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Substrate specificity analysis of semi-purified fibrinolytic protease of Metabacillus sp. CS-2 to support its potential as a wound debridement agent

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Abstract: Fibrinolytic proteases play an important role in the fibrin degradation process, which is crucial in the treatment of chronic wounds as a debridement agent. Fibrinolytics work by breaking down fibrin tissue that forms as part of a blood clot leading to cell necrosis. Metabacillus sp. CS-2 is known to be one of the potential sources of fibrinolytic protease production with high activity. This study aims to examine the activity and specificity of fibrinolytic proteases produced by Metabacillus sp. CS-2 before and after the ultrafiltration process by zymography. In this study, crude protease extraction was carried out from Metabacillus sp. CS-2 followed by ultrafiltration to improve the purity and activity of the enzyme. The obtained ultra-filtrate protease was then characterized for its specificity to 4 protein substrates, i.e. casein, gelatine, fibrin, and collagen. The results showed that the activity of the fibrinolytic protease enzyme from Metabacillus sp. CS-2 was improved by ultrafiltration from 0.342 □ 0.011 to 0.768□ 0.014 U/mL min-1. The obtained zymogram confirmed that the protease of Metabacillus sp. CS-2 can degrade all of the protein substrates tested. Interestingly, the most specific substrate for Metabacillus sp. CS-2 was fibrin evidenced by intense clear zone smeared from high to low enzyme sizes. In conclusion, ultrafiltration is proven to be effective in increasing the activity of fibrinolytic proteases. The ability to degrade fibrin and collagen substrates likely supports fibrinolytic protease of the strain to function as a debridement agent in wound healing treatment.

Keywords: *Debridement agent, Fibrinolytic protease, Metabacillus sp. CS-2, Ultrafiltration, Zymography.*

1. Introduction

Chronic wounds are a global problem that triggers infections that lead to sepsis, amputation, and death in various countries. According to the Wound Healing Society, the prevalence of chronic wounds in Germany and the United States is 2-3 million people, or 15% of the population experience chronic injuries caused by various factors [1]. The Asian continent such as India is predicted to have around 42 million people suffering from DM disease and experiencing chronic wound complications such as diabetic foot injuries by 15% [2].

Chronic injuries are still widely experienced by the people of Indonesia as 15% of the total population of Indonesia experience this, have a risk of amputation of around 30%, a mortality rate of around 32%, and chronic injuries are the cause of the most hospitalizations in about 80% [2]. Chronic wounds often progress to necrosis through a series of ongoing and complex pathological processes.

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Under normal conditions, fibrin plays an important role in the formation of blood clots to protect wounds. However, in chronic wounds, excessive accumulation of fibrin occurs. This build-up of fibrin can disrupt blood flow and oxygen supply to the tissues around the wound, which then leads to ischemia (lack of oxygen). Lack of enough oxygen and nutrients will damage tissue cells, leading to cell death or necrosis. This process makes chronic wounds increasingly difficult to heal without medical intervention, such as debridement to remove dead tissue and prevent the spread of infection [3].

Debridement aims to create an environment that supports wound healing by removing material that could be a source of infection or inhibit the growth of new tissue. Proteases are enzymes that play a role in breaking protein peptide bonds. Fibrinolytic proteases in particular can destroy fibrin clots, which form in response to injury or infection. Protease enzymes produced by bacteria can aid in the debridement process by destroying fibrin clots that can envelop and protect infected tissue [4].

Metabacillus sp. CS-2 has been screened by Nurhilaliyah et al. (2023) as able to produce fibrinolytic proteases *in vitro* [5-6]. Further research needs to be conducted to increase activity and to characterize further its specificity to substrates to explore its potential as a debridement agent. Ultrafiltration, as a molecular size-based separation method, can help improve the purity and concentration of these enzymes. Zymography on the other hand can help identify the specificity of fibrinolytic proteases against fibrin and other protein substrates [7]. Efforts to purify protease of *Metabacillus* sp. CS-2 by ultrafiltration and characterizing its specificity on various substrates by zymography has not previously been reported.

This study aims to examine the activity and specificity of fibrinolytic proteases produced by *Metabacillus* sp. CS-2 before and after the ultrafiltration process by zymography. Comparison of enzyme activity and specificity before and after ultrafiltration will be carried out quantitatively and qualitatively. Thus, this study was also intended to provide information about the effect of ultrafiltration on the characteristics of fibrinolytic protease enzymes from *Metabacillus* sp. CS-2 to support its potential as a debridement agent for chronic wounds.

2. Materials and Methods

2.1. Materials

The living materials used in this study included *Metabacillus* sp. CS-2 originated from the macro brown algae *Chnoospora* sp. Nurhilaliyah's (2023) [5]. The strain was stored at the Microbiology Laboratory of Universitas Muhammadiyah Semarang in glycerol at -20°C. Other materials include Nutrient Agar (NA), Skim Milk Agar (SMA), and Skim Milk Broth (SMB) bacterial media (both from Oxoid, UK), human blood from the blood bank (to prepare fibrin substrate), casein, gelatine, and collagen substrates (Sigma Aldrich) and reagents for enzyme activity and zymography tests [8]. **Ethica clearance**: This study has received ethical clearance document No. 192/ VI /2024/Komisi Bioetik from Medical Faculty of Universitas Islam Sultan Agung Semarang.

2.2. Methods

2.2.1. Bacterial Subculture

The bacterial isolate was rejuvenated as a confirmation test of Metabacillus sp. CS-2 sample on both NA and SMA media. The next step included a Gram-staining test to confirm colony purity as previously reported [5].

2.3. Enzyme Production

Bacterial protease production on NB and SMB incubated at 30°C for 24 h. The proteolytic enzymes were extracted by centrifugation at 12,000 rpm for 10 mins at 40°C, and then the supernatants obtained were used for subsequent enzyme activity tests, ultrafiltration of protease enzymes, and zymography [8-9].

2.4. Enzyme Ultrafiltration

The crude bacterial protease was concentrated using Vivaspin 10,000 molecular weight cut-off (MWCO). The first step was to clean the filter membrane with 8 mL Tris HCL 0.005M pH 8, which

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was centrifuged at 6,000 rpm for 10 mins. After that, Tris HCl as a cleaning liquid was discarded and then replaced with the crude enzyme. It was done by adding 10 ml of crude enzyme to the tube, then centrifuging at 6,000 rpm for 20 mins until an ultrafiltrate of purer enzyme result of 1 ml was obtained. The obtained semi-purified enzyme was transferred to a new tube [10-11].

2.5. Enzyme Activity Assay

Enzyme activity was measured using the Bergmeyer and Grab (1984) method with modifications. The sample was made by mixing 250 ul of Tris HCL 0.04 M pH 8, 250 ul of casein 1%, and 250 ul of enzymes, then incubated at 50°C for 10 mins. A total of 500 µL of 10% TCA was added to it, then re-incubated at 50°C for 10 mins. The mixture is then centrifuged at 4°C at 10,000 rpm for 10 mins. Supernatants were separated, and as many as 500 ul of supernatants were reacted with 1.2 ml of 0.4 M Na_2CO_3 sodium and 200 µl of Folin reagent and then re-incubated at 50°C for 30 mins. Absorbance is measured at a $\lambda = 660$ nm [8-9]. A unit of protease enzyme activity is defined as the amount of enzyme that can produce 1 mmol of tyrosine per minute under measurement conditions [8, 12-13]. Extract resulting from ultrafiltration of *crude protease* was regarded as protease concentrate and was subjected to concentration analysis by the modified Bradford method [12-13].

2.6. Zymography Assay

The ultrafiltrate protease sample obtained was characterized for its specificity towards casein, gelatine, fibrin, and collagen using zymography. Fibrin substate in particular was prepared from human blood. Blood plasma was extracted from 100 mL whole blood by centrifugation at 1500 x g for 10 mins. The obtained plasma was heated at 50 °C for days until dried. The resulting fibrin grains were ground using mortar to become fibrin powder.

To prepare zymography gel, each of 4 types of substrates was first mixed with sample buffers in a ratio of 1:4. The sample buffer contained SDS, glycerol, standard protein-dye (bromophenol blue), tris HCl 1.0 M pH 6.8, and ultrapure water (MQW) (Agrebi et al., 2009). Meanwhile, a 12% separating gel consisting of 3.4 ml of ddH_2O, 4 ml of acrylamide, and 0.1 ml of SDS 10%, 1.5 M Tris-HCl pH 8.8, 100 µl of ammonium persulfate (APS) 10%, and 10 µl of N,N,N′, N′-tetramethyl-ethylene-diamine TEMED were prepared as well as 4 target substrates of 1% casein, fibrin, gelatine, and collagen into the separating gel. The mixture of separating gel ingredients was homogenized quickly so that it did not harden immediately. The mixture was fed into the electrophoresis plate circuit with the help of a pipette, followed by the administration of MQW so that the gel surface was flat and there were no air bubbles. Space was left 1 cm from the top for stacking gel (upper gel). After the separating gel hardens, the remaining ultrapure water is discarded [12-13].

The 4% *gel* stacking mixture was prepared and homogenized quickly, the stacking gel had the same ingredient as the separating gel, consisting of 6.1 ml ddH_2O, 1.3 ml acrylamide, and 0.1 ml SDS 10%, 0.5 M Tris-HCl pH 6.8, 50 µl APS 10% and 5µl TEMED. The stacking gel mixture was inserted on top of the separating gel and the comb was immediately installed on top of the stacking gel to create a well. The gel was allowed to harden, and after the gel had hardened the comb and the trapper was removed from the glass [13].

The gel on the glass was attached to the electrophoresis device. The enzyme and protein marker samples that have been made are inserted into the comb hole (well), of 20 µl/well. The power supply was turned on and the protein separation by zymography was carried out with an electric current of 200 V for 90 mins. This process is stopped after the blue color reached the bottom of the gel approximately 1 to 2 h. The gel was removed from the glass [14].

The zymogram gel was observed using Coomassie blue after being reconditioned in Triton X-100 with shaking for 1 hour and incubated (digested) using 0.05 M buffer phosphate pH 8 at 60ºC for 30 minutes. After the staining process, the gel is immersed in a destaining solution containing 50% methanol, 10% glacial acetic acid, and aqueous [14]. The washing solution can be changed every 12 hours until a clear gel is obtained. The protease band appears as a clear zone surrounded by a dark blue color on the gel [13].

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3. Results and Discussion

3.1. Bacterial Subculture

Metabacillus sp. CS-2 colonies were rejuvenated and the result showed that the colonies grew in uniform with no signs of contamination. Microscopic characterization showed Gram-positive characteristics with bacilli-shaped purple cells. Observations on SMA media stained by Lugol confirmed that the bacterium was capable of producing protease enzymes as indicated by the formation of a clear zone around the colonies on *Skim Milk Agar* (SMA) media (**Fig. 1**), similar to previously reported [5,15].

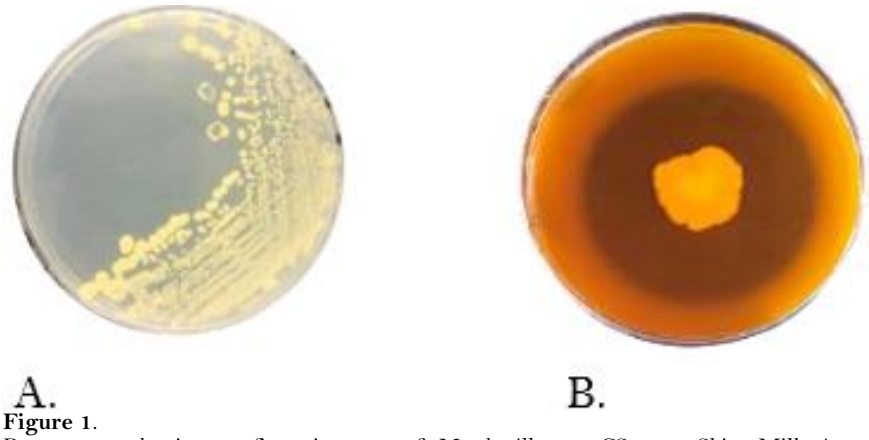

Figure 1.
Protease production confirmation test of *Metabacillus* sp. CS-2 on Skim Milk Agar (SMA) media, after the 7th day of observation. A. Yellow purified colony of *Metabacillus* sp. CS-2 on Nutrient Agar media B. A clear zone surrounding the bacterial colony at the center of the plate confirmed the presence of bacterial extracellular protease.

The results of the Gram staining test showed that the endophytic bacterial isolate of CS-2 isolate was gram-positive, which was characterized by purple cells. In addition, observations also revealed that the shape of bacterial cells is bacillus and the bacteria have a chain arrangement. Gram-positive bacteria have a cell wall composed of peptidoglycan that is insoluble in acetone alcohol, so the blue color of the crystal violet is retained, while the red dye safranin cannot penetrate the bacterial cells during staining. A persistent purple color indicates that the bacteria are Gram-positive [16].

The morphology of rejuvenated bacterial colonies on Nutrient Agar (NA) media (Figure 1) shows a circular shape with a diameter of 4 mm, flat edges, convex elevation, smooth consistency, and yellow color. The morphology observed had similarities with the colony morphology found by previous researchers so the rejuvenated colony is a colony of the same type of the bacterial colony *Metabacillus* sp. CS-2 was correctly used and reported in the previous study [5].

Bacterial isolate *Metabacillus* sp. CS-2 exhibited proteolytic activity around the colony, as seen in Figure 1. Observations were made for 7 days (Table 1), and the strain CS-2 exhibited coagulation of casein with varying diameters around the colony. This difference in diameter indicates the ability of the isolate to produce extracellular proteases that can clump casein [17]. The results showed that on day 7, the bacteria produced zones with a diameter of up to 50 mm (see Table 1).

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Table 1.
Proteolytic indexes of protease production by *Metabacillus* sp. CS-2 on Skim Milk Agar (SMA) by day within a week.

Day-	Colony diameter (mm)	Proteolytic (clear) zone diameter (mm)	Proteolytic index
1	6 ± 0.1	12 ± 0.1	2.00 ± 0.1
2	10 ± 0.2	21 ± 0.2	2.10 ± 0.2
3	11 ± 0.1	27 ± 0.2	2.45 ± 0.2
4	15 ± 0.2	35 ± 0.1	2.33 ± 0.2
5	15 ± 0.1	45 ± 0.3	3.00 ± 0.3
6	17 ± 0.3	45 ± 0.1	2.64 ± 0.3
7	22 ± 0.1	50 ± 0.1	2.27 ± 0.1

The ability of a microbe to change the substrate can be seen from the clear zone area formed on a growing medium. The larger the clear zone area that is formed, the more the microbe has a high ability to change the substrate contained in the medium [18]. Therefore, the protease content in *Metabacillus* sp. can be done quantitatively.

3.2. Enzyme Activity Before and After Ultrafiltration

After ultrafiltration using Vivaspin, a yellowish concentrated enzyme was obtained **Fig. 2**. The results of ultrafiltration using Vivaspin are usually in the form of an enzyme solution that has been separated and enriched. This process produces enzymes with higher concentrations than before ultrafiltration.

Figure 2.
Ultrafiltrate of crude protease of *Metabacillus* sp. CS-2 (appeared as orange intense color in the center of conical tube).

As seen in **Figure 2**, after ultrafiltration using Vivaspin, a yellowish concentrated enzyme was obtained. The results of ultrafiltration are usually in the form of an enzyme solution that has been separated and enriched. This process produces enzymes with higher concentrations than before ultrafiltration. The results of ultrafiltration using Vivaspin are usually in the form of a solution or

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liquid. The color and clarity of this solution depend on the nature of the protein or enzyme being processed. If the concentrated enzyme is colored or contains a specific pigment, then the result can also be similar or more intense in color [10-11].

Protease activity of *Metabacillus* sp. CS-2 was tested, showing that the enzymes of the isolate had different levels of protease activity before and after ultrafiltration. The results of the protease activity test are displayed in Table 2.

Table 2.
Activity (U/mL/min of crude and ultrafiltrate protease of Metabacillus sp. CS-2.

Bacterial protease sample	Absorbance at λ = 595 nm			Enzyme activity
	Sample active	Sample in-active	Total (y)	
Crude	1.629 ± 0.016	1.629 ± 0.022	1.629 ± 0.005	0.342 ± 0.011
Ultrafiltrate	1.752 ± 0.007	1.427 ± 0.015	0.325 ± 0.023	0.768± 0.014

Bovine Serum Albumin (BSA) standard solution was used to determine crude and ultrafiltrate proteases based on absorbance at λ = 595 nm following the Bradford method [8-9, 12-13]. The results showed that protease activity as ultrafiltrate is doubled compared to the crude extract (**Table 1**). Greater absorbance from ultrafiltrate (see **Table 1**) is directly related to the concentration of amino acids produced. Protease activity of *Metabacillus* sp. CS-2 was tested, showing that the enzymes of the isolate had different levels of protease activity before and after ultrafiltration.

The activity of crude protease was lower compared to semi-pure enzymes resulting from ultrafiltration. The activity in crude protease is 0.342 ± 0.011 while from ultrafiltrate reached 0.768± 0.014 U/ml. Such increased activity result is in line with that previously reported for protease of *Bacillus thuringiensis* [15]. This is likely because semi-pure enzymes have gone through an ultrafiltration process to remove impurities or other protein components. As a result, when reacted with casein as a substrate and TCA, only the protease enzyme will hydrolyze amino acids and peptides.

During ultrafiltration, inhibitory components, and unwanted contaminants are removed from the enzyme mixture, which allows the protease enzyme to function without interference from other proteins that can reduce its effectiveness. This purification process can also maintain the stability of the enzyme conformation, thereby increasing the ability of the enzyme to maintain its activity during the reaction. As a result, protease enzymes obtained from ultrafiltration exhibit better specificity to the substrate and higher efficiency in carrying out the proteolysis process compared to crude, which may contain various components that interfere with enzyme activity [19].

3.3. Specificity Assay by Zymography

Zymographic analysis of crude and ultrafiltrate protease of strain CS-2 enzymes showed that both are capable of degrading 4 substrates tested (casein, gelatine, fibrin and collagen) evidenced by the formation of clear zones on zymogram (**Figure 3**). Such clear zone represented degradation product of substrate by protease on a specific size.

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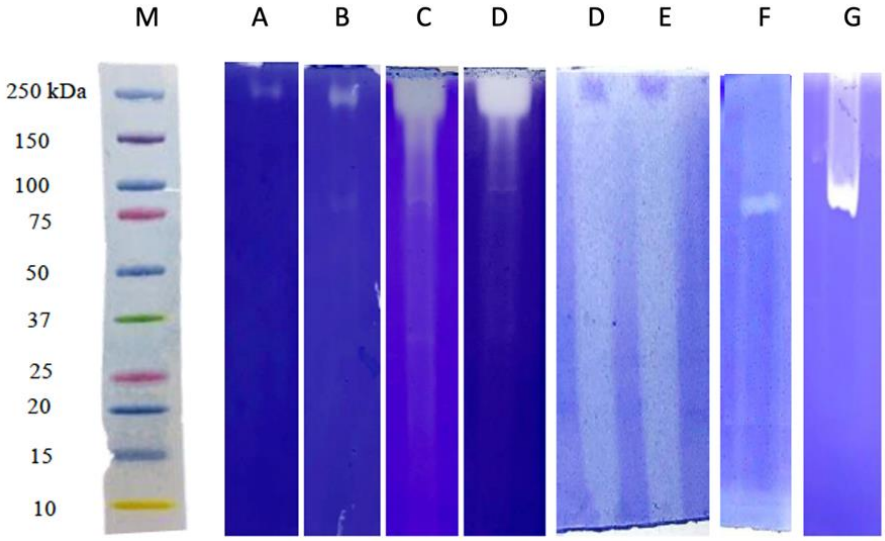

Figure 3.
A The zymogram showing the ability of fibrinolytic protease of *Metabacillus* sp. CS-2 both as crude extract and ultrafiltrate degrades four types of protein substrates. (A-B = crude and ultrafiltrate on casein; C-D crude and ultrafiltrate on gelatine; E-F = crude and ultrafiltrate on fibrin; E-F = crude and ultrafiltrate on collagen.

Using casein as substrate (Figure 2A) a single band with a molecular weight of 250 kDa was obtained from crude protease and so was it from, the semi-pure protease or ultrafiltrate (Figure 2B). Specificity of crude bacterial protease enzymes gelatin substrate was evidenced by the presence of clear 3 bands with molecular weights of 250, 150 and 100 kDa, while the ultrafiltrate showed clear zones with a molecular weight of 150 – 250 kDa (Figure 2C-D). Both crude and semi-pure protease of strain CS-2 using fibrin substrates showed uniform clear zone results ranging from protein-sized 10 kDa to 250 kDa. The results using crude protease enzyme with collagen substrate showed the presence of a clear band at a molecular weight of 75 kDa (Figure 2G-H).

In the zymography analysis, a separator gel is added to the substrate, in this case, casein, fibrin, gelatin, and collagen will be polymerized with acrylamide. The samples tested were crude enzymes and semi-pure enzymes. The results of the zimography showed the existence of protease activity which was characterized by the formation of a clear zone. Coomassie Brilliant Blue (CBB) dye will bind to proteins containing amino acid residues with aromatic side chains, producing a blue complex. The polymerized SDS-polyacrylamide gel with the substrate forms a clear zone as a result of degradation by protease enzymes. In zymography, wider or even clear zones indicate that the enzyme performed well in degrading the substrate and may reflect increased enzyme activity after the purification process [20].

Results of our study reflected that fibrin is the most suitable substrate of all for protease produced by strain CS-2 showing that likely more than one protease subunits work together in degrading fibrin. Along with the ability to degrade collagen at 75 kDa it can be referred that the potential of fibrinolytic protease of strain CS-2 could become more effective in the debridement of chronic wounds by erasing collagen as part of skin remnants that may present and block the oxygen or nutrients for healthy cells surrounding wounds.

4. Conclusions

Based on the study's results, it can be concluded that fibrinolytic protease enzyme activity from *Metabacillus* sp. CS-2 after ultrafiltration is higher than before ultrafiltration. Both crude enzymes and semi-purified enzymes are capable of degrading substrates of casein, gelatine, fibrin, and collagen at different molecular weights. Fibrin appeared to be the most suitable substrate for protease of strain CS_2 of all substrates tested in this study. Along with specificity to collagen substrate, high specificity

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to fibrin likely supports fibrinolytic protease of the strain to function as a debridement agent in wound healing treatment.

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