CHAPTER VI

Cloning, expression, purification and characterization of a recombinant bacterial fibrinolytic serine protease enzyme

6.1 Brief introduction

A number of approaches, including anticoagulant compounds, antiplatelet medications, fibrinolytic enzyme, and surgery, are utilized to treat thrombosis and remove blood clots [1]. Moreover, the wide range of adverse effects that can arise from the use of existing anti-thrombotic techniques is restricted by their high cost and various potential side effects. The main adverse consequences of such thrombolytic techniques on human health include reperfusion, utricaria (allergic reaction), and bleeding [2,3]. Headache, lighteadedness, ulcers, a longer clotting time, nausea, vomiting, etc. are some other side effects. Although urokinase type plasminogen activator, bacterial plasminogen activator, tissue type plasminogen, and other enzyme treatments are commonly used to manage thrombosis, their affinity and specificity for fibrin are low [4,5].

Recombinant DNA technologies are currently being used by researchers to produce an excess of fibrinolytic enzymes in a prokaryotic system. Genetic engineering has substantially aided in the development of the fibrinolytic enzyme in a range of bacterial host, such as the protease-deficient *Bacillus subtilis* strain WB800, *E. coli*, *Lactococcus lactis, Bacillus licheniformis,* and *Spodoptera frugiperda*. [6-11]. *Bacillus licheniformis* has been used to manipulate signal peptide and signal peptidase for the purpose of increasing nattokinase synthesis [11]. For in-vivo thrombolytic activity, eukaryotic systems like yeast have also been used to heterologously produce the nattokinase gene from *Bacillus* strains into *Pichia pastoris* strain [12]. However, due to its ease and low cost, *E. coli* is a widespread bacteria that produces proteins through fermentation procedures [13]. According to Weng et al. [14], *E. coli* can overexpress several bacterial fibrinolytic enzymes; however, the recovery of significant numbers of these enzymes is frequently tricky because the aggregation of recombinant proteins causes insoluble and inactive inclusion bodies [13,15]. Therefore, researchers have established different approaches to resolve this problem. Several refolding options have been studied to enhance the inclusion body renaturation of the fibrinolytic enzyme [15-18]. It is time-consuming to recover the active form of proteins from inclusion bodies. No method can be used universally to retrieve native proteins from solubilized inclusion bodies; instead, the ideal refolding conditions for each protein must be established through experimental research.

In this study, the protease gene sequence derived from *Bacillus subtilis* was codon optimized to be synthetically cloned into the pET26b(+) vector between the NcoI and XhoI restriction sites. The *E. coli* BL21 (DE3) competent cells were used to overexpress the synthetic plasmid and purified using affinity chromatography on a Ni-NTA column by three different approaches for analysing the best possible way to recover the active form of proteins from the inclusion bodies. Its expression and enzyme activity were analysed using SDS-PAGE analysis. The biochemical properties and the purified recombinant protease's in vitro and in vivo anticoagulant activity were characterized.

6.2 Results

6.2.1 Codon optimization and gene synthesis

The gene sequence encoding for fibrinolytic enzyme was codon optimized for the expression of recombinant protease gene by *E. coli* BL21 (DE3) cells (Figure 6.1 A). The optimized gene sequence, deprived of its signal sequence, was inserted downstream of the pelB leader sequence (which helps the expression of protein without its signal sequence) in the middle of the NcoI and XhoI restriction sites of the pET-26b(+) expression vector and also included an additional set of six His codons at the 3' end. The cloning of the gene in the vector is schematically shown in Figure 6.1 B.

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CCATGGAT
GCGGGCAAAAGCAACGGCGAGAAGAAGTACATCGTGGGTTTCAAGCAGACCATGAGCACCATGAGCGCGGCGAAG
AAGAAGGACGTGATCAGCGAGAAGGGTGGCAAAGTGCAGAAGCAATTCAAATACGTTGACGCGGCGAGCGCGACC
CTGAACGAGAAGGCGGTGAAAGAACTGAAGAAAGACCCGAGCGTTGCGTATGTGGAGGAAGATCACGTTGCGCAG
GCGTACGCGCAAAGCGTGCCGTATGGCGTTAGCCAGATTAAGGCGCCGGCGCTGCACAGCCAAGGTTTCACCGGC
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GACGGTAGCGGCCAATATAGCTGGATCATTAACGGTATCGAATGGGCGATTGCGAACAACATGGACGTGATCAAC
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GTTATTGCGGTGGGTGCGGTTAACAGCAGCAACCAGCGTGCGAGCTTTAGCAGCGTTGGCAGCGAACTGGATGTG
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AGCCCGCACGTGGCGGGTGCTGCGGCGCTGATTCTGAGCAAACACCCGAACTGGACCAACACCCAGGTTCGTAGC
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GCGCAG
CTCGAG
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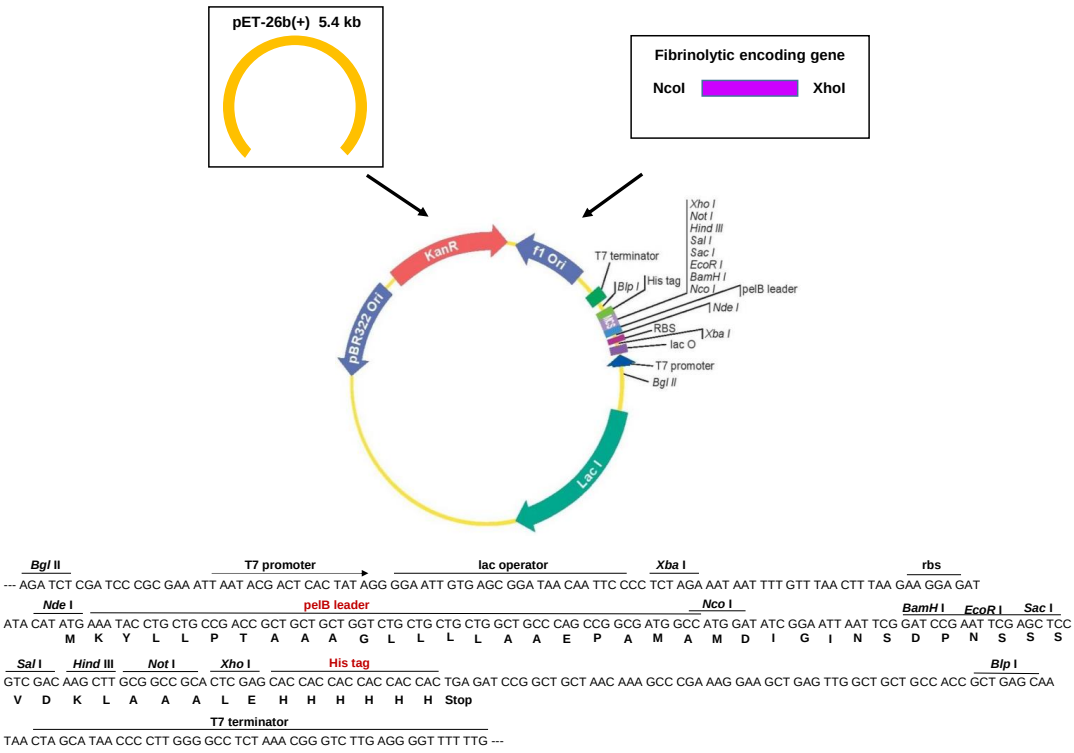
(A)



(B)

Figure 6.1: (A) Codon optimized sequence of protease gene. **(B)** Schematic diagram showing the construction of the recombinant protease gene in pET26b(+) expression vector.

6.2.2 Transformation of synthetic plasmid into BL21 competent cells and sequence analysis

The synthetic plasmid was transformed into *E.coli* BL21 competent cells (Figure 6.2 A) and its plasmid was isolated using GeneJET plasmid miniprep kit which was used as a template for double digesting it with NcoI and XhoI restriction enzymes that resulted in the visualization of two fragments which was ~5400 bp (vector) and ~1000 bp (insert), thus confirming the ligation of the insert with the pET-26b(+) vector in the synthetic plasmid (Figure 6.2 B).

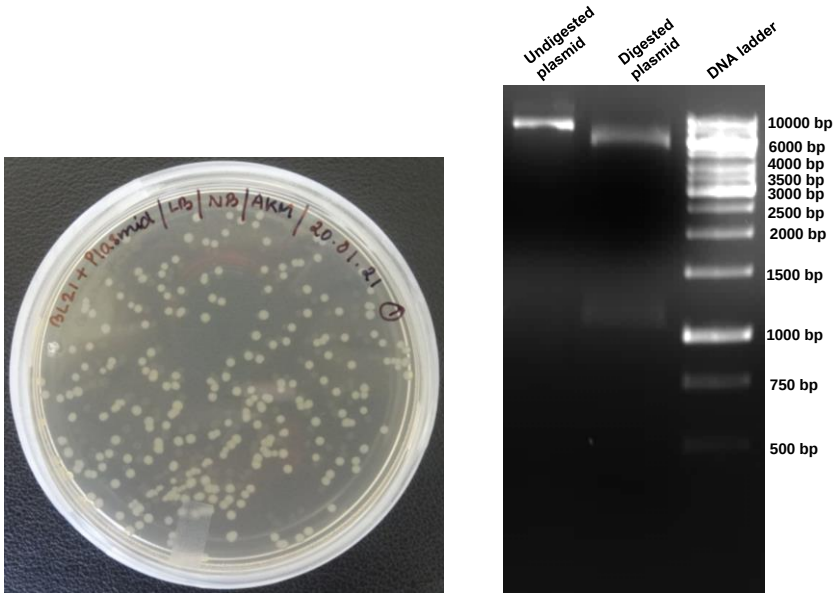


Figure 6.2: (A) Transformation of synthetic plasmid into BL21 (DE3) competent cells. **(B)** 1% agarose gel run of NcoI and XhoI enzyme digested plasmid.

The BLASTp analysis of the protease under study showed 100% identity with the amino acid sequence of more than 10 sequences, among which fibrinolytic enzyme AprE from *Bacillus subtilis* (Accession No. ATA67131.1), fibrinolytic enzyme AprE from *Bacillus pumilus* (Accession No. ATD12229.1) and S8 family peptidase from *Bacillus amyloliquefaeciens* group (Accession No. WP_217365432.1) were the top three sequences (Table 6.1). As shown in Figure 6.3, the three conserved amino acid residues Asp, His, and Ser necessary for the catalytic activity of serine proteases, are also present in the protease under study [19,20].

Table 6.1: Homologous search results of an amino acid sequence of the cloned plasmid using Basic Local Alignment Tool (BLASTp) tool from National Centre Biotechnology Information (NCBI).

Sl. No.	**Accession**	**Description of bacterium**	**Query coverage**	**Max identity**
1.	ATA67131.1	Fibrinolytic enzyme AprE [*Bacillus subtilis*]	100%	100%

2.	ATD12229.1	Fibrinolytic enzyme AprE [*Bacillus pumilus*]	100%	99.43%
3.	WP_217365432.1	S8 family peptidase [*Bacillus amyloliquefaciens* group]	100%	99.43%
4.	WP_077722018.1	S8 family peptidase [*Bacillus*]	100%	99.43%
5.	WP_025649555.1	S8 family peptidase [*Bacillus*]	100%	99.43%
6.	WP_272930824.1	S8 family peptidase [*Bacillus velezensis*]	100%	99.43%
7.	WP_206695523.1	S8 family peptidase [*Bacillus amyloliquefaciens* group]	100%	99.15%
8.	WP_061862038.1	S8 family peptidase [*Bacillus*]	100%	99.15%
9.	MBU0443824.1	S8 family peptidase [*Bacillus amyloliquefaciens*]	100%	99.15%
10.	WP_216631434.1	S8 family peptidase [*Bacillus amyloliquefaciens*]	100%	99.15%
11.	WP_178091937.1	S8 family peptidase [*Bacillus*]	100%	99.15%
12.	MCE4149016.1	S8 family peptidase [*Bacillus velezensis*]	100%	99.15%
13.	WP_021494939.1	S8 family peptidase [*Bacillus amyloliquefaciens* group]	100%	99.15%
14.	WP_003155195.1	S8 family peptidase [*Bacillus*]	100%	99.15%
15.	WP_221419317.1	S8 family peptidase [*Bacillus amyloliquefaciens* group]	100%	98.86%

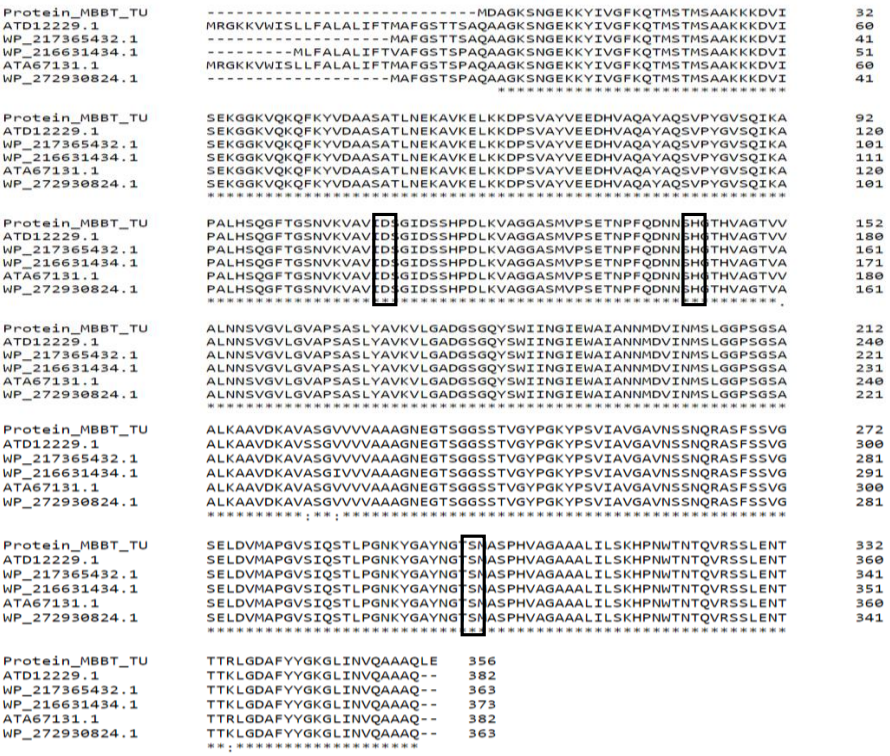


Figure 6.3: Amino acid sequence alignment of the fibrinolytic enzyme with several homologous proteins. The catalytic triad residues (Asp, His, and Ser) are highlighted within the box.

6.2.2 Expression and purification of recombinant protease gene

The T7 promoter of the pET26b(+) vector was used to transcribe the protease gene, and its secretion into the *E. coli* periplasm was guided by the vector's pelB signal sequence. Other fibrinolytic genes were also expressed in *E. coli* using the T7 promoter system [21,22]. The transformed *E. coli* BL21 (DE3) cells' culture was induced with 1.0 mM IPTG and analysed by three different approaches. This resulted in the production of a significant protein of ~40 kDa, as shown in Figure 6.4 A-C. The results of the SDS-PAGE analysis showed that all three methods expressed the recombinant protease gene; however, renaturation by dialysis was found to be the best method for recovery and refolding (renaturation) of the protease enzyme. The 6X His-tagged recombinant protease expressed by three approaches was purified using Ni-NTA affinity column chromatography (Figure 6.5 A-C).

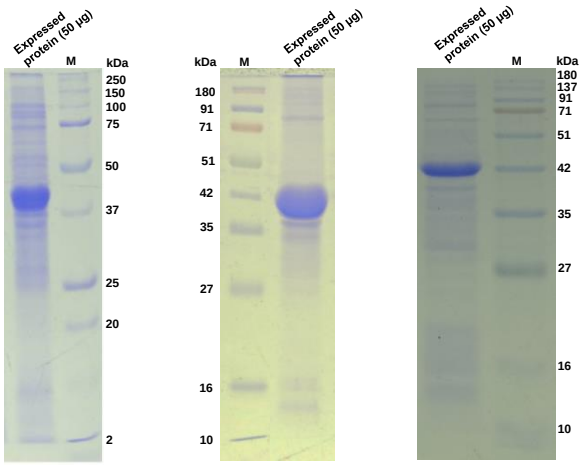


Figure 6.4: Determination of recombinant protein expression by 12.5% SDS-PAGE analysis under reducing conditions. (**A)** Expression of recombinant protease by the renaturation method of the recombinant protein by dialysis. **(B)** Expression of recombinant protease by renaturation by direct mixing for protein folding. **(C)** Expression of recombinant protein by the method of renaturation using urea.

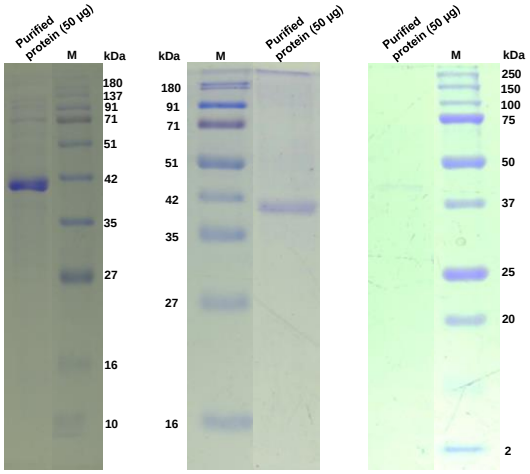


Figure 6.5: Determination of recombinant protein purification by affinity chromatography using Ni-NTA column by 12.5% SDS-PAGE analysis under reducing conditions. **(A)** purified protein is expressed by dialysis using the renaturation method of the recombinant protease eluted from 250 mM imidazole concentration. **(B)** Purified expressed protein by renaturation by direct mixing for protein folding eluted from 250 mM imidazole concentration. **(C)** Purified expressed protein is obtained by renaturation using urea eluted from 300 mM imidazole concentration.

6.2.3 The purified protease exhibited fibrinolytic activity but not fibrinogenolytic activity

12.5% SDS-PAGE analysed the kinetics of the recombinant purified protease enzyme for its fibrinolytic/fibrinogenolytic activity. The Aα, Bβ, and Cγ pairs of disulfide-bonded polypeptide chains make up fibrinogen, the precursor to fibrin [46]. The Aα-chain of human fibrin was found to be degraded by the purified recombinant protease enzyme by renaturation by dialysis and renaturation by direct mixing method for protein refolding within 90 min of incubation. Still, the Bβ- and Cγ-chains of fibrin could not be cleaved (Figure 6.6 A & B). Nevertheless, the purified recombinant protease enzyme obtained by in vitro renaturation using urea did not exhibit fibrin degradation activity (Figure 6.6 C). Moreover, the purified protease did not show fibrinogenolytic activity (Figure 6.7).

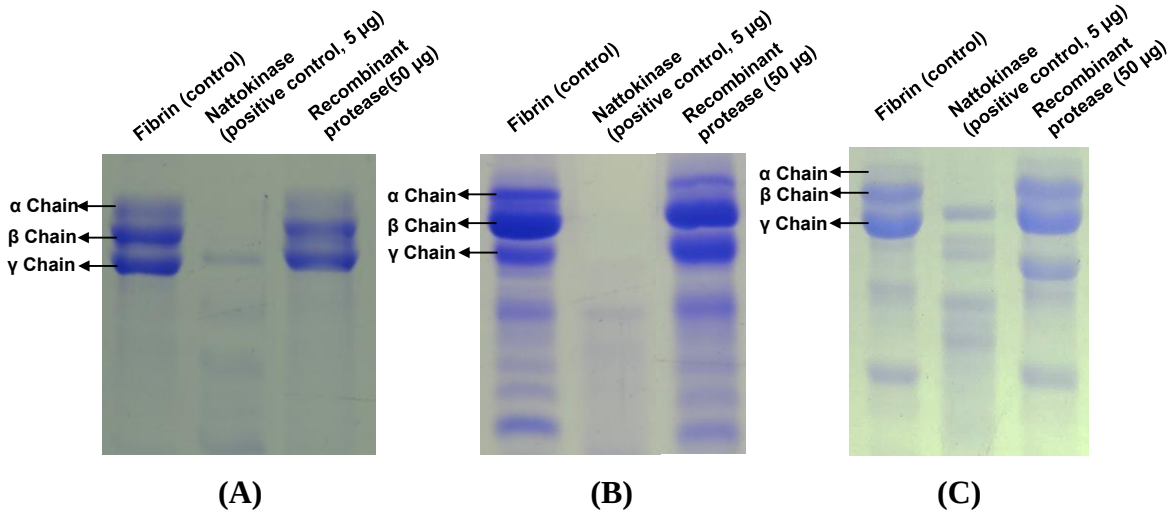
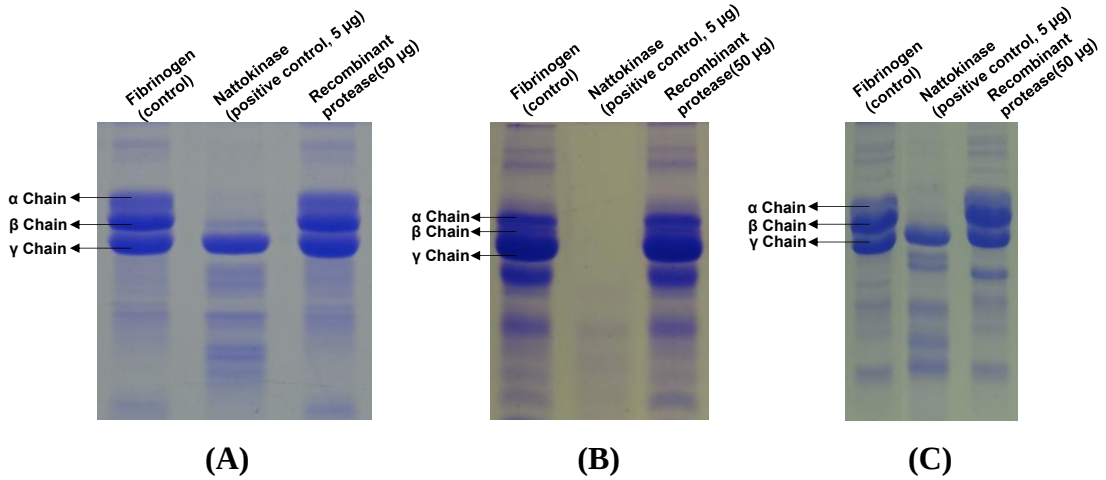


Figure 6.6: Comparison of fibrin degradation between expressed purified protease gene and nattokinase after 90 min incubation at 37°C under reducing conditions by 12.5% SDS-PAGE analysis. **(A)** Analysis of fibrin degradation by the method in vitro renaturation by dialysis. **(B)** Analysis of fibrin degradation by in vitro renaturation by dilution. **(C)** Analysis of fibrin degradation by the method of in vitro renaturation using urea.



(A) **(B)** **(C)**

Figure 6.7: Comparison of fibrinogen degradation between expressed purified protease gene and nattokinase after 90 min incubation at 37°C under reducing conditions by 12.5% SDS-PAGE analysis. **(A)** Analysis of fibrinogen degradation by the method renaturation of recombinant enzyme by dialysis. **(B)** Analysis of fibrinogen degradation by the method renaturation by direct mixing for refolding of protein. **(C)** Analysis of fibrinogen degradation by the method of renaturation using urea.

Based on the densitometric analysis of fibrin degradation products (by SDS-PAGE analysis), it was found that the recombinant protein expressed and purified by the method of renaturation by dialysis could degrade fibrin by 46.0%. In contrast, the recombinant protease expressed by the renaturation process directly mixing with a refolding solution could only degrade fibrin by 42.2%. The protein purified by the method of renaturation by dialysis demonstrated a distinct hydrolysis zone of 17 mm around the well on the fibrin-agarose medium plate (Figure 6.8).

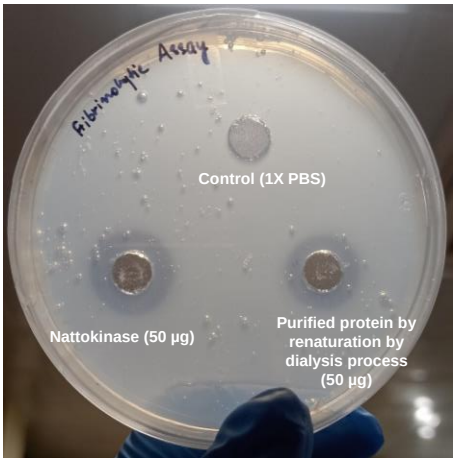
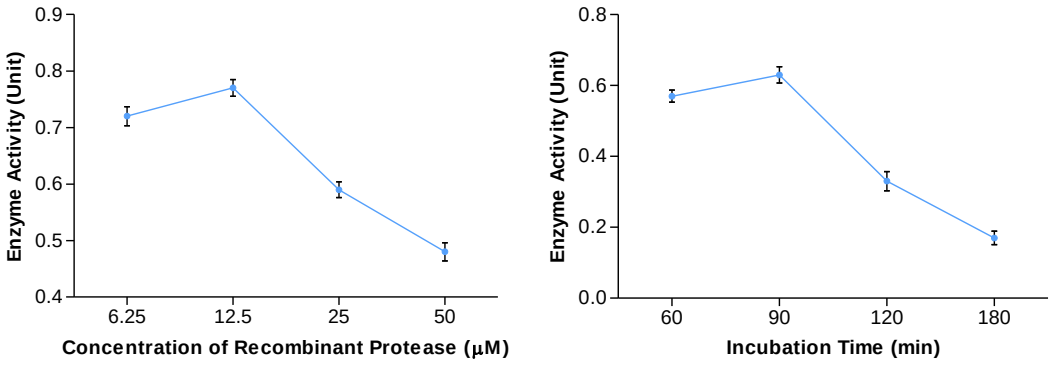


Figure 6.8: Zone of hydrolysis around the purified recombinant protease and nattokinase well on fibrin-agarose medium plate.

The purified recombinant protein shows optimum activity at 50 µg (equivalent to 12.5 µM) and 90 min of incubation at 37 °C (Figure 6.9 A & B).



(A) **(B)**

Figure 6.9: (A) Determination of dose-dependent fibrinolytic activity of recombinant protease post 90 min incubation at 37 °C by colorimetric method. **(B)** Determination of time-dependent fibrinolytic activity of recombinant protease post 90 min incubation at 37 °C by colorimetric method. Values are mean ± SD of triplicate experiments.

6.2.4 Inhibitory activity of recombinant protease

The tested metal ions could not influence the enzyme activity (Table 6.2).

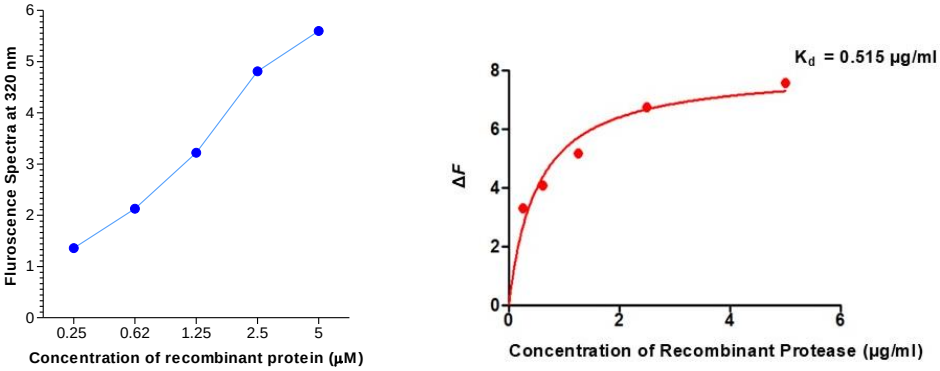
Table 6.2: Effect of various metal ions, if any, on the fibrinolytic activity of recombinant protease. Values are mean ± SD of three triplicates.

Metal ions (concentration)	**Relative Activity**
Control (without metal ion)	100
Ca^{2+} (4 mM)	98.45 ± 0.005
Mg^{2+} (4 mM)	98.68 ± 0.0028
Fe^{2+} (4 mM)	99.01 ± 0.0033
Co^{2+} (4 mM)	98.99 ± 0.001
Hg^{2+} (4 mM)	99.09 ± 0.0011

6.2.5 Recombinant protease shows interaction with thrombin and inhibits the catalytic activity of thrombin

In the presence of the purified recombinant protease, a gradual increase in the fluorescence intensity of thrombin was observed, indicating their interaction (Figure 6.10

A). The recombinant protease's dissociation constant (Kd value) value towards thrombin was determined at 0.5150 µg/ml, respectively (Figure 6.10 B).



(A) **(B)**

Figure 6.10: (**A-B)** Fluorescence spectra showing dose-dependent interaction of recombinant protease with a fixed thrombin concentration. The fluorescence spectra were obtained at an excitation wavelength of 280 nm, and the emission spectra were recorded from 300 to 450 nm.

The fibrinogen clotting time of thrombin was progressively extended by pre-incubating it with varying doses of the recombinant protease (Figure 6.11).

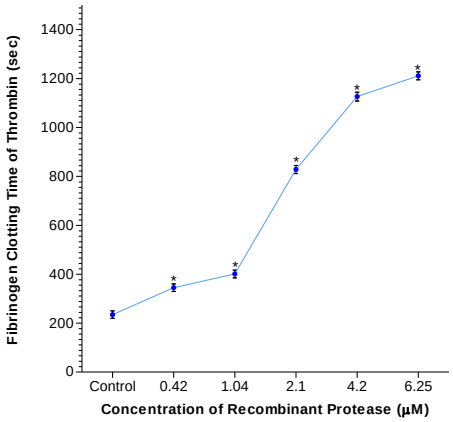


Figure 6.11: Inhibition of fibrinogen clotting activity of thrombin by recombinant protease (0.42-6.25 µM) at 37 °C for 3 h. Pre-incubation of thrombin with a graded concentration of the recombinant protease. Each value is a mean ± SD of triplicate determinations. Significance of difference with respect to control *p<0.05.

6.2.6 Dose-dependent anticoagulant activity of purified recombinant protease

The Ca2+ clotting time of PPP was dose-dependently extended by the purified recombinant protease (Figure 6.12). Additionally, it showed a dose-dependent rise in plasma's PT and APTT (Figure 6.13 A & B).

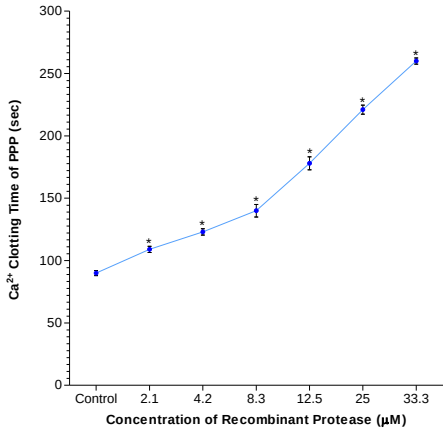
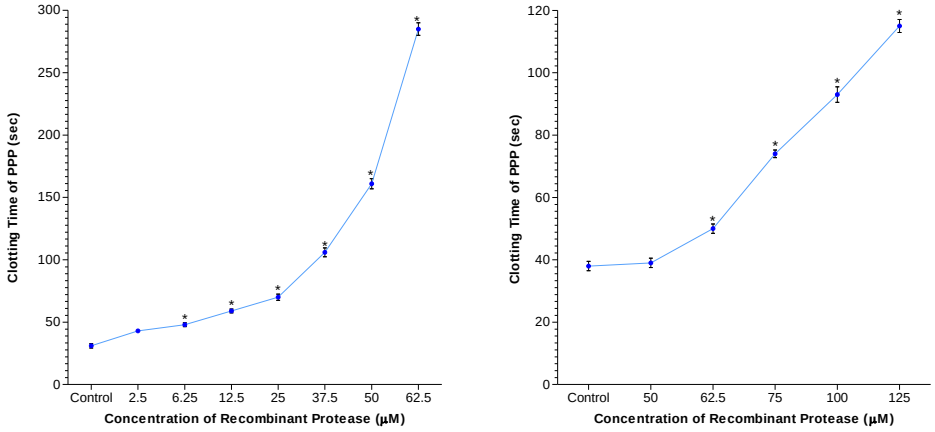


Figure 6.12: Dose-dependent *in vitro* anticoagulant activity of recombinant protease against goat PPP. The values are mean ± SD of triplicate determinations. Significance of difference with respect to control *p<0.05.



(A) **(B)**

Figure 6.13: (A) The *in vitro* effect of recombinant protease on PT time of goat PPP. **(B)** The *in vitro* effect of recombinant protease on APTT time of goat PPP. The values are mean ± SD of triplicate determinations. Significance of difference with respect to control *p<0.05.

6.2.7 Purified recombinant protease exhibited *in vitro* thrombolytic activity

The purified recombinant protease exhibited *in-vitro* blood clot lysis activity towards unheated blood clots; however, the recombinant protease's clot-bursting potency was reduced in dissolving the heat-treated blood clot (Figure 6.14)

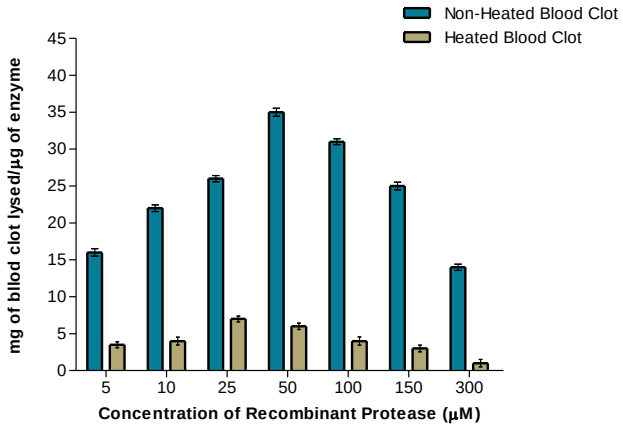


Figure 6.14: *In vitro* thrombolytic potency of recombinant protease. Different concentrations of test samples were incubated with non-heated and heated blood clot for 1 h at 37 °C. The values are mean ± SD of triplicate determinations.

6.2.8 Recombinant protease is deprived of hemolytic activity and cytotoxicity against NRK-52^{E} cells

At 0.15 mg/ml concentration, it showed very marginal (0.45%) hemolysis of goat erythrocytes (Figure 6.15). The recombinant protease at the tested doses did not show *in vitro* cytotoxicity in NRK-52^{E} cells (Figure 6.16).

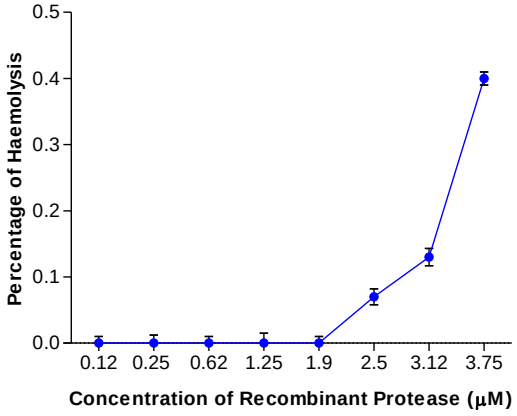


Figure 6.15: *In vitro* dose-dependent hemolytic activity of recombinant protease. The values are mean ± SD of triplicate determinations.

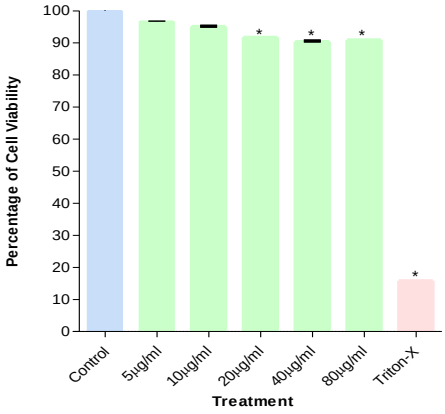


Figure 6.16: *In vitro* cell viability by MTT assay of recombinant protease on NRK-52^{E} cells. The values are mean ± SD of triplicate determinations. Significance of difference with respect to control *p<0.05.

6.2.9 Purified recombinant protease showed antiplatelet activity

The recombinant protease's dose-dependent platelet de-aggregation (antiplatelet) activity is illustrated in Figure 6.17.

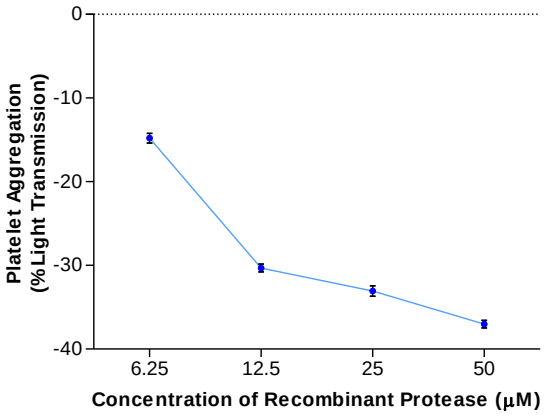
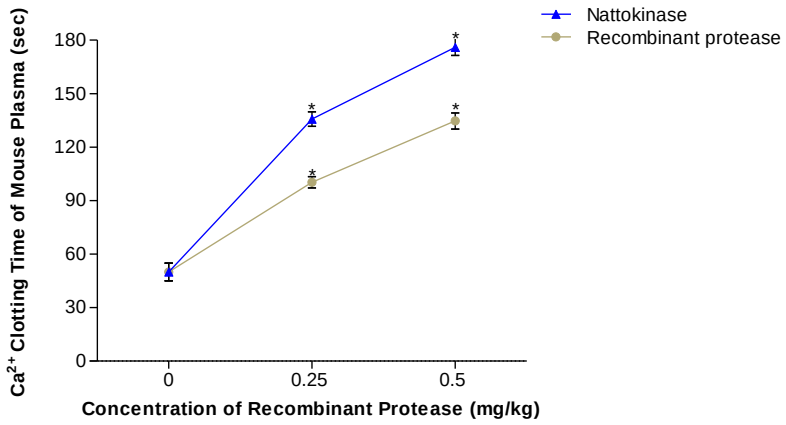


Figure 6.17: Platelet de-aggregation property of recombinant protease. The values are mean ± SD of triplicate determinations

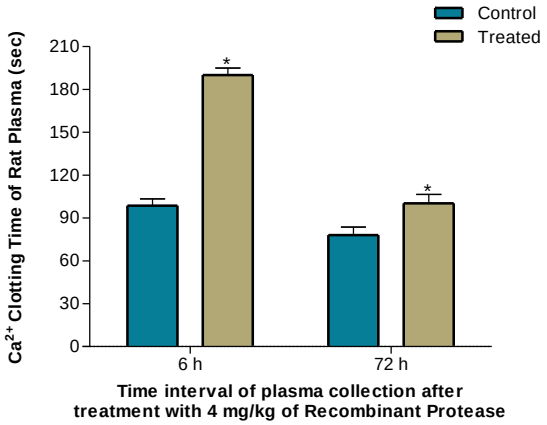
6.2.10 *In vivo* anticoagulant and toxicity assay of recombinant protease

Six hours after treatment in swiss albino mice, the recombinant protease-treated group showed a dose-dependent prolongation of the Ca^{2+} clotting time of PPP in comparison to the untretaed group of mice (Figure 6.18 A). However, the results were significantly lower than those of the control group of mice, which was treated with nattokinase.

Moreover, there was a decrease in the anticoagulant potency of the recombinant protease after 72 h of treatment in Wistar strain albino rats (Figure 6.18 B).



(A)



(B)

Figure 6.18: (A) Dose-dependent *in vivo* anticoagulant activity of the recombinant protease (0.25 and 0.5 mg/kg) after 6 h of i.v. administration in Swiss albino mice (n=6). **(B)** Dose-dependent *in vivo* anticoagulant activity of recombinant protease (4 mg/kg) after 6 h and 72 h of i.v. administration in Wistar strain rats (n=5). Data represent mean ± SD of triplicate experiments. Significance of difference with respect to control *$p<0.05$.

The recombinant protease was found to be non-toxic to rats at a dose of 4 mg/kg (i.v injection). It did not cause adverse effects or behavioural changes in recombinant protease-treated rats compared to the control group upto 72 hr of administration (Table 6.3).

Table 6.3: Comparison of some behavioural parameters of control and recombinant protease-treated rats. Values are mean ± S.D. of triplicate determinations.

Sl. No.	Parameter	Category	Start of Treatment	End of Treatment
1.	Survivability	Control	No death	No death
		Treated	No death	No death
2.	Body Weight (g)	Control	198 ± 6.00	206.67 ± 6.11
		Treated	195 ± 6.08	203.33 ± 6.81
3.	Rectal Temperature (°C)	Control	36.50 ± 0.53	36.83 ± 0.47
		Treated	36.27 ± 0.59	36.67 ± 0.49
4.	Grip Strength (s)	Control	146.67 ± 7.64	142.00 ± 7.21
		Treated	138.00 ± 7.00	137.67 ± 7.51
5.	Food Intake (g)	Control	5.48 ± 0.15	5.57 ± 0.17
		Treated	5.52 ± 0.09	5.64 ± 0.10
6.	Water Intake (ml)	Control	83.67 ± 3.21	84.00 ± 2.00
		Treated	84.00 ± 2.00	81.00 ± 1.00

6.2.11 Effect on serum biochemical parameters of recombinant protease-treated rat

When compared to the control rat group, the biochemical parameters of the serum from the blood of the treated rats (72 hours after treatment) did not exhibit any significant differences ($p>0.05$) (Table 6.4).

Table 6.4: Comparison some biochemical parameters of serum from Wistar rat intravenously treated with control (1X PBS) and recombinant protease (4 mg/kg) after 72 h. Values are mean ± S.D. of triplicate determinations

Sl. No.	Parameter	Unit	Value	
			Control	Treated
1.	SGPT	U/L	24 ± 2.8	19 ± 2.8
2.	SGOT	U/L	63.5 ± 3.5	58 ± 2.8
3.	Blood Urea Nitrogen	mg/dL	14 ± 4.2	12.5 ± 3.5
4.	Total Protein	g/dL	7.5 ± 3.5	5.7 ± 1.4
5.	Cholesterol	mg/dL	46.5 ± 4.9	44 ± 4.2
6.	LDL-Direct	mg/dL	10 ± 2.8	7.5 ± 2.1
7.	HDL-Direct	mg/dL	17 ± 4.2	17 ± 1.4
8.	Triglyceride	mg/dL	45 ± 4.2	65 ± 2.8
9.	Creatinine	mg/dL	0.25 ± 0.2	0.2 ± 0.1

6.2.12 Histopathological study of tissues obtained from recombinant protease-treated and control group of rat

When examined under a light microscope, the treated mice's liver, kidney, heart, lung, and ovary tissues exhibited no morphological changes or pathophysiological symptoms when compared to the same tissues from rat control group (Figure 6.19).

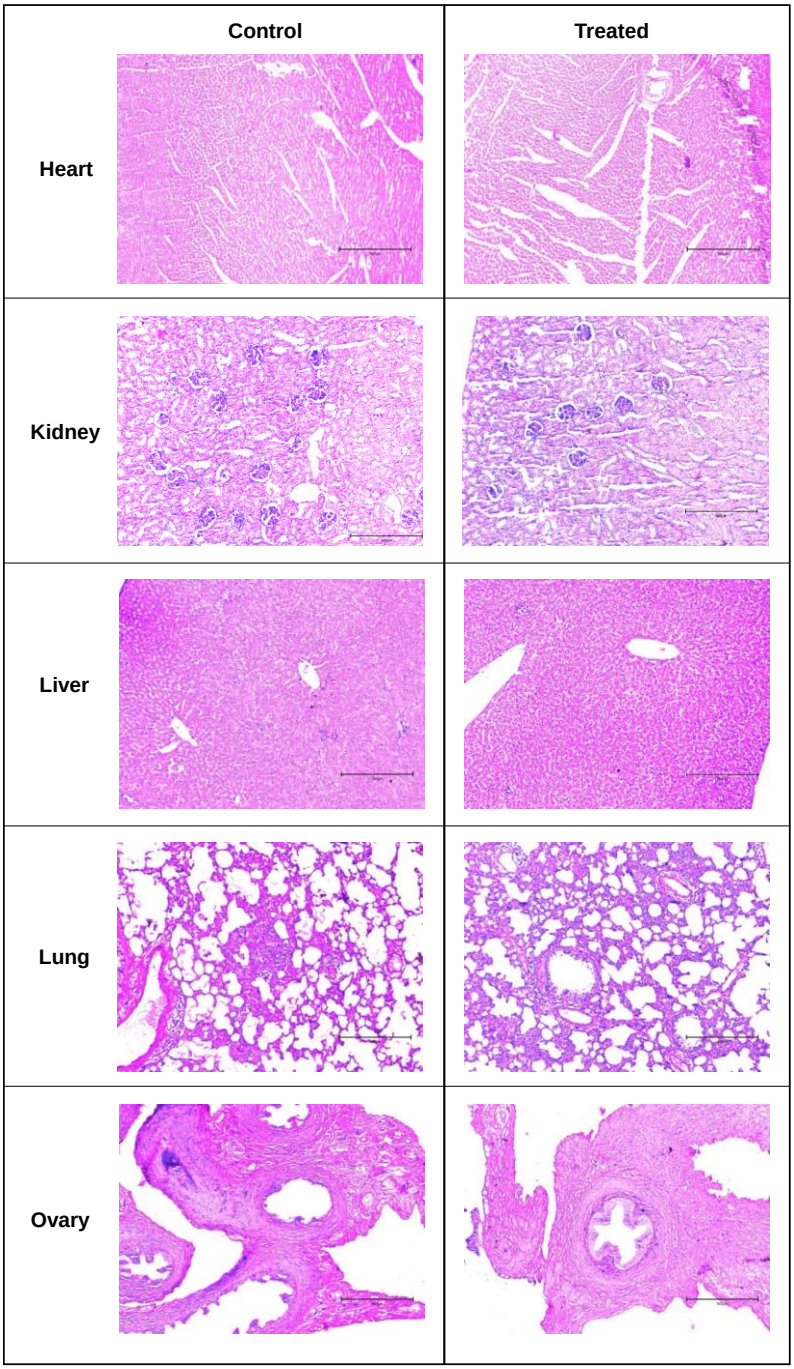


Figure 6.19: The effects of recombinant protease (4 mg/kg, i.v. injection) on the various organs of Wistar strain rat under light microscopy observation. The control group consisted of rats injected with 1X PBS. Hematoxylin-eosin stain was applied to the tissues after fixations, and they were examined under a light microscope at a 20X magnification bar of 300 µm

6.3 Discussion

The discovery of new therapeutics is vital for the management and prevention of cardiovascular diseases. Numerous experimental stages acquired the fibrinolytic protease

in this study, viz. (i) Codon optimization of the protease encoding gene sequence, (ii) cloning and expression of the protease gene in the *E. coli* expression system, (iii) optimization of a method for the efficient expression and renaturation of the enzyme, (iv) purification of recombinant protease, and validation of the enzyme activity, (v) characterization of some in vitro and in vivo pharmacological activity of the recombinant protease to assess it's efficacy, and (vi) assessment of in vitro and in vivo acute toxicity of the recombinant protease to determine its safety.

This study used IPTG to induce the recombinant protease gene expression under the T7 promoter's control. Both lactose and IPTG can be used for induction because the *E. coli* BL21 (DE3) strain generates functional lac permease (encoded by the lacY gene) and β-galactosidase (encoded by the lacZ gene). With the benefit of not being hydrolysed by β-galactosidase, IPTG is ideal for small-scale fermentation; however, because of its high cost and toxicity, it is unsuitable for large-scale fermentation. Lactose is a highly effective inducer as it can operate as an inducer and a carbon source, improving biomass and foreign proteins [23,24].

Prokaryotic systems like *E. coli* can easily overexpress fibrinolytic enzymes. However, it frequently produces proteins that cannot be dissolved and the creation of inactive aggregates known as inclusion bodies. Establishing better processes involving the body's isolation, solubilization, and refolding is obligatory to recover active proteins. Choosing appropriate cell disruption techniques is one of the most critical downstream processing issues [25]. The efficiency of various cell lysis methods, including lysozyme treatment, the French Press, freeze-thawing, and an amalgamation of lysozyme treatment and sonication, was assessed by Rodrguez-Carmona et al. [25]. They reported that a combination of non-mechanical (lysozyme treatment) and mechanical (sonication) approaches produced the highest yields of inclusion bodies and the most negligible contamination of viable cells. Centrifugation could be used to gather dense inclusion bodies after cell disruption. But this pellet must be frequently washed with detergents like Tween, Triton, and SDS or denaturants like urea and GdnHCl. This procedure can significantly lower the level of contaminants [26].

Affinity chromatography was used to purify 6 His-tagged recombinant protease in this study. The resins in the column could easily identify the protease gene carrying the 6 His-tag as the end of the vector was histidine-tagged. This chromatography column

purified the recombinant proteins due to these resins' affinity for the 6 His-tag and produced the recombinant protein with relative protease activity. However, additional purification techniques should be considered for the large-scale manufacturing of enzymes for industrial uses, as this method might not be cost-beneficial.

When assessing the fibrinolytic enzyme's potential as a treatment, specificity towards fibrin becomes crucial. Most microorganisms that can produce a fibrinolytic enzyme also have a fibrinogenolytic enzyme, thereby reducing the fibrin-specificity of the enzyme [27,28]. The effectiveness of fibrinolytic enzymes extracted from innumerable sources for direct hydrolysis of fibrinogen and fibrin has been established. The 340 kDa glycoprotein known as human fibrinogen is divided into two symmetrical portions, each with three polypeptide chains: Aα, Bβ, and γ. During tissue damage, these chains polymerize to create fibrin [29,30]. Fibrinogen degradation can favorably happen at three potential sites, i.e., Aα, Bβ, and γ chains, simultaneously or separately [31]. A similar fibrin degradation mechanism has been described at the α, β, and γ chains [32].

Fibrinolytic enzymes are divided into Aα- and/or Bβ- fibrinogenases established on their specificity for the Aα-chain and Bβ-chain fibrin/fibrinogen [33]. Therefore, the recombinant protease enzyme purified by the two methods, i.e., renaturation of the recombinant protein by dialysis and renaturation by direct mixing method for protein refolding, can be categorized as an α-fibrinogenase enzyme based on the degradation pattern of fibrin. Most fibrinolytic enzymes displayed potent Aα fibrinogenase, followed by Bβ- and γ-chains fibrinolysis. However, no γ-chain lysis was present in the fibrinolytic enzymes isolated from *B. subtilis* JS2 [34] and *B. amyloliquefaciens* CB1 [35]. The lone bacterium that synthesized fibrinolytic enzyme with high Bβ fibrinolytic activity was *Bacillus sp*. nov. SK006 [36].

In thrombus development, platelets play an essential role [37-39]. Since platelets make up the majority of the cells in arterial thrombi, which are created in the presence of increased shear stress at the locations of atherosclerotic vascular damage and disrupted blood flow, it follows that inhibiting platelet aggregation is a crucial additional method of preventing blood coagulation [40]. The recombinant protease exhibits antiplatelet action to lessen thrombus formation in addition to its fibrinogenolytic activity. Therefore, antiplatelet activity also contributes its in vivo anticoagulant activity.

The anticoagulant effect of the recombinant protease was demonstrated by its prolonged clotting time in both PT and APTT, indicating that the blood coagulation cascade's intrinsic and extrinsic routes mediate its action. *In vitro* erythrocyte hemolysis was not demonstrated by the recombinant protease. This outcome is consistent with other *Bacillus sp.* fibrinolytic enzymes, all of which have good safety records [33,41,42]. Anticoagulants, sometimes referred to as blood thinners, are typically necessary for patients who acquire atrial fibrillation to reduce the risk of thrombus formation, which could otherwise lead to the onset of cardiovascular illnesses [43]. It has been found that the recombinant protease delays the progressive coagulation of blood like many other fibrinolytic enzymes [33,44], both *in vitro* and *in vivo*, indicating that it is an anticoagulant enzyme. The purified recombinant protein binds to thrombin, inhibiting its enzymatic activity.

The Ca-clotting time of PPP was dose-dependently prolonged in the recombinant protease-treated group in Swiss albino mice six hours post-treatment. But, compared to the mice in the nattokinase-treated control group, the results were significantly lower. Since the recombinant protease is a fibrinolytic enzyme that functions by converting plasminogen to plasmin, it then breaks down fibrin in clots. Whereas, Nattokinase not only target fibrin but also acts on fibrinogen, enhancing their ability to manage both existing clots and reducing the potential for new clot formation, so the anticoagulant potency of the recombinant protein was less than nattokinase. A 4 mg/kg intravenous infusion of the recombinant protease (at a eight times higher dose at which it shows anticoagulant activity) did not alter the behavioural or serum biochemical characteristics of the group of control rats. Furthermore, the rats treated with the purified recombinant protease did not exhibit any signs of toxicity or fatality in the histological analysis of the chosen organs. This result unequivocally shows that the recombinant protease is safe to use in clinical settings to treat cardiovascular illnesses.

In summary, the recombinant protease demonstrated *in vitro* fibrinolytic, anticoagulant, thrombin-inhibition, and thrombolytic activities and also exhibited dose-dependent in vivo anticoagulant activity in Wistar strain rats. These characteristics significantly predict a promising medication prototype for treating thrombotic diseases.

By modifying the refolding conditions, and selecting a more beneficial promoter and host system, the expression and recovery rates of the recombinant protease gene need

to be additionally improved. Bioprocess development and the economic exploitation of genetically modified bacteria depend on a potent promoter, stable expression system, and suitable refolding conditions. The need for a protease enzyme with high activity and stability is noteworthy for industrial applications. An enzyme's stability, activity, or specificity for industrial purposes can be considerably improved using modern genetic techniques, including metabolic engineering, molecular breeding and its alterations, and combinatorial biosynthesis. Error-prone PCR, which has served as the standard method to create modifications at arbitrary positions in a sequence and, as a result, to produce randomly mutated enzymes, a few of which might exhibit superior characteristics compared to the parent enzyme, is one way to introduce gene mutagenesis and recombination among the various techniques. Further in-depth preclinical study with purified recombinant protease is necessary to establish its therapeutic potential before clinical trials.

Bibliography

[1] Kotb, E. The biotechnological potential of fibrinolytic enzymes in the dissolution of endogenous blood thrombi. *Biotechnology progress*, 30(3): 656-672, 2014.

[2] Nazari, J., Davison, R., Kaplan, K., and Fintel, D. Adverse reactions to thrombolytic agents: implications for coronary reperfusion following myocardial infarction. *Medical Toxicology Adverse Drug Experience*, 2: 274-286, 1987.

[3] Bonnard, T., Law, L., Tennant, Z., and Hagemeyer, C. Development and validation of a high throughput whole blood thrombolysis plate assay. *Scientific reports*, 7(1): 2346, 2017.

[4] Bhargavi, P. L. and Prakasham, R. A fibrinolytic, alkaline and thermostable metalloprotease from the newly isolated Serratia sp RSPB11. *International journal of biological macromolecules*, 61: 479-486, 2013.

[5] Lal, V. Fibrinolytic drug therapy in the management of intravascular thrombosis, especially acute myocardial infarction-A review. *Pharmacol Clin Res*, 2(4): 555593, 2017.

[6] Nagel, F.-J., Oostra, J., Tramper, J., and Rinzema, A. Improved model system for solid-substrate fermentation: effects of pH, nutrients and buffer on fungal growth rate. *Process Biochemistry*, 35(1-2): 69-75, 1999.

[7] Liu, J., Xing, J., Chang, T., Ma, Z., and Liu, H. Optimization of nutritional conditions for nattokinase production by Bacillus natto NLSSE using statistical experimental methods. *Process Biochemistry*, 40(8): 2757-2762, 2005.

[8] Wagner, J., Gruz, P., Kim, S.-R., Yamada, M., Matsui, K., Fuchs, R. P., and Nohmi, T. The dinB gene encodes a novel E. coli DNA polymerase, DNA pol IV, involved in mutagenesis. *Molecular cell*, 4(2): 281-286, 1999.

[9] Koene, R. J., Prizment, A. E., Blaes, A., and Konety, S. H. Shared risk factors in cardiovascular disease and cancer. *Circulation*, 133(11): 1104-1114, 2016.

[10] Wang, C. T., Ji, B. P., Li, B., Nout, R., Li, P. L., Ji, H., and Chen, L. F. Purification and characterization of a fibrinolytic enzyme of Bacillus subtilis DC33, isolated from Chinese traditional Douchi. *Journal of Industrial Microbiology Biotechnology*, 33(9): 750-758, 2006.

[11] Yuan, J., Yang, J., Zhuang, Z., Yang, Y., Lin, L., and Wang, S. Thrombolytic effects of Douchi Fibrinolytic enzyme from Bacillus subtilis LD-8547 in vitro and in vivo. *BMC biotechnology*, 12: 1-9, 2012.

[12] Mahajan, P. M., Gokhale, S. V., and Lele, S. S. Production of nattokinase using Bacillus natto NRRL 3666: media optimization, scale up, and kinetic modeling. *Food science biotechnology*, 19: 1593-1603, 2010.

[13] Tungekar, A. A., Castillo-Corujo, A., and Ruddock, L. W. So you want to express your protein in Escherichia coli? *Essays in Biochemistry*, 65(2): 247-260, 2021.

[14] Weng, Y., Yao, J., Sparks, S., and Wang, K. Y. Nattokinase: an oral antithrombotic agent for the prevention of cardiovascular disease. *International journal of molecular sciences*, 18(3): 523, 2017.

[15] Ni, H., Guo, P.-C., Jiang, W.-L., Fan, X.-M., Luo, X.-Y., and Li, H.-H. Expression of nattokinase in Escherichia coli and renaturation of its inclusion body. *Journal of Biotechnology*, 231: 65-71, 2016.

[16] Jeong, S.-J., Cho, K. M., Lee, C. K., Kim, G. M., Shin, J.-H., Kim, J. S., and Kim, J. H. Overexpression of aprE2, a Fibrinolytic Enzyme Gene from Bacillus subtilis CH3-5, in Escherichia coli and the Properties of AprE2. *Journal of microbiology biotechnology*, 24(7): 969-978, 2014.

[17] Meng, Y., Yao, Z., Le, H. G., Lee, S. J., Jeon, H. S., Yoo, J. Y., and Kim, J. H. Characterization of a salt-resistant fibrinolytic protease of Bacillus licheniformis HJ4 isolated from Hwangseokae jeotgal, a traditional Korean fermented seafood. *Folia Microbiologica*, 66(5): 787-795, 2021.

[18] Yao, Z., Meng, Y., Le, H. G., Lee, S. J., Jeon, H. S., Yoo, J. Y., Kim, H.-J., and Kim, J. H. Cloning of a novel vpr gene encoding a minor fibrinolytic enzyme from Bacillus subtilis SJ4 and the properties of Vpr. *Journal of microbiology biotechnology*, 30(11): 1720, 2020.

[19] Nakamura, T., Yamagata, Y., and Ichishima, E. Nucleotide sequence of the subtilisin NAT gene, aprN, of Bacillus subtilis (natto). *Bioscience, Biotechnology, Biochemistry*, 56(11): 1869-1871, 1992.

[20] Siezen, R. J. and Leunissen, J. A. Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Science*, 6(3): 501-523, 1997.

[21] Ghasemi, Y., Dabbagh, F., and Ghasemian, A. Cloning of a fibrinolytic enzyme (subtilisin) gene from Bacillus subtilis in Escherichia coli. *Molecular biotechnology*, 52: 1-7, 2012.

[22] Lee, S.-Y., Yu, S.-N., Choi, H.-J., Kim, K.-Y., Kim, S.-H., Choi, Y.-L., Kim, C.-M., and Ahn, S.-C. Cloning and characterization of a thermostable and alkaline fibrinolytic enzyme from a soil metagenome. *African Journal of Biotechnology*, 12(45): 6389, 2013.

[23] Howhan, P. and Pornbanlualap, S. Cloning and effective induction of Escherichia coli nucleoside diphosphate kinase by lactose. *ScienceAsia*, 29: 347-353, 2003.

[24] Zhang, H., Wang, Y., Hu, X., Zhu, H., and Wei, Z. Effect of different culture conditions for dextransucrase production in Escherichia coli using lactose as inducer. *African Journal of Biotechnology*, 8(8), 2009.

[25] Rodríguez-Carmona, E., Villaverde, A., and García-Fruitós, E. How to break recombinant bacteria: does it matter? *Bioengineered Bugs*, 2(4): 222-225, 2011.

[26] Middelberg, A. P. Preparative protein refolding. *Trends in biotechnology*, 20(10): 437-443, 2002.

[27] Mander, P., Cho, S. S., Simkhada, J. R., Choi, Y. H., and Yoo, J. C. A low molecular weight chymotrypsin-like novel fibrinolytic enzyme from Streptomyces sp. CS624. *Process Biochemistry*, 46(7): 1449-1455, 2011.

[28] Yogesh, D. and Halami, P. M. A fibrin degrading serine metallo protease of Bacillus circulans with α-chain specificity. *Food bioscience*, 11: 72-78, 2015.

[29] Macrae, F. L., Duval, C., Papareddy, P., Baker, S. R., Yuldasheva, N., Kearney, K. J., McPherson, H. R., Asquith, N., Konings, J., and Casini, A. A fibrin biofilm covers blood clots and protects from microbial invasion. *The Journal of clinical investigation*, 128(8): 3356-3368, 2018.

[30] Weisel, J. W. and Litvinov, R. I. Fibrin formation, structure and properties. *Fibrous proteins: structures mechanisms*: 405-456, 2017.

[31] Yang, H., Yang, L., Li, X., Li, H., Tu, Z., and Wang, X. Genome sequencing, purification, and biochemical characterization of a strongly fibrinolytic enzyme from Bacillus amyloliquefaciens Jxnuwx-1 isolated from Chinese traditional Douchi. *The Journal of General Applied Microbiology*, 66(3): 153-162, 2020.

[32] Choi, J.-H., Sapkota, K., Kim, S., and Kim, S.-J. Starase: A bi-functional fibrinolytic protease from hepatic caeca of Asterina pectinifera displays antithrombotic potential. *Biochimie*, 105: 45-57, 2014.

[33] Mukherjee, A. K., Rai, S. K., Thakur, R., Chattopadhyay, P., and Kar, S. K. Bafibrinase: A non-toxic, non-hemorrhagic, direct-acting fibrinolytic serine protease from Bacillus sp. strain AS-S20-I exhibits in vivo anticoagulant activity and thrombolytic potency. *Biochimie*, 94(6): 1300-1308, 2012.

[34] Yao, Z., Kim, J. A., and Kim, J. H. Properties of a fibrinolytic enzyme secreted by Bacillus subtilis JS2 isolated from saeu (small shrimp) jeotgal. *Food science biotechnology*, 27: 765-772, 2018.

[35] Heo, K., Cho, K. M., Lee, C. K., Kim, G. M., Shin, J.-H., Kim, J. S., and Kim, J. H. Characterization of a fibrinolytic enzyme secreted by Bacillus amyloliquefaciens CB1 and its gene cloning. *Journal of microbiology biotechnology*, 23(7): 974-983, 2013.

[36] Hua, Y., Jiang, B., Mine, Y., and Mu, W. Purification and characterization of a novel fibrinolytic enzyme from Bacillus sp. nov. SK006 isolated from an Asian traditional fermented shrimp paste. *Journal of agricultural food chemistry*, 56(4): 1451-1457, 2008.

[37] Choi, J.-H., Park, S.-E., Kim, S.-J., and Kim, S. Kaempferol inhibits thrombosis and platelet activation. *Biochimie*, 115: 177-186, 2015.

[38] Kamath, S., Blann, A. D., and Lip, G. Platelet activation: assessment and quantification. *European heart journal*, 22(17): 1561-1571, 2001.

[39] Ruggeri, Z. M. and Mendolicchio, G. L. Adhesion mechanisms in platelet function. *Circulation research*, 100(12): 1673-1685, 2007.

[40] Schafer, A. I. Antiplatelet therapy. *The American journal of medicine*, 101(2): 199-209, 1996.

[41] Majumdar, S., Dutta, S., Das, T., Chattopadhyay, P., and Mukherjee, A. K. Antiplatelet and antithrombotic activity of a fibrin (ogen) olytic protease from Bacillus cereus strain FF01. *International journal of biological macromolecules*, 79: 477-489, 2015.

[42] Majumdar, S., Sarmah, B., Gogoi, D., Banerjee, S., Ghosh, S. S., Banerjee, S., Chattopadhyay, P., and Mukherjee, A. K. Characterization, mechanism of anticoagulant action, and assessment of therapeutic potential of a fibrinolytic serine protease (Brevithrombolase) purified from Brevibacillus brevis strain FF02B. *Biochimie,* 103: 50-60, 2014.

[43] Lopez-Sendon, J., Lopez de Sa, E., Bobadilla, J., Rubio, R., Bermejo, J., and Delcan, J. Cardiovascular pharmacology (XIII). The efficacy of different thrombolytic drugs in the treatment of acute myocardial infarct. *Revista espanola de cardiologia,* 48(6): 407-439, 1995.

[44] Lu, F., Lu, Z., Bie, X., Yao, Z., Wang, Y., Lu, Y., and Guo, Y. Purification and characterization of a novel anticoagulant and fibrinolytic enzyme produced by endophytic bacterium Paenibacillus polymyxa EJS-3. *Thrombosis Research,* 126(5): e349-e355, 2010.