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Cloning and Expression of Serratiopeptidase protein and assessment of its activity for clot lysis

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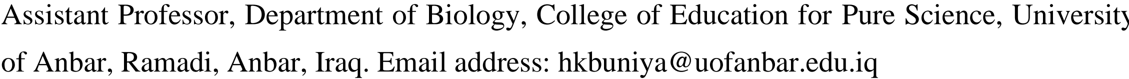

Abstract

Objective

Cardiovascular diseases (CVDs) remain a leading cause of morbidity and mortality worldwide, with blood thrombosis playing a critical role in conditions such as myocardial infarction and stroke. Thrombolytic agents, including tissue plasminogen activators and streptokinase, are commonly used to dissolve blood clots and restore normal blood flow. However, these agents have several limitations, including high cost, short half-life, immune responses, and potential hemorrhagic complications, which restrict their widespread clinical use. Serratiopeptidase, a zinc-dependent metalloprotease produced by *Serratia marcescens*, has gained attention for its fibrinolytic, anti-inflammatory, and analgesic properties. This enzyme has demonstrated effective fibrin clot degradation with minimal side effects, making it a promising candidate for thrombolytic therapy. Additionally, its ability to bind to alpha-2-macroglobulin may help mask its antigenicity, potentially reducing immune-related adverse reactions. Despite these advantages, large-scale recombinant production of Serratiopeptidase is necessary to enhance its availability and therapeutic applications. In this study, we aimed to clone and express the *Serratiopeptidase* (STP) gene in *Escherichia coli* BL21(DE3) and evaluate the thrombolytic activity of the recombinant protein. Using molecular cloning techniques, followed by protein expression, purification, and fibrinolytic assays, we assessed the efficacy of recombinant Serratiopeptidase in clot degradation.

Materials and Methods

The *STP* gene was isolated from a pathogenic *Serratia marcescens* strain. The gene was digested with *EcoR1*, ligated into the expression vector pGEM®-3Zf, and transformed into *E. coli* BL21(DE3). Expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG). The

recombinant STP protein was analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fibrinolytic activity of the expressed Serratiopeptidase protein was assessed using blood clot lysis assays.

Results

The PCR product of the *STP* gene, approximately 1500 bp in size, was confirmed via agarose gel electrophoresis and sequencing. Successful cloning in *E. coli* was verified using 12% SDS-PAGE, which showed a protein band corresponding to the expected molecular weight of 52 kDa. Functional analysis demonstrated that the recombinant Serratiopeptidase effectively lysed human blood clots, showing complete clot degradation compared to the control.

Conclusion

Our findings indicate that recombinant Serratiopeptidase exhibits significant thrombolytic activity, effectively dissolving human blood clots. These results suggest that Serratiopeptidase could serve as a promising alternative to existing thrombolytic agents. Further studies are needed to optimize its production, assess its stability and safety, and explore its clinical applications for cardiovascular disease management.

Keywords: cloning, *Serratia marcescens*, Serratiopeptidase, thrombolysis enzymes

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Introduction

Enzymes are valuable therapeutic tools used in modern medicine to treat various medical conditions. They interact with specific molecules to facilitate the restoration of the body's normal physiological state (Natarajan & Subashkumar 2023). As essential biocatalysts, enzymes play a crucial role in metabolic reactions. Additionally, they can be sourced from various organisms, including microorganisms, enabling large-scale production (Doshi et al. 2020). The genus *Serratia* comprises Gram-negative, rod-shaped bacteria that are ubiquitously distributed. These

bacteria belong to the order *Enterobacterales* (Zivkovic Zaric et al. 2023). The genus includes at least 14 species, with *Serratia marcescens* being the most clinically significant due to its association with human infections (Iguchi et al. 2014). *Serratia* is medically important as it is implicated in various diseases, including bacteremia, sepsis, and brain abscesses. It can also affect the respiratory and urinary tracts and is associated with wound infections, particularly those related to burns. Furthermore, *Serratia* can lead to keratitis and, in rare cases, meningitis and endocarditis (Mykhailenko et al. 2020). The characteristic red pigmentation observed in many *Serratia* species is due to the production of the pigment prodigiosin; however, some species do not produce this pigment (Kashash et al. 2022). *Serratia marcescens* secretes a metalloprotease enzyme known as serralysin, which exhibits proteolytic activity. This enzyme is also referred to as serratiopeptidase (STP) and was initially purified from *Serratia marcescens* E15, which was first isolated from the intestine of the silkworm *Bombyx mori* (Araghi et al. 2019). Serratiopeptidase is a metalloprotein containing zinc at its active site, with a molecular weight ranging from 45 to 60 kDa and consisting of 470 amino acids. Additionally, it can bind to alpha-2-macroglobulin, allowing it to evade immune detection. Notably, this enzyme lacks sulfur-containing amino acids such as cysteine and methionine.

Clinically, serratiopeptidase is widely utilized for its anti-inflammatory, anti-edematous, and analgesic properties. It is also marketed as a health supplement for cardiovascular disease prevention and is believed to have anti-atherosclerotic effects (Bhagat et al. 2013). Despite the availability of effective and safe preventive strategies, cardiovascular diseases remained the leading cause of death in Asia in 2019, accounting for approximately 10.8 million deaths—around 35% of all fatalities in the region (Zhao 2021). Thrombotic diseases pose significant health risks, as they are characterized by the formation of fibrin clots that obstruct blood flow, leading to oxygen deprivation, ischemia, and potential tissue or organ necrosis (Martin et al. 2020). Disseminated intravascular coagulation (DIC) is a complex condition involving multiple pathophysiological mechanisms influenced by triggering events, the body's response, and associated pathological conditions. This disorder leads to the obstruction of small and medium-sized blood vessels, potentially resulting in organ dysfunction. DIC-associated thrombosis is often secondary to severe infections, malignancies, or trauma (Levi & Scully 2018; Bick 1994). Thrombolytic agents are crucial in dissolving blood clots within the cardiovascular system, and numerous studies are focused on improving the efficacy and specificity of fibrinolytic therapy (Tough 2005). In recent decades, microbial thrombolytic enzymes have garnered significant medical interest. Serratiopeptidase, in particular, has demonstrated the ability to degrade atherosclerotic plaques associated with cardiovascular disease. This enzyme selectively targets non-living tissue, effectively removing fatty deposits, cholesterol, cellular waste products,

calcium, and fibrin from arterial walls (Tiwari 2017). The commercial production of serratiopeptidase through various biotechnological approaches is essential to enhance enzyme activity while minimizing production costs. Many researchers are focusing on optimizing microbial strains to increase yield and efficiency. Current investigations are exploring the production of serratiopeptidase in *Serratia marcescens* isolated from diverse sources, which may offer a promising strategy for large-scale production (Natarajan & Subashkumar 2023). Additionally, the epigenome, which includes mechanisms such as DNA methylation, chromatin remodeling, histone tail modifications, microRNAs, and long non-coding RNAs, interacts with environmental factors such as nutrition, pathogens, and climate to influence gene expression and phenotypic outcomes (Shahsavari et al. 2023; Mohammadabadi et al. 2023). Multi-level interactions between the genome, epigenome, and environmental factors play a significant role in gene regulation (Amiri Roudbar et al. 2020). Furthermore, growing evidence suggests that epigenomic variation affects both health and productivity (Alavi et al. 2022). The expression of eukaryotic genes is temporally and spatially regulated, with only a fraction of the genome being expressed in specific tissues depending on developmental stage and physiological needs (Heidarpour et al. 2011; Khabiri et al. 2023). Gene expression in eukaryotes is highly tissue-specific (Mohammadabadi & Asadollahpour Nanaei 2021; Mohammadabadi et al. 2024), and the regulation of gene expression is influenced by both intra- and inter-tissue interactions (Bordbar et al. 2022). Thus, the aim of this study was to amplify the STP gene (1500 bp) from *Serratia marcescens*, clone it in *Escherichia coli* as an expression host, and evaluate the effectiveness of the expressed serratiopeptidase protein as a thrombolytic agent for dissolving blood clots.

Materials and methods

Bacterial Strain, Cloning Vector, and Reagents: The cloning vector pGEM®-3Zf was obtained from Promega (USA) and used to clone the *STP* gene for subsequent expression in *E. coli* BL21(DE3). The restriction enzyme EcoRI was purchased from Promega (USA) and used for DNA digestion following the manufacturer's instructions. Primers were obtained from Macrogen (Korea) (Table 1). EcoRI restriction sites were incorporated into both the forward and reverse primers (bold). Specific primers were designed using Primer-BLAST (NCBI). The master mix was obtained from Abm (Canada), the T4 DNA ligase enzyme from Solarbio (China), and the DNA extraction kit from Geneaid (Taiwan).

Serratia marcescens Isolation: The Serratia marcescens strain was isolated from patients attending the Women and Maternity Hospital in Ramadi City. Samples were initially cultured in nutrient media and then transferred to the postgraduate laboratory for further confirmation.

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Identification was based on cultural characteristics and biochemical tests, including the catalase test, indole production, methyl red test, citrate utilization, and urease test.

Table 1. Primers used in the study

Accession code	Primers name	Primers Sequence 5' ⟶ 3'	Product size (bp)
STP	Forward	GGGC***GAATTC***CAACCGGCTACGATGCTGTA	1500
	Reverse	GGGC***GAATTC***AAAGTCCGTGGCGACGTCTA	

Table 2. Polymerase chain reaction for amplification of the STP gene

Steps	**Time**	**Temperature**	**No. of cycles**
Initial denaturation	5 min	95 °C	1
Denaturation	1 min	95 °C	30
Annealing	30 sec	57 °C	
Extension	45 sec	72 °C	
Final extension	5 min	72°C	1
Hold temperature	Overnight	20 °C	

DNA Extraction: Genomic DNA was extracted from S. marcescens and subjected to electrophoresis on a 1% agarose gel. Electrophoresis was performed at 70 V for 45 minutes to confirm the presence of DNA (Green & Sambrook 2012).

PCR Amplification of the Serratiopeptidase (STP) Gene: Specific primers were used to amplify the STP gene using PCR and a thermal cycler. The reaction mixture (25 µL) was prepared in a 0.2 mL microtube and contained 1 µL of forward (F) and reverse (R) primers (10 pmol each), 1 µL of extracted DNA (final concentration 1.1 ng/25 µL), 12.5 µL of master mix, and 9.5 µL of nuclease-free water. PCR was performed under the thermal cycling conditions described in Table 2. Following amplification, PCR products were electrophoresed on a 1.2% agarose gel for 45 minutes.

Bacterial Transformation: The pGEM®-3Zf cloning vector was used for bacterial transformation to introduce the STP gene into *Escherichia coli* BL21(DE3) for protein expression, following the procedure established by Mandel and Higa (Mandel & Higa, 1970). Both the vector and STP gene were digested with the restriction enzyme *EcoRI* to generate complementary sticky ends. The gene was then ligated into the vector using T4 DNA ligase, forming recombinant DNA. The hybrid DNA was introduced into *E. coli* BL21(DE3) cells, which

were cultured in LB broth supplemented with ampicillin (100 mg/L) at 37°C for 24 hours with shaking at 200 rpm.

Induction of Protein Expression: After successful transformation and bacterial growth in LB broth supplemented with ampicillin, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 μL/mL. The bacterial cultures were incubated in a water bath at 37°C for three hours with shaking at 200 rpm. Following incubation, bacterial cells were lysed using sonication to extract the expressed protein.

SDS-PAGE Analysis of Protein Expression: SDS-PAGE was performed to analyze the expression of the recombinant STP protein. Protein samples (40 μL) were mixed with 10 μL of 5X protein loading buffer, incubated at 100°C for 10 minutes to denature proteins, cooled to room temperature, and centrifuged at 12,000 rpm for 5 minutes. Electrophoresis was performed using a 10% resolving and stacking gel for three hours, alongside a molecular weight marker. The gel was stained using Coomassie Brilliant Blue, and de-staining was carried out to enhance visualization. The molecular weight of the expressed protein was estimated to be within the range of 45–60 kDa.

Thrombolytic Activity Assay: The thrombolytic activity of Serratiopeptidase was assessed to evaluate its potential as a thrombolytic agent. Venous blood (500 μL) was collected from healthy individuals, and 50 μL of blood was transferred into sterile centrifuge tubes. The tubes were incubated until complete clot formation. The serum was carefully removed by pipetting, and the remaining clot was washed with saline solution. Then, 100 μL of cell lysis buffer was added to the experimental tubes, while distilled water was added to the control tube. The samples were incubated at 37°C for 24 hours, and the results were recorded (Buniya et al. 2023).

Results and discussion

Isolation and Purification of *Serratia marcescens:* *Seratia marcescens* cells grown on different media were identified based on their morphological characteristics after 24 hours of incubation at 37°C. The colonies appeared smooth, slightly convex, and round but did not produce the prodigiosin pigment. The isolates were also negative for Gram staining, the indole test, the methyl red test, the catalase test, and the citrate test. These characteristics confirm that the isolates belong to *S. marcescens*, as described by Prokop and Kashash (Kashash et al. 2022; Procop et al. 2017). Non-pigment-producing strains of *S. marcescens* are frequently isolated from humans and pose a significant risk of hospital-acquired infections, whereas the pathogenicity of pigment-producing strains remains uncertain (Grimont & Grimont 1978; Grimont & Grimont 2006). The

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absence of prodigiosin production may be attributed to glucose-mediated catabolic repression or a reduction in pH during growth and fermentation (Sole et al. 1994).

DNA Extraction and *Serratiopeptidase* Gene Amplification: Genomic DNA was extracted, and the *Serratiopeptidase* (*STP*) gene was amplified using specific primers. Electrophoresis of genomic DNA and PCR products on a 1.2% agarose gel (Figures 1 and 2) revealed the presence of the *STP* gene at approximately 1500 base pairs, consistent with the findings reported by Srivastava et al. (2019).

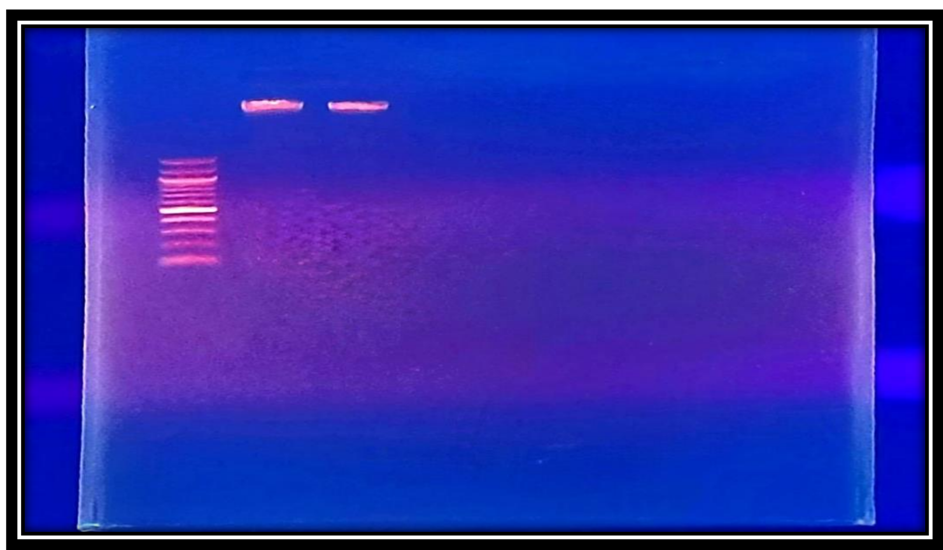

Figure 1. Agarose Gel Electrophoresis forthe extracted DNA at a concentration of 1% and a voltage of 70 for 45 minutes.

Protein Expression: Serratiopeptidase expression was induced by adding IPTG to the culture, prompting the cells to produce the protein. The induced samples were then subjected to electrophoresis on a 12% SDS-PAGE gel. The results revealed distinct protein bands with an approximate molecular weight of 52 kDa (Figure 3), which aligns with the expected size based on the cloned DNA fragment. Mohankumar and KrishnaRaj reported that 37 °C is generally the optimal temperature for protein production (Mohankumar et al. 2011).

Thrombolytic Activity: The experimental results demonstrated that the Serratiopeptidase protein could completely dissolve the blood clot within 24 hours. In contrast, the control tube containing only a saline solution showed no clot dissolution. Similarly, no thrombolytic activity was observed in the bacterial sample lacking the *STP* gene (Figure 4). Proteases are increasingly utilized as therapeutic agents for various diseases due to their ability to break down proteins through proteolysis. They are used as oral digestive aids, solvents for dissolving internal protein

deposits, and as anti-inflammatory and thrombolytic agents. Recently, the U.S. Food and Drug Administration (FDA) has approved several proteases for medical use in treating conditions such as stroke, hemophilia, and acute myocardial infarction (Doshi et al. 2020).

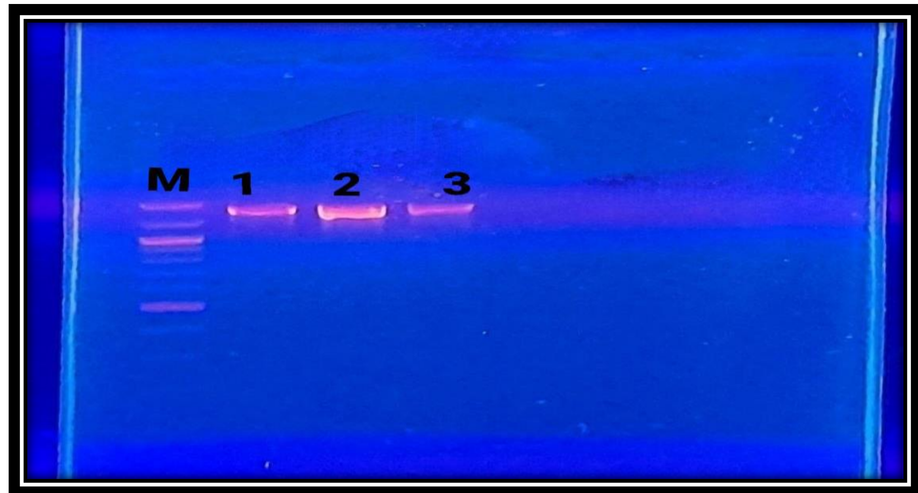

Figure 2. Agarose gel Electrophoresis for the PCR products of the STP gene at a concentration of 1.2% and 70 volts for 45 minutes, M: 100 bp DNA Ladder, Lanes 1, 2, and 3 represent the STP gene

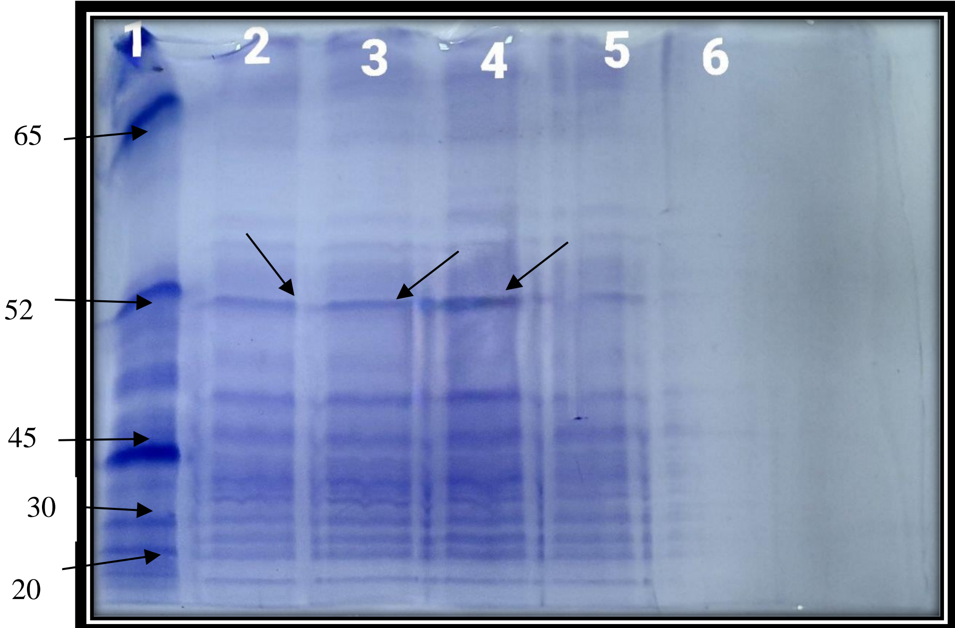

Figure 3. Expression analysis of the native Serratiopeptidase protein in *Escherichia coli* BL21 (DE3). The cell lysate samples were analyzed on 10% SDS–PAGE and stained with Coomassie brilliant blue G-250. Lane 1, low molecular weight protein marker, lane 2, 3 and 4 induced host, Lane 5 and 6 uninduced host. The expression of recombinant Serratiopeptidase is indicated by an arrow

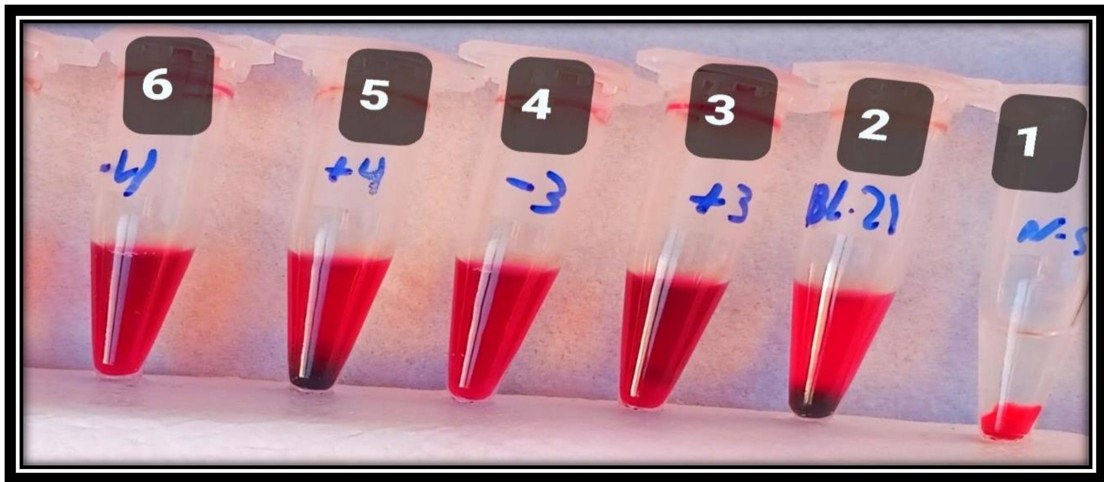

Figure 4. Activity of expressed Serratiopeptidase on human blood clots (photo taken after 24 h): Tube 1- PBS, Tube 2- Expression host without target gene, Tube 3, 4, 5 and tube 6 induced expression host. The figure shows the efficiency of Serratiopeptidase in complete dissolution of the human blood clots in all tubes except tube No. 5 the clot was not completely broken down.

Microbial enzymes, particularly those with thrombolytic capabilities such as streptokinase, staphylokinase, and recombinant streptokinase, have garnered significant interest over conventional thrombolytic agents due to their ease of production and lower cost (Kotb 2013; Buniya et al. 2014; Buniya et al. 2023). The production of recombinant proteins, including therapeutic proteins, in prokaryotic expression hosts—particularly *Escherichia coli*—remains a key challenge in biotechnology. *E. coli* is widely used as an expression host due to extensive knowledge of its genetics, physiology, and complete genomic sequence, which greatly facilitates gene cloning and cultivation. Specifically, *E. coli* BL21 (DE3) is ideal for use with the T7 promoter-based expression system (Jana & Deb 2005; Shrivastava et al. 2013). Serratiopeptidase has demonstrated anticoagulant properties and thrombolytic activity in human blood samples (Natarajan & Subashkumar 2023). As a fibrinolytic drug, it aids in dissolving clots and restoring blood flow within blood vessels. It has a strong affinity for fibrin and possesses notable analytical properties (Krishnamurthy et al. 2018; Kotb 2014). Consequently, serratiopeptidase is used to break down fibrin and degrade dead or damaged tissue, thereby dissolving clots and atherosclerotic plaques (Santhosh 2018). Additionally, it helps remove deposits of fatty substances, cholesterol, and cellular waste from arteries while mitigating issues related to blood viscosity, reducing the risk of stroke and thrombophlebitis (Tiwari 2017). Serratiopeptidase has also been reported to enhance blood circulation, which may aid in hemostasis (Hogan et al. 2017). Furthermore, it decreases capillary permeability caused by histamine and serotonin and facilitates

the breakdown of secretions and proteins, improving their absorption through the lymphatic system and bloodstream. It has been shown to support thrombolysis by effectively removing arterial cysts and plaques (Nair 2022).

Conclusions: In this study, we successfully cloned the *stp* gene responsible for encoding serratiopeptidase in an expression host. The biological activities of the expressed serratiopeptidase were analyzed and found to exhibit high *in vitro* thrombolytic activity. As a future direction, large-scale purification of serratiopeptidase is necessary, and its *in vivo* efficacy must be assessed using animal models to achieve complete characterization.

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Conflict of Interest: There is no conflict of Interest

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شبیه سازی و بیان پروتئین سراتیوپپتیداز و ارزیابی فعالیت آن برای لیز لخته

ندا الفهداوی

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چکیده

هدف: بیماری‌های قلبی عروقی (CVDs) همچنان یکی از علل اصلی مرگ و میر در سراسر جهان هستند و ترومبوز خون نقش مهمی در شرایطی مانند انفارکتوس میوکارد و سکته دارد. عوامل ترومبولیتیک، از جمله فعال کننده‌های پلاسمینوژن بافتی و استرپتوکیناز، معمولاً برای حل کردن لخته‌های خون و بازگرداندن جریان خون طبیعی استفاده می‌شوند. با این حال، این عوامل دارای چندین محدودیت هستند، از جمله هزینه بالا، نیمه عمر کوتاه، پاسخ‌های ایمنی، و عوارض خونریزی دهنده بالقوه، که استفاده گسترده بالینی آنها را محدود می‌کند. Serratiopeptidase یک متالوپروتئاز وابسته به روی تولید شده توسط Serratia marcescens است، که به دلیل خواص فیبرینولیتیک، ضد التهابی و ضد درد آن مورد توجه قرار گرفته است. این آنزیم تخریب موثر لخته فیبرین را با حداقل عوارض جانبی نشان داده است و آن را به یک کاندید امیدوارکننده برای درمان ترومبولیتیک تبدیل کرده است. علاوه بر این، توانایی آن برای اتصال به آلفا-۲-ماکروگلوبولین ممکن است به پوشاندن آنتی ژنی آن کمک کند و به طور بالقوه واکنش‌های جانبی مرتبط با ایمنی را کاهش دهد. با وجود این مزایا، تولید نوترکیب در مقیاس بزرگ Serratiopeptidase برای افزایش در دسترس بودن و کاربردهای درمانی آن ضروری است. در این مطالعه، هدف شبیه سازی و بیان ژن Serratiopeptidase (STP) در Escherichia coli BL21 (DE3) و ارزیابی فعالیت ترومبولیتیک پروتئین نوترکیب بود. همچنین با استفاده از تکنیک‌های شبیه‌سازی مولکولی، و به دنبال آن بیان پروتئین، خالص‌سازی و سنجش‌های فیبرینولیتیک، کارآیی Serratiopeptidase نوترکیب در تخریب لخته ارزیابی شد.

مواد و روش‌ها: ژن STP از سویه بیماری‌زای Serratia marcescens جدا شد. ژن با EcoR1 هضم شد، به ناقل بیان pGEM®-3Zf متصل و به E. coli BL21 (DE3) تبدیل شد. بیان با استفاده از ایزوپروپیل-β-D-1- تیوگالاکتوپیرانوسید (IPTG) القا شد. پروتئین نوترکیب STP از طریق الکتروفورز ژل سدیم دودسیل سولفات–پلی آکریل آمید (SDS-PAGE) آنالیز شد. فعالیت فیبرینولیتیک پروتئین Serratiopeptidase بیان شده با استفاده از روش لیز لخته خون ارزیابی شد.

نتایج: محصول PCR ژن STP با اندازه تقریبی ۱۵۰۰ جفت باز از طریق الکتروفورز ژل آگارز و تعیین توالی تایید شد. شبیه‌سازی موفقیت‌آمیز در E. coli با استفاده از SDS-PAGE دوازده درصد تأیید شد که یک نوار پروتئینی مربوط به وزن مولکولی مورد انتظار ۵۲ کیلو دالتون را نشان داد. تجزیه و تحلیل عملکردی نشان داد که Serratiopeptidase نوترکیب به طور موثر لخته های خون انسان را لیز می‌کند و تخریب کامل لخته را در مقایسه با شاهد نشان می‌دهد.

نتیجه‌گیری: یافته‌های این پژوهش نشان می‌دهد که Serratiopeptidase نوترکیب فعالیت ترومبولیتیک قابل‌توجهی را نشان می‌دهد و به طور موثر لخته‌های خون انسان را حل می‌کند. این نتایج نشان می دهد که Serratiopeptidase می‌تواند به عنوان یک جایگزین امیدوار کننده برای عوامل ترومبولیتیک موجود باشد. مطالعات بیشتری برای بهینه سازی تولید آن، ارزیابی پایداری و ایمنی آن، و کشف کاربردهای بالینی آن برای مدیریت بیماری‌های قلبی عروقی مورد نیاز است.

واژه‌های کلیدی: آنزیم‌های ترومبولیز، سراتیا مارسسنس، سراتیوپپتیداز، کلونینگ

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