig. 2. Thromboelastography analysis of fibrinolytic protease and Mg^{2+}. A: R value (reaction time - the time of blood placed in TEG until initial fibrin formation). B: K value (coagulation time - the time to clot formation). C: α value (angle - the velocity of clot formation). D: MA value (maximum amplitude - maximum strength of the developed clot). Control: citrated blood mixed with PBS; Mg: citrated blood mixed with $MgCl_2$ (2.5 mM); 0.6 μg and 1.2 μg: citrated blood mixed with 0.6 μg and 1.2 μg *S. commune* fibrinolytic protease respectively; 0.6 μg&Mg: citrated blood mixed with 0.6 μg *S. commune* fibrinolytic protease and 2.5 mM $MgCl_2$. Alphabetical lowercase means significant variation between other treatments, a difference was considered statistically significant at $p < 0.05$. TEG values were expressed relative to the control sample (at 100%).

exhibits a digestive activity mainly on blood clotting fibrins but neither on platelets nor plasminogen. Thus, it is likely to function as a fibrin-specific thrombolytic agent, which prevents systemic fibrinolysis and acute bleeding.

Magnesium therapy in coronary heart disease has recently been proposed and beneficial effects have been documented in clinical trials (Shechter et al., 1999). In experiments of model animals, infusion of $MgSO_4$ significantly reduces stent thrombosis in dogs and swine (Rukshin et al., 2001; Rukshin et al., 2002). Magnesium supplementation with an antithrombotic agent inhibits platelet activation and alters outcomes in patients by inhibition of platelet-dependent thrombosis (Whiss and Andersson, 2002). Intravenous magnesium infusion singly was observed with no significant differences on coagulation or fibrinolysis processes of citrated blood (Ravn et al., 2001). Similar results were also observed in the present study. From our results, magnesium supplementation could enhance *S. commune* fibrinolytic protease activity to reduce blood clot amplitude. The effect suggests that the protease and magnesium may be considered as an adjuvant to standard thrombolytic therapy in the future, while further researches are still needed.

In conclusion, magnesium ion enhanced the activity of *S. commune* fibrinolytic protease, which was proven to degrade blood clot effectively in this study. A regulatory manipulation in this fibrinolytic protease action by magnesium supplementation can also be expected in the future. Additionally, magnesium supplementation may reduce the cost of thrombolytic therapy by providing an alternative to the currently used fibrin-specific antithrombotic agent. The up-regulation mechanism provides a niche to the development of new antithrombotic therapy protocol with magnesium for clinical application.

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鎂離子提升裂褶菌 (*Schizophyllum commune*) 溶纖蛋白酶之活性影響

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摘要：近年來，已發展國家中的人們對於預防以及治療血栓性疾病，皆投注以相當高的關注，而同時，科學家們也希望由研究中，可再找到更經濟與安全性更高的抗血栓藥物，來治療血栓性疾病。由本實驗室先前的研究發現，自菇蕈類-裂褶菌 (*Schizophyllum commune*) 醱酵培養物中，有一個具備溶解纖維蛋白活性的活性蛋白酶 (fibrinolytic protease)；於本篇研究中，此溶纖蛋白酶再被證明其具有效溶解血液凝塊的作用；並且，由實驗篩選出對此溶纖蛋白酶活性，具有刺激效果的二價金屬離子，進一步使用溶纖蛋白酶與二價金屬離子共同之處理，觀察其對大鼠血液血栓彈力圖譜 (thromboelastography, TEG)之影響，研究血液凝固作用的進行，與當中各項參數變化。由結果發現，鎂離子具有最明顯提升溶纖蛋白酶活性，且於大鼠血液血栓彈力圖譜分析，鎂離子與溶纖蛋白酶的添加亦顯著地降低血液凝固強度 (clotting strength)，而無抑制血液凝固發生與進行速度之影響。因此，總結以上結果，鎂離子提升作用具有提高溶纖蛋白酶抗血栓形成的有利影響性；而此，本篇文章為首度以血液血栓彈力圖譜分析，觀察真菌溶纖蛋白酶對抗血栓形成影響的研究，並且也指出鎂離子對於提昇裂褶菌溶纖蛋白酶抗血栓活性的有效性。

關鍵詞：溶纖蛋白酶、裂褶菌、鎂離子、抗血栓形成、血液血栓彈力圖。