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Original Article

EXPRESSION AND ENZYMATIC PROPERTIES OF A UNIQUE RECOMBINANT ANTICOAGULANT AND FIBRINOLYTIC ENZYME FROM *ACINETOBACTER BAUMANNII* TU04

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ABSTRACT

Objective: The objective of this research is to clone and express a new fibrinolytic enzyme encoding serine protease gene in *Escherichia coli* thus, characterize such purified recombinant.

Methods: The recombinant clone was successfully expressed in Lemo21 system and purified using immobilized nickel cation affinity chromatography on a His•bind resin®, followed by ammonium sulfate precipitation and protein filtration in combination. General properties of the purified enzyme were investigated, including the molecular weight, effects of inhibitors and metal ions, substrate specificity, amylolytic activity, fibrinolytic activity and effect of anticoagulant activity *in-vitro.*

Results: The recombinant clone was expressed in Lemo21 system in the cytoplasm in a soluble and active form. The resulting enzyme, SERpro was purified to homogeneity with a purification of 19.35-fold and recovery yield of 4.85%. The enzyme exhibited maximal activity at 37 °C and at pH7.4, respectively. The molecular weight of the purified enzyme was 82 kDa, determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fibrinogenolysis peptide sequence analysis revealed that SERpro degraded Bβ chain of Fibrin at a much lower rate but cleaved Aα and γ-chain extensively. The enzyme was activated by metal ions such as Mg^{2+}, $Fe^{3+}$and Zn^{2+}, and was inhibited strongly by PMSF. The clotting time of human blood serum in the presence of 1U SERpro reached a relative partial thromboplastin time of 13.9% with a 1.14-fold increase.

Conclusion: The study deduced SERpro as a new protease with anti-thrombotic activity from *Acinetobacter baumannii* TU04.

Keywords: Fibrinolytic enzyme, *Acinetobacter baumannii*, subtilisin-like serine protease, Lemo21 expression host, Anticoagulant.

INTRODUCTION

Cardiovascular diseases (CVDs) are caused by the disorders of heart and blood vessels [1]. According to health data statistic published by World Health Organization (WHO) 2013, CVDs are projected to remain as the single leading cause of mortality, accounting for 23.3 million deaths in 2030 [2-3]. Thromboembolism is the most prevalent type of CVD [4]. Thrombolytic therapy is the best way to achieve recanalization for this complication [5-8]. Thrombolytic agents characterized by its ability to hydrolyze thrombi via direct degradation or plasmin activation reveals a significant importance in the prevention and treatment of thrombosis [8]. Various potent thrombolytic agents have been successfully identified and characterized from animals [9-11], plants [12] and microorganism [7, 13-15].

Recently, studies have also demonstrated the efficacy of thrombolytic agents such as tissue-type plasminogen (t-PA) [16-17], single-chain urokinase-type plasminogen activator (scu-PA or pro-urokinase, Pro-UK) [18] and plasmin-like protein [19] in the recanalization and reperfusion of thrombo-embolism. However, their hemorrhagic side-effects, low specificity, modest reliability and benefits [8] have prompted a quest for alternative agents, from other sources that are safer and more potent to be developed into therapeutic drugs for thrombo-embolism.

In general, fibrinolytic agents derived from Asian traditional fermented foods can function as an adjunct to conventional cardiovascular disease therapy through their incorporation as food additives and nutraceutical application, which can help in preventing cardiovascular disease [6, 14]. In this study, a bacterial strain isolated from *Tapai Ubi*, a traditional Malaysian fermented food, identified as *Acinetobacter baumanii* TU04 [20] were analyzed for its thrombolytic potential. Prior to our report, fibrinolytic proteases had not yet been reported from *Acinetobacter baumannii,* therefore, in this study we describe the molecular cloning and expression of a new fibrinolytic enzyme encoding serine protease gene in *Escherichia coli* and the characterization of the purified recombinant enzyme.

MATERIALS AND METHODS

Chemicals

Human plasma fibrinogen, Thrombin (EC 3.4.21.5) from bovine (Calbiochem Cat# 605157), Plasmin from human plasma (10 602 361 001) [5U], Plasminogen, Perfect Protein™ Marker (Novagen, 69149-3) and Prestained Protein Marker, (NEB#P7702) were used. Specific kits for sample extraction and purification were purchased from Promega and Qiagen. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) set and reagents were bought from Bio-Rad Co. (Richmond, CA, USA). Chromatography column and His® Bind resin were purchased from GE Healthcare. Other fine chemicals and reagents used were of analytical grade.

Bacterial strains and growth conditions

Acinetobacter baumannii TU04 isolated from *Tapai Ubi*, a traditional cassava-fermented food, was grown at 37 °C, 200rpm for 16 h in basal medium containing 1% (w/v) tryptone, 0.5% (w/v) beef extract, 1% (w/v) NaCl, pH 7.0±0.2 (Accession No: KP204010). *Escherichia coli* DH5α was used in cloning steps and Lemo21 (DE3), which contains the T7 RNA polymerase gene under the IPTG-inducible *lac*UV5 promoter, was used for the expression of the enzyme under T7 promoter control. *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium supplemented with ampicillin of 100 µg/ml at 37 °C when necessary.

Construction of protease cloning and expression system

Genomic DNA of *Acinetobacter Baumannii* TU04 was isolated by the procedure as described Sambrook (1989) [21]. Two degenerate oligonucleotide primers were synthesized to amplify DNA fragments encoding the subtilisin-like serine protease gene: forward primer, AsLf*Eco* R1: 5' GCC GCA GAA TTC ATG ACA AGG ATA ATC GTA GCA TCC 3' (with *Eco* R1 restriction site as underlined) and reverse primer, AsLrXho 1: 5' CTG GCT TCT CGA GCT AAA CAA CTG GGT AAG TCT GAT 3' (with Xho 1 restriction site shown as underlined). These primers were synthesized based on sequences of the conserved

regions of previously cloned fibrinolytic proteases of *Bacillus*, collected from GenBank. The PCR mixture was prepared in a total volume of 50 µl containing 5U FirePol DNA polymerase, 10X buffer B, 25 mM $MgCl_2$, 20 mM dNTP mix, 10 µM forward and reverse primer each and approximately 3-5ng of genomic DNA of *Acinetobacter baumannii* as the template. The PCR product of about ~2.2 kb was gel extracted, and its nucleotide sequence was determined.

The amplicons were digested with Eco R1 and Xho 1, and the isolated fragments ligated into Eco R1 and Xho 1 digested pET-22b (+) vector (Novagen, Darmstadt, Germany). The resulting plasmids were named as pET22SERpro and was introduced into *E. coli* DH5α. DNA manipulations including digestion with restriction enzymes, purification of DNA fragments, ligation with T4 DNA ligase and transformation were performed as described by Sambrook (1989) [21]. The analysis of sequence data and sequence similarity searches were performed using the BLASTn program of the National Center for Biotechnology Information (NCBI). Homology alignment was performed with the CLUSTALw program using MEGA Version 6.06 software. The plasmid DNA prepared from these clones was then sub-cloned into the expression host, Lemo21 (DE3).

Gene expression of SERpro

E. coli cells strain Lemo21 (DE3) (New England Biolabs, C2528H) harboring the recombinant pET22b-SERpro were grown in Luria Bertani medium supplemented with 50 µg/ml of ampicillin, 100 µg/ml chloramphenicol and 1000 µM L-rhamnose at 37 °C, with shaking until growth reached $A_{600\ nm}$~0.65. Expression of the gene was induced by addition of IPTG to a final concentration of 0.4 mM and the incubation was further extended for an additional 19 h at 30 °C. The cells were harvested by centrifugation at 5,500 rpm for 30 min at 4 °C, washed twice in 1X PBS buffer (pH8.0) containing 5 mM $MgCl_2$, resuspended in the same buffer with the addition of 5 mM lysozyme and lysed by sonication for 10 min with a 5 sec pulse. The samples were then centrifuged at 8,100 rpm for 20 min to separate and remove the insoluble fractions.

Purification of the recombinant SERpro enzyme

The cell-free extract from the lysate from 3L of induced cells harboring pET22b-SERpro was used for purification of recombinant protein. All purification steps were carried out at 4 °C. The extracted protein were adjusted to the final concentration of 50 mM Phosphate buffer (pH7.4) and were pre-incubated with His•bind resin® at 4 °C with slow agitation for at least an hour though generally not longer than 3 h. Following incubation, the resin containing bound SERpro+His protein was transferred to a gravity flow column® (2.6 cm X15 cm) and washed with 20 column volume (CV) of buffer A (50 mM Phosphate buffer; pH7.4 with 1% Triton X-100). The protein of interest was eluted using elution buffer (buffer A containing 250 mM imidazole) at a flow rate of 1.0 ml/minute. Fractions containing the protein were pooled and dialyzed against buffer B (50 mM Phosphate buffer; pH7.4) and concentrated using VivaFlow 10, 000 MWCO, PES membrane. The concentrated eluates were pooled and subjected to 60% ammonium sulphate precipitation. The precipitated enzyme was dissolved in buffer B and dialyzed against buffer B to remove salt in excess, and analyzed for purity by SDS-PAGE. Protein concentration was determined according to Bio-Rad protein assay with bovine serum albumin (BSA) as the standard. A blank was prepared in same conditions, but enzyme was replaced with buffer. The assays were performed in triplicates.

Enzyme activity assay

Fibrinolytic enzyme activity was determined colorimetrically, using synthetic fibrin clot methods [22-23] with minor modifications. The reaction mixture containing 1% (v/v) fibrinogen human plasma, 10U thrombin from bovine, 50 mM phosphate buffer (pH 7.4±0.2) and purified enzyme was incubated at 37 °C for 180 min. The reactions were stopped by the addition of 750 µl of 15% (w/v) trichloroacetic acid (TCA) solution (0.11M TCA, 0.22M Sodium acetate and 0.33M acetic acid) for 30 min at room temperature. The reaction mixture was centrifuged at 5,500 rpm for 10 min and 100 µl of supernatant was incubated with 2 ml of 0.55M Na_2CO_3 and 1 ml of Folin-Ciocalteau (Sigma Aldrich; St. Louis, MO, USA) at 37 C for 30 min. The absorbance was recorded at 660 nm. Caseinolytic activity was assayed according to Lee, (2006) [22]. One unit of fibrinolytic or caseinolytic activity (CU) was defined as the amount of enzyme releasing 1 µmol of tyrosine equivalent per min under the assay conditions.

Amidolytic activity was assayed using Kim (1996) [7] methods with minor modifications. One unit of amidololytic activity (AU) was expressed as micromoles of substrates hydrolyzed per minute per milliliter by the enzyme. The kinetic constant was determined using Hanes-Woolf plots based on the initial reaction rates.

SDS-PAGE analysis and Immunoblotting

SDS-PAGE was performed on 12% running gel as described by Laemmli (1970) [24], and the resolved proteins were visualized by coomassie staining. For western blot analysis, protein samples were subjected to SDS-PAGE and then wet-transferred onto nitrocellulose membrane. The membrane was blocked with 3% (v/v) skim milk in PBST (20 mM PBS, pH 8.0, 0.1% Tween-20) at room temperature for at least 1 h, and then incubated with mouse anti his: hrp conjugated antibody in PBST with 1% (v/v) skim milk (diluted 1:1000). Immunodetection was performed using the Opti 4CN substrate kit according to manufacturer's instructions.

Effect of pH on fibrinolytic activity and stability

The following buffers were used to investigate the effect of pH upon the purified enzyme activity: 100 mM sodium acetate buffer, pH 4.0-5.0, 100 mM sodium phosphate buffer; pH 6.0-7.0, 100 mM Tris-HCL; pH 8.0-9.0 and 100 mM glycine-NaOH buffer; pH 10.0-11.4. The optimal pH for the purified enzyme was determined by incubating the enzyme at 37 °C with different buffers and the residual activities were determined. The effect of pH on enzyme stability was determined by pre-incubating the purified enzymes in buffers with different pH values for 24 h at 37 °C.

Effect of temperature on fibrinolytic activity and stability

The optimal temperature for the purified enzyme was determined over the range of 08-60 °C by spectrophotometric assay. The thermal stability was determined by incubating the purified enzyme at various temperatures for 60-240 min at pH 7.4, and the residual activity were measured.

Effect of metal ions and protease inhibitors

The effect of metal ions on the enzyme activity was determined using $CoCl_2$, $MgSO_4$, $ZnSO_4$, $MnCl_2$, $Fe_2(SO_4)_3$, KCL, $CuSO_4$ and $CaCl_2$. The purified enzyme was pre-incubated with various metal ions at a concentration of 5 mM for 6 hr at 37 °C and the residual activities were determined. The activity of the enzyme in the absences of metal ions was taken as 100%.

The effect of protease inhibitors were investigated using 5 mM of diamine tetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, ethylene glycol tetraacetic acid (EGTA) and Dithiothreitol (DTT). The enzyme was pre-incubated with the protease inhibitors at 37 °C for 30 min and the residual activity was determined spectrophotometrically.

Fibrinogenolytic activity analysis

A reaction mixture consisting of 30 µl 1% (w/v) human fibrinogen solution in 50 mM Tris-HCl buffer, pH7.8 containing 0.1M sodium chloride was prepared. 30 µl of the purified enzyme was incubated with the reaction mixture at 37 °C for 24 h. At each interval of 3 h, the reaction was stopped by adding 90 µl of denaturing solution (4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol and 10M Urea). The polymerizations of fibrinogen monomer were observed by turbidity changes with time and were separated on 12% SDS-PAGE gel according to Laemmli method. Fibrinogen without SERpro enzyme was pre-checked as a negative control.

***In vitro* coagulation assays**

Anticoagulant activity *in vitro* was determined as an increase in the relative partial thromboplastin time (RPTT) using a Batomunkueva

and Egorov (2001) [25] modification of the method described by Strukova (1989) [26]. The reaction mixture containing 0.1 ml of the human blood serum, 0.1 ml of a thromboplastin suspension and 0.1 ml of kaolin was incubated in a water bath at 37 °C for 2 min and then rapidly mixed with 0.1 ml of 0.3% (w/v) $CaCl_2$. The time of the blood serum clotting was determined using a stopwatch after addition of 0.1 ml of pure enzyme. The control mixture contained an equivalent amount of 0.86% (w/v) physiological saline solution. The relative PTT was calculated using the formula [(A-B)/B] X 100%, where A and B are the time (in seconds) of the blood serum clotting in the presence and absence of the enzyme, respectively. The data was expressed as mean values of three replications.

RESULTS AND DISCUSSION

Cloning and sequencing of the protease gene

To obtained the clones with fibrinolytic enzyme gene of *Acinetobacter baumannii* TU04, potential serine protease encoding fibrinolytic enzyme gene sequence of *Bacillus* was aligned with the chosen subtilisin-like serine protease gene of *Acinetobacter baumannii* obtained from GenBank of NCBI. Sequences with high similarity; the subtilisin-like serine protease gene from *A. baumannii* MDR-ZJ06 deposited by Zhou (2011) [27] with the Accession No: YP_005526853.1 were referred as the potent fibrinolytic protease gene and were subjected as the study template for cloning. The full-length sequence of the gene of interest was intensively analyzed (data not shown) and cloned using pET-22b cloning/expression vector. The obtained recombinant clone was introduced into *E. coli* DH5α and the open reading frame (ORF) of the DNA fragment was analyzed.

The gene sequence analysis of the plasmid pET-22b of about 5,943bp with gene insert of 2.184kb revealed an open reading frame (ORF) starting at bp 360 with an ATG codon and ending at a TGA codon at 2214, thereby encoding a protein of 739 amino acids. The nucleotide sequence and the deduced amino acid sequence were shown in fig.1**.** GenBank accession number for this sequence is KP204011. The ORF translated protein has a predicted molecular mass and iso-electric point of about 76,028 Da and 4.6, respectively.

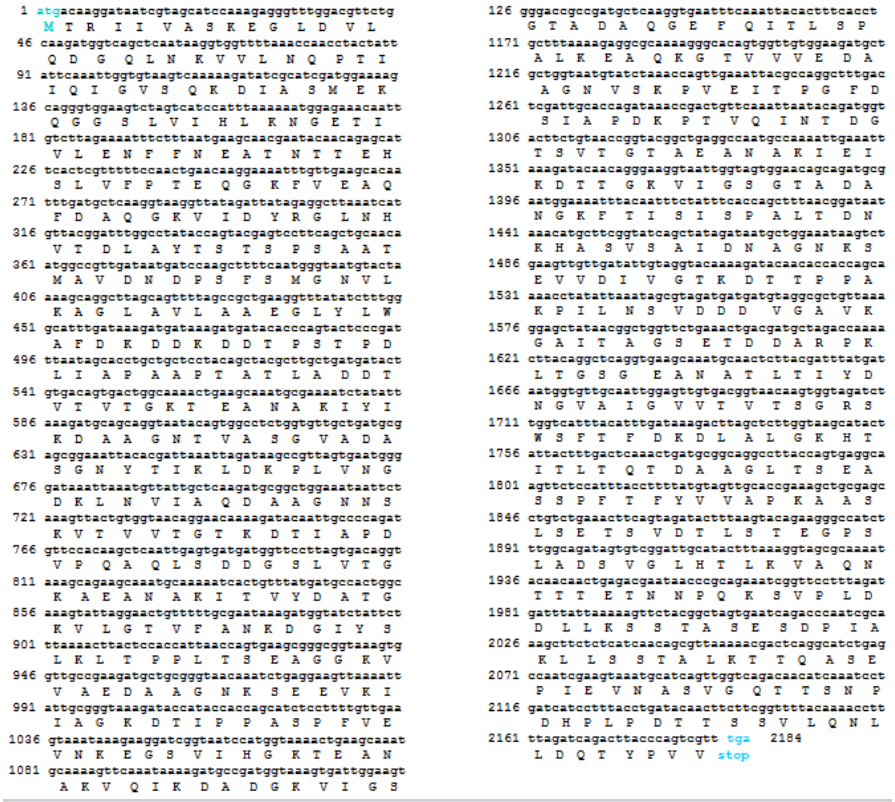


Fig. 1: The nucleotide and deduced amino acid sequences of genomic DNA encoding SERpro clone

A BLASTx search of the completely deduced amino acid sequence of the gene (739 amino acid; Fig.1) showed a high homology of about 98% (1370 total score) with subtilisin-like serine protease of *Acinetobacter baumannii* [Gen Bank accession number WP_002000057.1]. The recombinant clone do not have any signal peptide sequence based on its amino acid composition and hydrophobicity profile, a common characteristic of the extracellular protease.

The deduced amino acid sequence of the gene (fig. 1) was also aligned with other established fibrinolytic enzymes; protein sequences obtained from the protein database of NCBI using CLUSTALw program using MEGA Version 6.06 software. The resulting phylogenetic tree was illustrated in fig. 2. The dendrogram analysis showed an analogous sequence similarity of about 89% with other established fibrinolytic proteases. The apparently high level of sequence similarity suggests that this protein could be a new protease with fibrinolytic function.

Over-expression and purification of the recombinant clone

Plasmid pET22b-SERpro was transformed into Lemo21 (DE3) system to express the target protein, regulated by the IPTG-inducible *lac*UV5 promoter. SDS-PAGE analysis showed that SERpro-His-tagged protein was successfully expressed after induction with 0.4 mM IPTG for 19 h at 30 °C (fig. 3, lanes 3 and 5). A new protein band corresponding to the expected size (~82kda) was observed in total cell extract and soluble fraction of induced cells. The amount of expressed protein, SERpro was approximately 6.8% of the total soluble proteins of the intracellular fraction. These results indicated that this protein was not efficiently over-expressed in the cytoplasm of Lemo21 although optimization was carried out in the presence of increasing concentration of L-rhamnose in standard Luria-Bertani broth (result not shown).

SERpro was purified from the intracellular fraction of Lemo21 using a combination of chromatographic steps summarized in table 1. The

measured protein concentration, fibrinolytic activity and SDS-PAGE analysis were used as indices of purification. The recombinant SERpro purification steps gave an overall purification of 19.35 fold. The overall activity yield of the purified SERpro was 4.85% with a specific activity of 2.07 U/mg of protein. The purified SERpro was delineated to be homogeneous based on SDS-PAGE (fig. 3, lane 7). Western blotting revealed that the band indeed corresponded to a recombinant his-tagged protein (fig. 3, lane 8). The apparent molecular weight of the enzyme was calculated based on the bands separations on SDS-PAGE using a standard curve established with the proteins of known molecular weight. The predicted molecular weight of SERpro 76,028 kDa correlates with the purified SERpro molecular weights of 82 kDa thus indicates that the enzyme is monomeric.

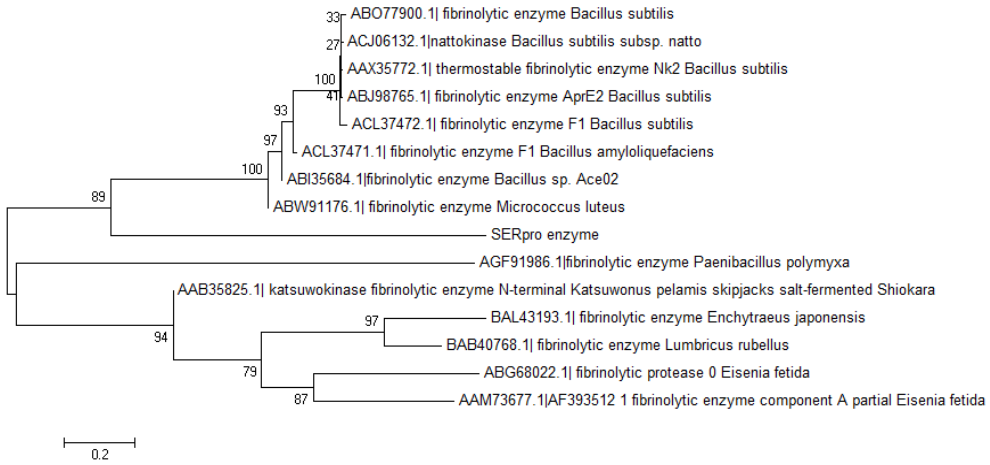


Fig. 2: Dendrogram generated based on the protein sequence alignment of the SERpro protease gene of *Acinetobacter baumannii* TU04 with references fibrinolytic enzymes using bootstrap replication of 1,000. Reference fibrinolytic protease sequences are shown with their respective gene bank accession number

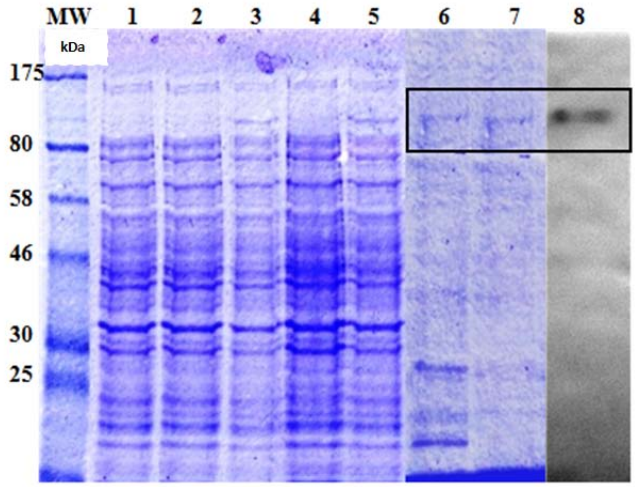


Fig. 3: SDS-PAGE analysis illustrating expression and purification of recombinant SERpro. Lane M: Perfect Protein™ marker; Lanes 1 and 2: IPTG-induced and uninduced whole cell lysate without SERpro gene insert, respectively; Lanes 3 and 4: IPTG-induced and uninduced whole cell lysate with recombinant SERpro gene, respectively; Lane 5: The extracted membrane protein from the 3L lysate of induced pET22b-SERpro transformant; Lane 6: Purified sample using His-bind resin; Lane 7: Purified SERpro after subjected to 60% ammonium sulfate precipitation; Lane 8: Immunoblotting assay using mouse anti his: hrp conjugated antibody at the C-terminus of the recombinant SERpro

Table 1: Purification summary of the SERpro enzyme from Lemo21

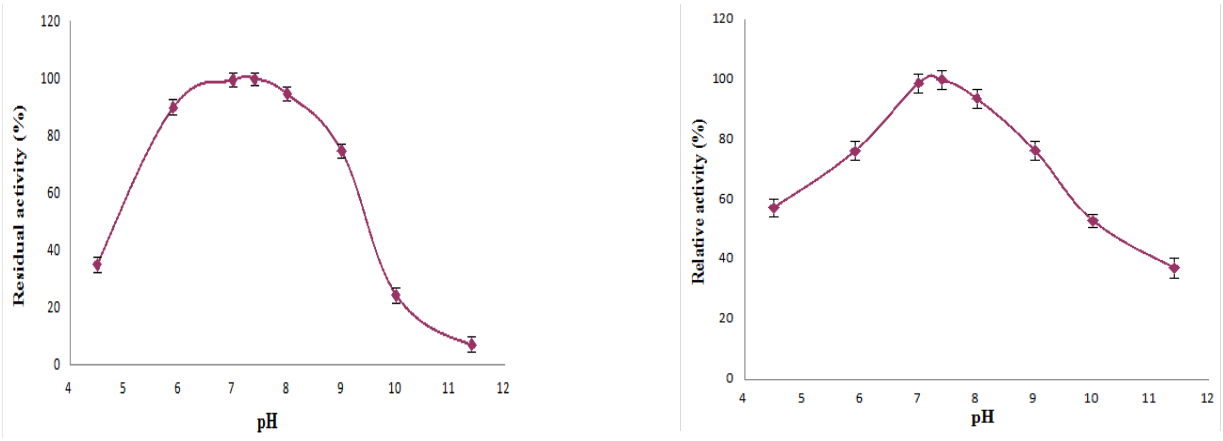
Sample	Total enzyme activity (U/ml)[a]	Total protein (mg)	Specific activity (U/mg)	Yield (%)[b]	Purification factor (fold)
Crude lysate	73.8	690.84	0.107	100	1
His•bind resin	53.13	51.25	0.833	71.99	7.785
Ammonium sulfate precipitation	6.77	6.528	1.037	9.17	9.692
VivaSpin, 50kDa	3.58	1.73	2.07	4.85	19.346

[a]One unit of total activity (U/ml) was determined by measuring the liberated 1 µmoles of L-tyrosine/ml/min at 37 °C, [b]Yield was calculated based on total enzyme activity (U/ml)

Effect of pH and temperature on fibrinolytic activity

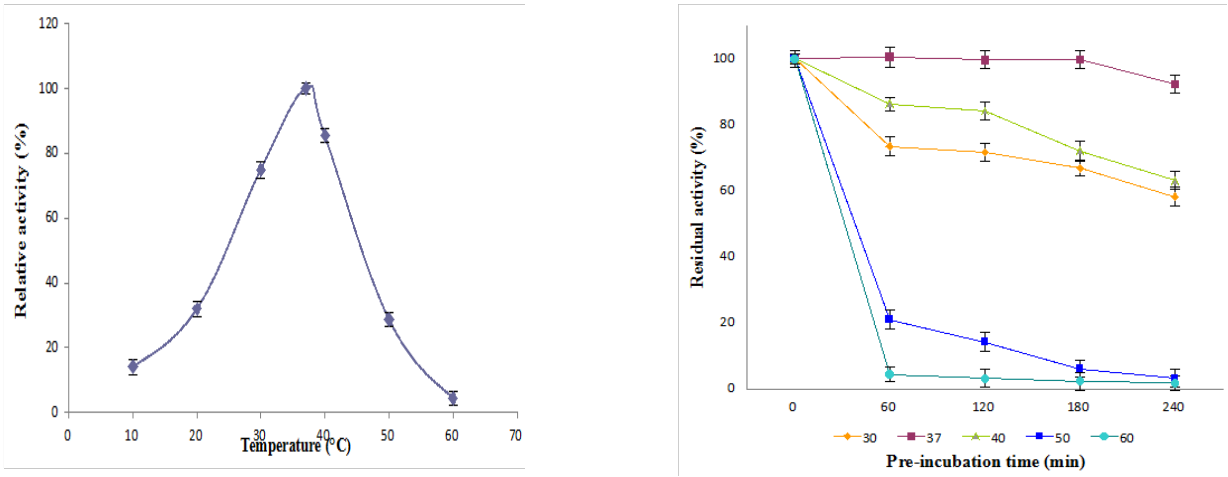
The purified SERpro showed optimal catalytic activity at pH7.4 (fig. 4a) and retained more than 60% of its activity at 37 °C for up to 24 h in various buffers over a pH range of 6.0-9.0 (fig. 4b).

The optimum temperature for SERpro activity was 37 °C (fig. 5a). The thermal stability profile showed that the SERpro retained 90% of its initial activity after 4 h incubation at 37 °C and possessed<5% residual activity after 4hr incubation at 50 °C and 60 °C, respectively (fig. 5b).



(a) (b)

Fig. 4: Effect of pH on fibrinolytic enzyme (a) activity and (b) stability of SERpro



(a) (b)

Fig. 5: Effect of temperature on the fibrinolytic enzyme (a) activity and (b) stability of SERpro

Effect of metal ions and protease inhibitors on fibrinolytic activity

The effect of metal ions and various protease inhibitors on the SERpro activity is as shown in fig. 6. The SERpro residual activity was calculated on the basis of the activity of enzyme without any metal ion or inhibitor under the same reaction condition. The SERpro activity increased significantly in the presences of 5 mM Mg^{2+}, $Fe^{3+}$and Zn^{2+}but was completely inactivated by the 2 mM PMSF, which is a well-known serine protease inhibitor. The enzyme was also inhibited by EDTA and EGTA with 45% of its original activity was lost. However, the other inhibitors such as 2-mercaptoethanol and DTT only cause mild inhibition, 16.9-29.9% respectively, indicating the importance of the serine residue of this enzyme for enzyme activity. The data was expressed as mean values of three replications with their corresponding standard deviation.

Substrate specificity of SERpro

The fibrinolytic activity and caseinolytic activity of SERpro was measured and the ratio of fibrinolytic to caseinolytic activity was calculated. The SERpro enzyme hydrolyzed both casein and fibrin at 0.357 U/ml and 0.5584 U/ml, respectively and with substrate specificity (F/C) of 1.56. This result concludes that SERpro has relatively high specificity for fibrin as substrate.

The amidolytic activity of SERpro was investigated using several synthetic substrates (table 2). The SERpro enzyme exhibited the highest degree of specificity for the substrate N-Succinyl -Ala-Ala-Pro-Phe-*p*NA (for subtilisin and chymotrypsin), similar with other established fibrinolytic enzymes such as subtilisin natto (27.7 kDa) [13], subtilisin DFE from *Bacillus amyloliquefaciens* [15], IMR-NK1 from *Bacillus subtilis* [28], and subtilisin DJ-4 from *Bacillus* sp. DJ-4 [29].

The Michaelis-Menten constant, *Km* and *Kcat* for the hydrolysis of N-succinyl-Ala-Ala-Phe-*p*NA were calculated to be 0.55 mM and 16.8 S^{-1}, respectively. The *Km* and *Kcat* value for the hydrolysis of Pyro-Glu-Gly-Arg-*p*NA was not determined due to the relatively low level of the amidolytic activity (AU), which were non-significant for SERpro characterization.

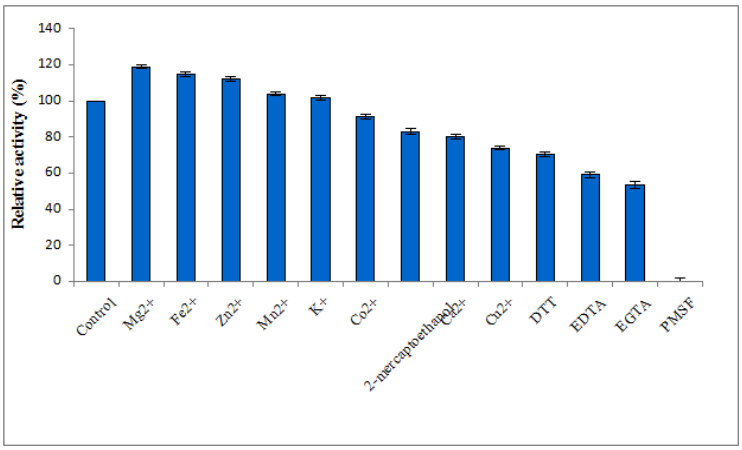


Fig. 6: Effect of metal ions and protease inhibitors on the fibrinolytic activity of SERpro enzyme

Table 2: Amidolytic activity of SERpro enzyme [a] with different synthetic substrates

Synthetic substrate	Characteristic	Concentration (mM)	Amidolytic activity (AU)
H-D-Val-Leu-Lys-*p*NA[b]	Plasmin	0.5	59.17±0.71
Pyro-Glu-Gly-Arg-*p*NA	Urokinase	0.5	1.53±0.02
N-Succinyl-Ala-Ala-Pro-Phe-*p*NA	Subtilisin or Chymotrypsin	0.5	136.42±0.41

[a]Data are expressed as mean values of three replications with their corresponding standard deviation, [b]*p*NA, *p*-nitroanilide

Fibrinogenolytic activity analysis

The fibrinogenolytic mode and rate of hydrolysis for the SERpro was elucidated using SDS-PAGE. As illustrated in fig. 7, the SERpro rapidly degraded the Aα and γ-chain but hydrolyzed Bβ chains at a much lower rate. The peptide (MW~37.5kDa) remained uncleaved even after 24 h of incubation. This remnant peptide could be γ' species originated from polypeptide fragment D. This species was less susceptible to SERpro enzyme may be due to the fact that the three subunit chains of fibrinogen differed in their physical and/or chemical compositions [30]. As a summary, the fibrinogenolysis pattern of SERpro was determined to be Aα->Bβ->γ-, which was found to be similar with PPFE-1 [8] and subtilisin DJ-4 [29].

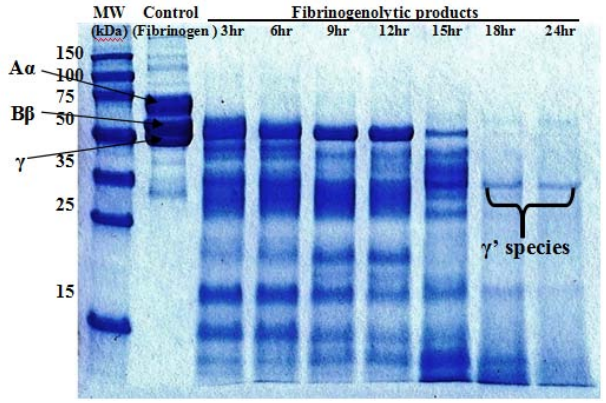


Fig. 7: Fibrinogen hydrolysis by the SERpro fibrinolytic enzyme. Lane 1: Fibrinogen without SERpro enzyme after 24h incubation; referred as negative control; Lanes 2-8: Fibrinogenolytic products of SERpro incubated at 37°C; reaction stopped at every 3hr interval for 24h

Clotting time

The coagulation mechanisms of the SERpro was analyzed by the relative partial thromboplastin time (RPTT), which is a common clinical test for measuring the efficacy of the both extrinsic and the common coagulation pathways [1, 25]. The anticoagulant activity of SERpro enzyme was expressed as relative PTT (%) (table 3). Based on the table 3 results, the clotting time of human blood serum was substantially prolonged with SERpro fibrinolytic enzyme to 94 s in the presences of 1.0U of the purified SERpro; thus relative PTT reached 13.9% with the 1.41-fold increase. Similar value has been reported by Wesam (2011) [31] for fibrinolytic enzyme K42 from *Bacillus subtilis* K42. However, a higher dosage of about 100U/0.1 ml enzyme was used to analyze the anti-thrombotic effect, resulting RPTT of 241.7%. Correlatively, if a higher dose of SERpro enzyme were envisaged, it is presumed that SERpro would be the pre-eminent. This feature resolute SERpro as a potent anticoagulant and/or thrombolytic agent.

Table 3: *In vitro* application of SERpro fibrinolytic enzyme as anticoagulant

Dose (U/0.1 ml)	Clotting time (s)	RPTT (%)
Controls	82.5±0.71	0.0
0.5	87±0.5	5.5
1.0	94±0.83	13.9

CONCLUSION

The overall findings of SERpro with respect to the molecular weight, effects of inhibitors and metal ions, substrate specificity, fibrinogenolytic activity and effect of anticoagulant indicate SERpro as a new fibrinolytic enzyme. In conclusion, the present results suggest SERpro fibrinolytic enzyme obtained from *Acinetobacter baumannii* TU04 may become a new source of fibrinolytic agents and further studies on its safety and functional evaluations are required and underway.

ABBREVIATION

BSA, bovine serum albumin; CBB, Coomassie brilliant blue; IPTG, isopropyl-β-D-1-thiogalactopyranoside; *p*NA, *p*-nitroanilide, PMSF, phenyl methane sulfonyl fluoride; EDTA, ethylene diamine tetra acetate; MW, molecular weight; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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CONFLICTS OF INTERESTS

We declared that we have no conflict of interest

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