Expression and Biological Activity of Serratiopeptidase in *E.coli* BL21(DE3)

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ABSTRACT

Background: Humans are constantly exposed to a variety of microbes, making them vulnerable to numerous diseases. Among these, biofilm-associated infections form the largest category of diseases. Serratiopeptidase, recently referred to as the "miracle enzyme," has been widely utilized in Europe and Asia as a therapy to combat biofilms. **Objective:** This study aimed to clone and express the *stp* gene and to test the effectiveness of the expressed Serratiopeptidase as an antimicrobial and antibiofilm agent. **Methodology**: The gene encoding Serratiopeptidase was isolated from a pathogenic isolate of *Serratiamarcescens* after amplification of the *stp* gene, followed by confirmation through sequencing. Bacterial transformation was performed using the cloning vector pGEM-3Zf $_{(+)}$ after amplification and ligation of the gene encoding the protein. Then, it was transformed into the expression host *E. coli* BL21 (DE3) bacteria. IPTG was used to induce the bacterial culture to produce the protein. The presence of the Serratiopeptidase protein was confirmed through SDS-PAGE. **Results**: The anti-biofilm activity was validated using a titer plate assay. The results demonstrated significant effectiveness as an anti-biofilm agent. However, the expressed protein showed no antimicrobial activity against bacterial and fungal species. **Conclusion**: Expressed protein has anti-biofilm formation, but has no activity against pathogenic bacteria and fungi.

Keywords: Serratiopeptidase, *Serratia marcescens*, Antimicrobial, Anti-biofilm.
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INTRODUCTION

Serratia species are opportunistic Gram-negative bacteria in the large family, Enterobacteriaceae. *Serratia* are widespread in the environment, but are not a common component of the human fecal flora. *Serratia marcescens* is the primary pathogenic species of *Serratia*. They are capable of thriving in diverse environments, including water, soil, and the digestive tracts of various animals (1).

S. marcescens is a significant human pathogen. It has a long and interesting history in taxonomy, medical experimentation, and human clinical infection. Intensive care units are often affected by epidemics of *S. marcescens* colonization and infection. The important reservoirs in epidemics are the digestive tract, the respiratory tract, the urinary tract, and the artificial nails of adults and health care workers. Medical equipment, lotions, antiseptics, medications, blood products, and sinks have also been described as the sources of epidemics (2).

Biofilms are self-constituting ecosystems consisting of compact aggregates of microbial cells. Their unique structure aids bacteria in adhering to fixed surfaces. In response to environmental signals, bacteria form biofilms to ensure their survival (3,4). Biofilms allow bacteria to evade the host immune system, resist medications, and protect themselves from the harmful effects of sterilizers (5). According to National Institutes of Health statistics, biofilm formation is associated with approximately (65%-80%) of microbial and chronic diseases (6).

Biofilms pose a significant threat to global health due to their capacity to resist antibiotics, host defense systems, and external stresses. This resistance contributes to the persistence of chronic infections (7). To prevent the emergence of antibiotic resistance, there has been significant interest in developing new anti-infective therapies (8). Enzymatic therapies have been crucial in treating various diseases, mainly through the use of proteases. These enzymes are considered among the most promising agents for breaking down biofilms, as they break down proteins into smaller units that can easily pass through cell membranes and be metabolized (9, 10).

Serratiopeptidase is a metalloprotein containing zinc in its active site. Its molecular weight ranges from 45 to 60 kDa. The enzyme consists of 470 amino acids and is classified under the EC number 3.4.24.40. It can also bind to alpha-2-macroglobulin, which helps it mask its antigens. This enzyme is free of sulfur-containing amino acids, such as cysteine and methionine (11). Serratiopeptidase belongs to the serine protease family, which first attracted the interest of Japanese biochemists more than 25 years ago. Since then, it has been widely used in healthcare in Asian and European countries (12).

The anti-biofilm properties of Serratiopeptidase depended on its ability to modulate the expression of adhesion molecules. It also reduced the expression of cell-surface proteins in bacteria. Serratiopeptidase not only prevents the formation of biofilms but also promotes the breakdown of pre-existing biofilms. This ability enhances antibiotic penetration into resistant biofilms, thereby increasing their sensitivity to antibiotics (13). The enzyme can alter the binding of virulent strains to biofilms and has demonstrated potent activity against established biofilms. Interestingly, the enzyme can interact with cell adhesion molecules that form the biofilm (11). The aim of this study was to clone and express the Serratiopeptidase protein and assess its activity as an antibiofilm and an antimicrobial agent.

METHODOLOGY

Bacterial strain, clone vector, and reagents

The cloning vector pGEM®-3Zf was obtained from Promega Company, USA, to clone the *stp* gene and subsequently express it in *E. coli* BL21 (DE3). The restriction enzyme *Eco*R1 was obtained from Promega, USA, and prepared according to the manufacturer's instructions. Meanwhile, the Master mix was obtained from Abm Company, Canada, and the T4 DNA Ligase enzyme was obtained from Solarbio Company, China. The DNA extraction kit was obtained from Geneaid Company, Taiwan.

Table 1. Primers used in the study (14)

Company	**ProductSize**	**Primer Sequence** 5′ → 3′	**Target**
Macrogene	1500 bp	F:5`GGGCGAATTCCAACCGGCTACGATGCTGTA3` R:5`GGGCGAATTCAAAGTCCGTGGCGACGTCTA3`	Ser

Table 2. Polymerase chain reaction (PCR) for amplifying the *stp* gene

Steps	Time	Temperature	No. of cycles
Initial denaturation	5 min	95 ℃	1
Denaturation	1 min	95 ℃	35
Annealing	30 Sec	58 ℃	
Extension	45 Sec	72 ℃	
Final extension	5 min	72 ℃	1
Hold temperature	Overnight	20 ℃	

***Serratia marcescens* isolation:**

The bacterium strain *S. marcescens* was isolated from patients attending the Women and Maternity Hospital in Ramadi city. Samples were cultured in nutrient media. They were then transferred to the postgraduate laboratory. There, they were confirmed through culture traits and biochemical tests.

Ethical approval:

Ethical approval for this study was obtained from the ethical approval Committee at the University of Anbar (Ref. No.258, dated 26/12/2024). The approval confirms that the research was conducted according to approved ethical and scientific standards.

Extraction of Genomic DNA:

DNA was extracted from the bacteria *S. marcescens*. Next, it was electrophoresed with a 1% agarose gel prepared by adding 0.3 g of agarose and 30 ml of TBE under standard conditions of 70 volts for 45 minutes to confirm the presence of DNA (15). The specific primers shown in Table 1 were used to amplify the gene encoding Serratiopeptidase (*stp*) by PCR using a Thermal Cycler. Samples were prepared by adding one µL of forward (F) and reverse (R) primers to one µL of DNA isolated from *S. marcescens* bacteria, which was added to a 0.2 ml tube, in addition to 12.5 µL of Master mix and 9.5 µL of nuclease-free water to obtain 25 µL of the reaction mixture. Then, the polymerase chain reaction was performed under the conditions described in Table 2. Samples were electrophoresed on 1.2% agarose gel for 45 min.

Bacterial transformation:

The pGEM®-3Zf clone vector was used to perform bacterial transformation and insert the *stp* gene into *E. coli* BL21(DE3) to express the recombinant protein according to the procedure used by Mandel and Higa (16). Both the cloning vector and the *stp* gene were digested with the restriction enzyme *Eco*RI to obtain the cohesive ends. The gene was ligated into the vector using T4 DNA ligase to generate an integrated hybrid DNA. This hybrid DNA was then transferred into genetically engineered *E. coli* BL21(DE3) bacteria. The bacteria were cultured in LB broth supplemented with Ampicillin at 37°C for 24 hours, shaking at 200 rpm, to propagate the hybrid DNA.

Protein production

To stimulate protein production, Isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to the growth media. One microliter of IPTG (1mM) was added per milliliter of bacterial culture. The bacterial cultures were then incubated for three hours in a water bath at 37°C, shaking at 200 rpm. Then, the bacterial cells were destroyed to obtain the protein.

Protein electrophoresis using SDS-PAGE:

The SDS-PAGE kit was used to investigate the expression of the hybridized protein by bacterial cells. Protein samples were prepared by adding 40 μL of each sample to a centrifuge tube, adding 10 μL of 5X protein loading buffer, and mixing. The samples were placed in a water bath at 100 °C for 10 minutes to denature the protein. They were then cooled to room temperature. Afterward, the samples were centrifuged at 12,000 rpm for 5 minutes. Electrophoresis was conducted by preparing a 12% separation gel and a 5% stacking gel. The samples were electrophoresed for three hours in accordance with the protein size marker. A staining solution was applied to the gel to visualize the proteins, followed by a destaining solution to remove the excess stain and achieve a clear image of the gel. The molecular weight of the protein was determined based on the protein size marker in the 45-60 kDa range.

Antibacterial and antifungal Activity

The activity of the protein Serratiopeptidase was tested against several bacterial and fungal genera using the Kirby-Bauer solid disk media diffusion method, as described by Vandepitte *et al*., (17). The microbial suspension was diluted. Then, a cotton swab was passed over the previously prepared culture media to obtain homogeneous growth. Wells, each with a diameter of 6 mm, were made in each dish. An equal amount of protein was placed in each well. The uninduced sample was used as a positive control. The dishes were incubated at 37 °C for 24 hours, and the results were recorded afterward.

The anti-biofilm formation

According to Almeida and his group (18), flat microtiter plate assays were used along with crystal violet stains. The results were then read using an ELISA device. Brain heart broth (BH) media was inoculated with *Staphylococcus aureus* colonies and incubated at 37°C for 24 h. Samples were added to the titer plate, which was incubated for 24 h at 37°C. The wells' contents were washed with saline solution several times, and when the plate was dry, 0.1% crystal violet stain was added. After 20 min, the stain was removed with distilled water, and ethanol was added. The optical density of the wells was measured by ELISA at 630 nm, and the results were recorded.

RESULTS

S. marcescens Isolation and purification:

S. marcescens growing on different nutrient media was identified based on its morphological characteristics. These characteristics were observed after 24 hours of incubation at 37°C. The colonies appeared smooth, small, convex, and circular, with no visible prodigiosin pigmentation. The bacteria were also negative for Gram stain and indole test, positive for methyl red, and positive for catalase and citrate tests. These characteristics confirm that the isolates belong to *S. marcescens*, as described by (19,20). Non-pigment-producing strains of *S. marcescens* are commonly isolated from human clinical samples and are associated with nosocomial infections. In contrast, the pathogenicity of pigment-producing strains remains uncertain (21,22). Glucose may inhibit prodigiosin production through catabolic repression or low pH during growth and fermentation (23).

DNA extraction and Serratiopeptidase gene insertion

DNA was extracted from bacterial cells, and the gene was amplified with a polymerase chain reaction (PCR). Gel electrophoresis on a 1.2% agarose gel confirmed the presence of the *stp* gene, with bands observed at approximately 1500 bp. This is consistent with the result reported by Srivastava *et al*. (2019) (24), as shown in Figures (1) and (2)

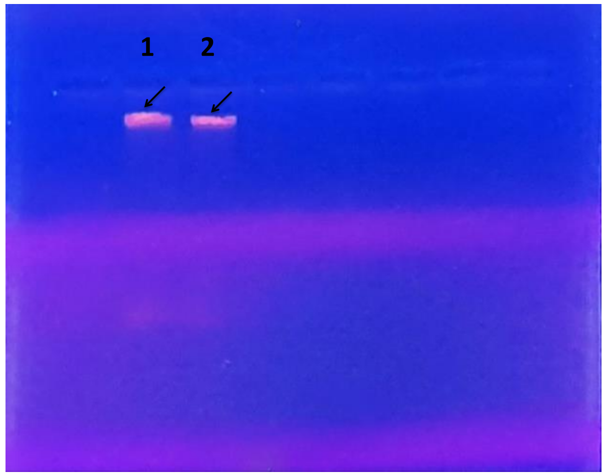

Figure 1. Agarose gel electrophoresis for extracted Genomic DNA at a concentration of 1% and a voltage of 70 for 45 hours.

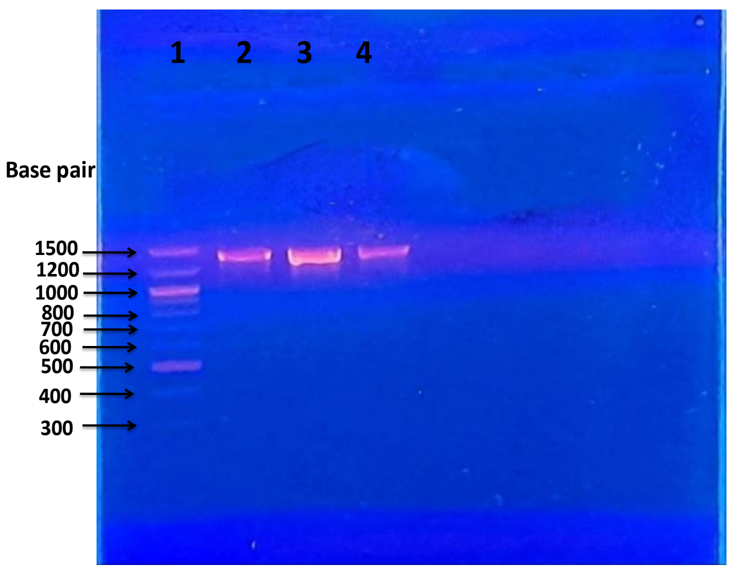

Figure 2. Agarose gel electrophoresis for PCR products of the *stp* gene at a concentration of 1.2% and a voltage difference of 70 volts for 45 minutes, lane 1:100 bp DNA ladder, 2, 3, and 4 represent the *stp* gene.

Electrophoretic results indicated that the primers used were ideal for amplifying the 1500 bp *stp* gene, consistent with findings from several researchers who amplified the Serratiopeptidase gene under the same conditions (23, 14, 25).

Protein expression:

Serratiopeptidase protein was expressed by inducing cells with IPTG. The induced samples, along with a protein size marker, were electrophoresed on a 12% polyacrylamide gel (SDS-PAGE). The electrophoresis results showed clear protein bands with a molecular weight of approximately 52 kDa, consistent with the size of the DNA fragment loaded onto the clone vector. Mohankumar and Krishna Raj (26) reported that 37 °C is generally the most suitable temperature for protein production. Illustrated in Figure (3).

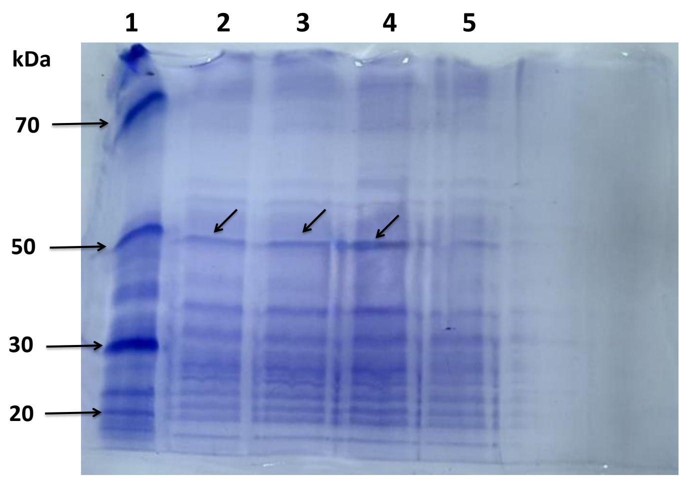

Figure 3: Expression analysis of the native Serratiopeptidase protein in *Escherichia coli* BL21 (DE3). The cell lysate samples were analyzed on 10% SDS–PAGE and stained with Coomassie brilliant blue G-250. Lane 1, low molecular weight protein marker, lane 2, 3, and 4 induced host, Lane 5 uninduced host.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

Effectiveness of protein as an antimicrobial:

The antimicrobial assay demonstrated that none of the tested bacterial or fungal strains exhibited susceptibility to the Serratiopeptidase protein. Bacteria have evolved numerous antibiotic resistance mechanisms that enable them to survive despite antimicrobials. One of these mechanisms is the chemical modification of antibiotics, which occurs through enzymatic processes, changing the chemical structure of antibiotics without disrupting the metabolism process. The mechanism of antibiotic resistance involves modifying aminoglycoside antibiotics by adding a phosphate group, which reduces their affinity for bacterial targets. *Pseudomonas aeruginosa* possesses a set of N-acetylase enzymes that modify these antibiotics at various positions, thereby reducing their effectiveness against bacteria. Additionally, specific modification processes can introduce nucleotides into the antibiotic's reaction centers, rendering them ineffective (27-29). Modifications to the protein that regulates the movement of materials into and out of the cell can restrict the absorption of antibiotics and decrease bacterial susceptibility to them(30,31). Bacteria also have efflux pumps that expel antibiotics and other drugs from the cell (32).

Anti-biofilm Activity

A titer plate approach was used to assess the efficacy of the protein serratiopeptidase as an anti-biofilm agent against biofilms formed by *S. aureus*, which is well known for its potent capacity to generate biofilms. Biofilm inhibition assays indicated that Serratiopeptidase significantly reduced or completely inhibited biofilm formation compared to untreated controlsin samples where it was added, as illustrated in Figure (4).

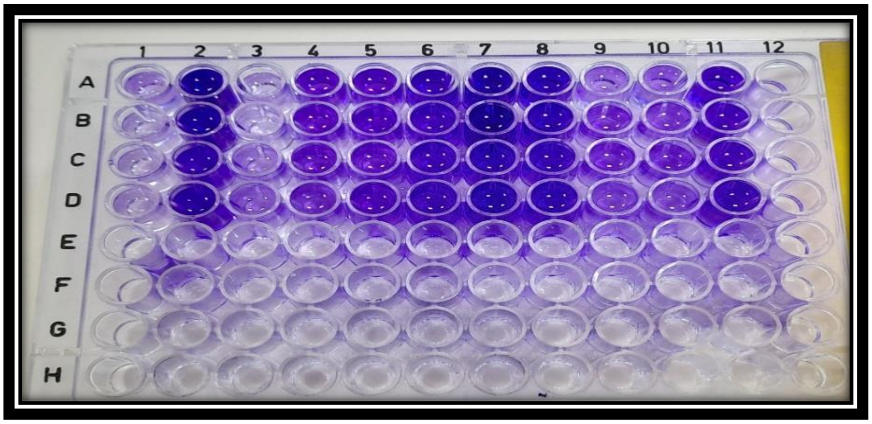

Figure 4. The effectiveness of Serratiopeptidase as an anti-biofilm is shown, where the columns represent: 1: the control; 2: it contains *S. aureus* without adding the enzyme; columns 3, 4, 5, 6, 8, 9, 10, and 11 contain bacterial isolates in addition to the enzyme, indicating that biofilm formation is almost non-existent compared to the control. Column 7, bacterial isolates, in addition to the uninduced *E. coli* BL21 (DE3), show biofilm formation in the absence of the Serratiopeptidase enzyme.

These results were confirmed by measuring the percentage of biofilm formation with an ELISA device at a wavelength of 630 nm, as listed in Table 3.

Table 3. Absorbance values at a wavelength of 630 nm for the *S. aureus* isolate

No	Isolates	Absorption values	Level of biofilm
1	BRH media	0.067	No biofilm
2	*S. aureus*	0.658	Veryhighly biofilm
3	*S.aureus+* Serratiopeptidase	0.072	No biofilm forming
4		0.140	Weakly biofilm
5		0.147	Weakly biofilm
6		0.272	Moderately biofilm
11		0.269	Moderately biofilm
8		0.376	Moderately biofilm
9		0.113	No biofilm
10		0.108	No biofilm
7	*S. aureus+BL21*	0.501	Highly biofilm

DISSCUSION

Biofilms comprise various carbohydrate molecules and proteins, particularly amyloid (surface proteins) and peptidoglycan. Phosphatases are also crucial components of cell biofilms (33,34). Serratiopeptidase is an enzyme that breaks down these proteins within the cell due to its proteolytic properties. This action reduces or prevents bacterial biofilm formation, thus helping protect host cells from invasion (35,36).

Selan *et al*. (13) reported similar results, concluding that Serratiopeptidase weakens the ability of bacteria to form or disperse biofilms because it is able to lyse the peptide bonds among amino acids in biofilm. Many bacteria gain their pathogenic potential through biofilm formation, which conceals their pathogenic molecular patterns and protects them from detection by the body's immune cells, resulting in recurrent microbial infections. According to the National Institutes of Health, 65-80% of chronic microbial infections are associated with biofilm formation (23).

Proteases are considered the most promising enzymes for removing biofilms because they break down membrane-forming proteins into smaller units that can be transported across the biofilm and metabolized. Once the cells are stripped of their biofilms, they become vulnerable to antibiotics. Therefore, it is essential to administer biofilm-breaking agents alongside antibiotics to combat the membranes and effectively prevent bacterial regrowth (9).

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الفعالية الحيوية لبروتين سيراتيوبيبتيداز المعبر عنه في *E.coli* BL21(DE3)

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قسم علوم الحياة- كلية التربية للعلوم الصرفة - جامعة الانبار – الرمادي – الانبار - العراق

الخلاصة:

خلفية عن الموضوع: يتعرض البشر باستمرار لمجموعة متنوعة من الميكروبات وهذا يجعلهم عرضة للعديد من الأمراض. من بينها، العدوى المرتبطة بالأغشية الحيوية التي تشكل أكبر فئة من الأمراض. تم استخدام سيراتيوبيبتيداز، الذي يشار إليه مؤخرًا باسم "الإنزيم المعجزة"، على نطاق واسع في أوروبا وآسيا كعلاج لمكافحة الأغشية الحيوية. **الهدف من الدراسة:** تهدف هذه الدراسة إلى كلونة وتعبير الجين stp واختبار فعالية سيراتيوبيبتيداز المنتج في مضائف التعبير كمضاد للميكروبات ومضاد للأغشية الحيوية. **المواد وطرق العمل:** تم عزل الجين المشفر لبروتين السيراتيوبيبتيداز من العزلة المسببة للأمراض من Serratia marcescens بعد تضخيمها لجين stp ثم تأكيد ذلك عن طريق تحديد التتابعات للجين المحدد. تم إجراء التحول البكتيري باستخدام ناقل الاستنساخ pGEM®-3Zf (+) بعد تضخيم وربط الجين الذي يشفر البروتين به. ثم تم تحويله إلى بكتيريا BL21 (DE3) المضيفة. تم استخدام IPTG لتحفيز المزارع البكتيرية لإنتاج البروتين. تم تأكيد وجود بروتين Serratiopeptidase من خلال الترحيل الكهربائي باستخدام .SDS-PAGE **النتائج:** تم التحقق من صحة نشاط مضاد للأغشية الحيوية باستخدام اختبار لوحة العيارية, أظهرت النتائج فعاليته الكبيرة كمضاد للأغشية الحيوية. من ناحية أخرى، لم يُظهر البروتين المعبر عنه أي نشاط مضاد للميكروبات ضد الأنواع البكتيرية والفطرية. **الاستنتاجات:** البروتين المعبر عنه له تكوين مضاد للأغشية الحيوية في حين أنه ليس له نشاط ضد البكتيريا المسببة للأمراض والفطريات.

الكلمات المفتاحية : سيراتيوبيبتيداز ، Serratia marcescens ، مضاد للبكتريا ، مضاد لتكوين الاغشية الحيوية .