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Degradation of Artificial Wound Eschar (AWE) by Recombinant Protease

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Abstract
Eschar is a major problem in wound healing process which promotes the bacterial growth, prevents formulation of granulation tissue, causing infections and the removal of eschar or necrotic tissue is needed for wound healing process which involves surgical debridement. Surgical debridement result in destroying healthy tissue, inflammation and may limit the surgery to be performed at certain body parts. Finally, enzymatic debridement was applied by employing proteolytic enzymes such as protease in facilitating the wound healing process plus, recombinant enzyme shows a better proteolytic activity which contribute as debriding agent to digest necrotic tissue. In this study, the recombinant protease was being expressed and purified to observe its ability in degrading the protein in AWE substrate (fibrin, collagen, elastin).
Keywords: debridement; eschar; recombinant protease

Introduction

A wound is commonly characterized as disruption of the epithelial lining or mucus produced by physical or thermal injury which can be categorized as acute or chronic wounds based on their severity [1]. Eschar is a thick and adherent dead tissue or non-healing wounds such as pressure ulcers, diabetic foot ulcers, burn injury including ulcers from primary rheumatologic diseases [2]. The ideal eschar removal technique should selectively remove nonviable tissue with minimal blood loss in providing faster wound healing through conservative treatment [3]. Debridement is the most widely recognized method used for wound management in promoting re-epithelization and removal of necrotic tissue within wounds. Debridement can be done in surgical, biological, autolytic and enzymatic debridement [4]. Commonly, surgical debridement had been applied in the removal of eschar however, the used of sharp instrument when performing surgery may result in destroying healthy tissue, infection and inflammation. The considerable efforts in discovering the suitable technique in digesting devitalized tissue from eschar without is through the enzymatic debridement.

Proteolytic enzymes such as protease is widely used in health sector and the alternative to surgical debridement in applying proteolytic enzymes to wounded area which aid in the natural healing .The bromelain protease derived from pineapple had shown promising effects in eschar debridement however in industry application, bacterial protease are typically favoured over animal and plant proteases due to their various physiological properties, cost-effective and comparatively simple genetic modification [5]. Among several protease producers, *Bacillus sp*. is the most widely employed bacteria for proteases. Here we study the expression and purification of recombinant protease in *E.coli* BL21 (DE3). The recombinant enzyme was found to have high proteolytic activity with substrate which contribute in degrading wound eschar efficiently as well as potentially be an alternative component of wound debridement dressings.

Materials and methods

Expression of Recombinant Protease using *E.coli* BL21 (DE3)

Protease used was first isolated from *B.pumilus* from fermented food which designated as SPBP gene. Purified recombinant protease had successfully cloned and expressed in *E.coli* BL21 (DE3) by previous project (Nurulfarhana Hussin, 2018) (Unpublished data). Glycerol stock of recombinant *E. coli* BL21 (DE3) carrying vector pET-21b-SerPro was thawed and dipped with pipette tip. The dipped pipette tip pipette tip harboring the expression of recombinant protease was grown on the autoclaved LB broth supplemented with 100 µg/mL of ampicillin, allowed for incubation at 37˚C for 16 hours with shaking at 200 rpm as starter culture. The starter culture was inoculated (10% inoculated size) into LB broth supplemented with 100 µg/mL ampicillin for the purpose of overexpression. The condition for expression of recombinant protease using *E. coli* BL21(DE3) was set at temperature 37˚C with shaking at 200 rpm and the cell growth was observed until OD_{600nm} reached 0.6-0.8. The culture was induced with 0.4 mM of IPTG at 37˚C for 3 hours while uninduced culture acted as a control. Induced cell were harvested & subjected to cell lysis using sonicator along with SDS-PAGE.

Purification of Recombinant Protease using Chromatography Technique

For the first step of purification of recombinant serine protease, nickel affinity chromatography was used with column material of HisTrap HP, 1mL charged with 0.1M $NiSO_4$ on AKTA^{TM} Pure Machine. Prior to loading the sample into the column, the column containing soluble crude extract was washed with binding buffer and the bound serine protease on the column was eluted using elution buffer. Fraction of partially purified serine protease were concentrated using buffer exchange by Amicon 30 molecular weight cutoff (MWCO) device. The eluted fraction containing recombinant protease was transferred into Amicon tube and the fractions was concentrated by centrifugation at 4000 rpm at 4°C until the protein become concentrated down to 500µL. SDS-PAGE was conducted to check the purity and molecular weight of serine protease. SDS-PAGE analysis was being conducted on crude lysate of transformed *E.coli* BL21 (DE3) upon induction at 37˚C for 3hr, fraction of partially purified protein using nickel affinity chromatography with column material of HisTrap HP 1ml and protein concentrated using buffer exchange by Amicon 30 MWCO.

Protease Assay

Protease activity assay was conducted by using casein solution as a substrate. Three centrifuge tubes were added with 0.65% casein solution in 50mM potassium phosphate buffer (pH 8). Then, 10 µM of serine protease was added into the three centrifuge tubes followed by incubation at 37˚C for 10 minutes. The reaction mixture was terminated by addition of 5mL of trichloroacetic acid (TCA) into each tube and incubated at 37˚C for 30 minutes. Next, the mixture were centrifuged at 12000 rpm for 5 minutes. 2mL of the supernatant from each vials including the blank was transferred into a new tube and mixed with 5 mL of 500 mM sodium carbonate (Na_2CO_3) and 1 mL of 0.5 mM Folin-Ciocalteu reagent. The mixture was then incubated at 37˚C for 30 minutes followed by centrifugation at 12000 rpm for 5 minutes. The amount of tyrosine released in the mixture was measured by using spectrophotometer at absorbance 660 nm. One unit of the enzyme activity (U) was defined as the amount of enzyme needed to release 1 µmol of tyrosine equivalent per minute under the assay conditions. The standard curve was made by using 1.1 mM tyrosine as standard. Protein estimation was done by running 100 µL of sample on a nanodrop machine to check protein concentration (mg/ml) at absorbance 280 nm.

Preparation of Artificial Wound Eschar (AWE)

Artificial Wound Eschar (AWE) was prepared by mixing fibrinogen, collagen, fibrin and then clotting with thrombin. Briefly, 325 mg collagen and 50 mg fibrin were mixed thoroughly in 10 mL Tris buffer (50 mM Tris base, 100 mM NaCl, 10 mM $CaCl_2$), titrated to pH 7.4. The fibrinogen solution was prepared by dissolving 100 mg fibrinogen in 10 mL Tris buffer. The two mixture were completely mixed and 0.25 mL thrombin solution was added. Then, the mixture was quickly mixed and poured onto the

petri dish containing 47mm nylon membrane filter. Clotting of proteins was observed by a formation of a soft layer on top of membrane filter as a result of the thrombin-induced fibrinogen polymerization. The clotted hydrogel was gently rinsed with distilled water for 3 times. The membrane filter was dried with tissue paper and continued dried completely for 3 days [6].

Degradation Analysis of AWE

An AWE degradation analysis was completed through modified method from published method that based on [6] where the AWE substrate had been labeled with fluorescent tagged dye however, similar method following protease assay was used to observe the degradation analysis due to the limitation of substance and financial status. Protease activity was conducted by using dried AWE as a substrate. Briefly, dried prepared AWE substrate was cut into smaller pieces and gently put into four different 15 mL centrifuge tubes and one was act as blank. The analysis was observed in 1 hour, 6 hour and 24 hour. The reaction mixture contained AWE substrate in 50 Mm of potassium phosphate buffer (pH 8). 10 µM of serine protease concentrated using buffer exchange was added into three of the vials and incubated at 37°C for respective hours. The reaction was terminated by the addition of trichloroacetic acid (TCA) and incubated at 37°C for 30 minutes. After that, the mixture was centrifuged at 12000 rpm for 5 minutes. 2 mL of supernatant was transferred into new tube and mixed with 5mL of 500 mM sodium carbonate (Na_2CO_3) and 1 mL of 0.5 mM Folin-Ciocalteu reagent. The mixture was incubated at 37°C for 30 minutes followed by centrifugation at 12000 rpm for 5 minutes. The degradation analysis of AWE component (collagen, fibrin, fibrinogen) was measured using spectrophotometer at absorbance 660 nm.

Results and discussion

Expression of Recombinant Protease in *E.coli* BL21 (DE3)

Recombinant *E. coli* BL21(DE3) carrying vector pET-21b-SerPro that has been thawed at –80^{0}C was grown on autoclaved LB broth supplemented with 100 µg/ml ampicillin as demonstrated in Figure 1. *E.coli* is among the most well-known cell factories for producing recombinant proteins which giving the rapid growth rate and achieve high cell density with doubling time about 20 minutes, offers a low-cost recombinant protein expression system as well as contributing in the easiness of genome manipulation [7; 8]. Recombinant *E.coli* in LB agar supplemented with ampicillin able to grow properly as it carry an ampicillin-resistance gene as well as have major antimicrobial resistance towards ampicillin which hinders the action of ampicillin for the cell to survive [9]. Addition of ampicillin as a best selection marker has been commonly used for protein expression in *E.coli* which contribute in affecting the growth of *E.coli* as well as maximize the expression of serine protease [10].

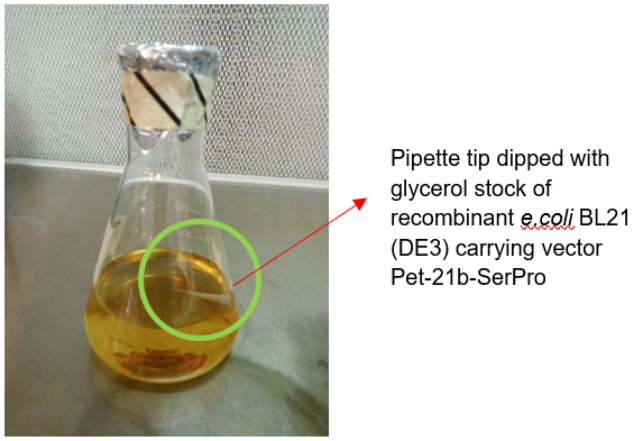


Figure 1 Starter culture of recombinant *E.coli* BL21 (DE3) carrying vector pET-21b-SerPro supplemented with 100 µg/mL ampicillin.

As shown in Figure 1, the prepared starter culture was inoculated into 5 conical flasks containing fresh 1000 mL LB broth supplemented with 100 µg /mL ampicillin for the purpose of overexpression. The sample was cultured until optical density reached 0.6-0.8 as it is the best condition for expressing serine protease and followed by optimized induction condition for expression of serine protease with 0.4 mM of IPTG at 37^0C for 3 hours. The absorbance reading of the culture was measured at OD_{600nm} and the culture took 3 hours to reach absorbance reading of 0.6-0.7 as illustrated in Figure 2.

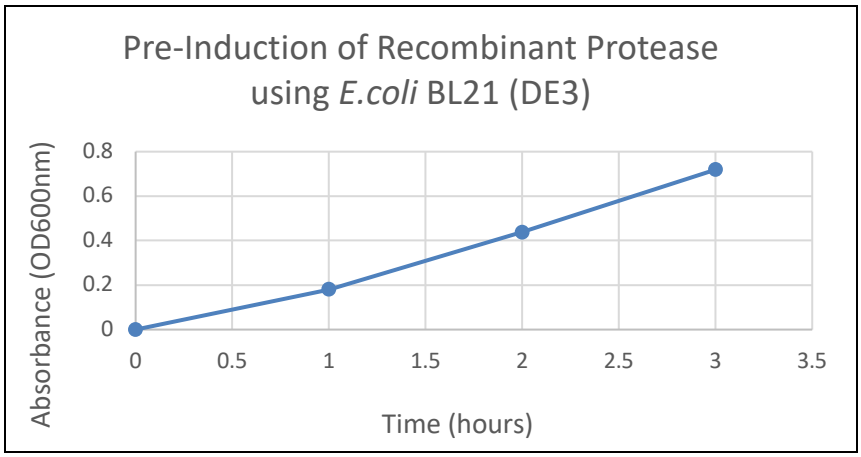


Figure 2 Time taken for 5 conical flask containing overexpressed culture of recombinant protease in *E.coli* BL21 (DE3) to reach OD_{600nm} = 0.7

After the absorbance reading of culture reached 0.7, the sample was induced with 0.4 mM IPTG at 37^0C and preserved at post-induction temperature for 3 hours. Presence of IPTG provides induction of protein synthesis with great selectivity and activity because it able to activate the T7 promoter which influences high recombinant protein expression [7]. The induced cells were then harvested by centrifugation as displayed in Figure 3.

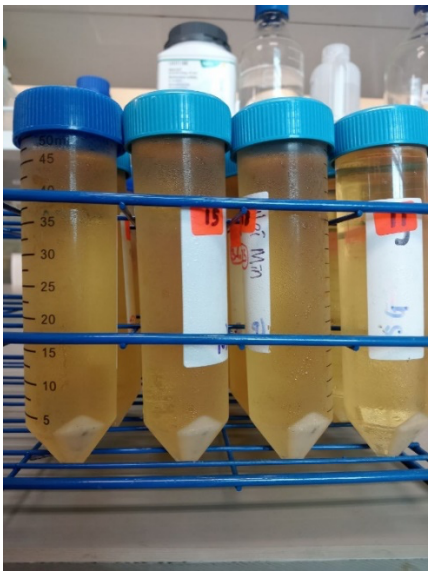


Figure 3 Induced cells that was harvested through centrifugation which separate supernatant and pellet

The harvested cell pellet was weighed to obtain the total mass pellet for the cell lysis to be made. Serine protease isolated from *B.pumilus* is an intracellular serine protease, the protease should be inside the pellet prior to cell lysis. The total mass pellet obtained was recorded in Table 1. In addition, the cell lysis was performed in accordance with the recommended ratio of 1g of cell pellet was lysed in 10 mL of lysis buffer. For 1000 mL of overexpressed culture, total 12.0431g of cell pellet were harvested.

Table 1: Total mass cell pellet

Falcon Tube	Mass of Empty Falcon Tube (g)	Mass of Falcon Tube Containing Pellet (g)	Mass of pellet (g)
1	13.8537	14.4445	0.5908
2	13.7733	14.1303	0.357
3	13.1800	13.8127	0.6327
4	13.3170	14.0336	0.7166
5	13.3807	13.9756	0.5949
6	13.4316	14.2491	0.8175
7	13.5727	14.0832	0.5105
8	13.2048	13.7482	0.5434
9	13.2124	13.9118	0.6994
10	13.2440	13.9915	0.7475
11	13.2116	13.9214	0.7098
12	13.4480	13.9333	0.4853
13	13.7712	14.3695	0.5983
14	13.7606	14.2629	0.5023
15	13.9275	14.6209	0.6934
16	13.6515	14.2368	0.5853
17	13.3382	14.0306	0.6924
18	13.2532	13.8648	0.6116
19	13.6707	14.1092	0.4385
20	13.5607	14.0766	0.5159

SDS-PAGE analysis was conducted to determine the effects of transformation and IPTG induction on the expression of serine protease. SDS-PAGE is a protein separation technique based on molecular weight. It is a method for separating protein molecules based on their molecular weight and electrophoretic mobility which determine the rate of protein migration through a gel matrix [11]. Smaller proteins migrate faster due to less resistance from the gel matrix when separated through a gel matrix. The structure and charge of the proteins also have an effect on the rate of migration through the gel matrix [12]. The solution used in SDS-PAGE was prepared to denature proteins by boiling with the anionic detergent called sodium dodecyl sulphate (SDS) and β-mercaptoethanol. The combination of detergent and heat are enough to break the noncovalent bonds that hold protein folds together while the addition of β-mercaptoethanol contribute in breaking covalent bonds between cysteine residues.

N,N-methylene bisacrylamide was used as a cross-linking reagent which control the pore size of gel through its chemical polymerization that occurs due to the reaction of free oxygen radicals

generated from the reaction ammonium persulfate (APS) with N,N,N'N'-tetramethylethylenediamine (TEMED). TEMED is used as a catalyst with APS to catalyse acrylamide polymerization [13]. In this study, SDS-PAGE was performed with 4% (w/v) stacking and 12% (w/v) separating gels, which suitable concentration of acrylamide and bisacrylamide for the optimal separation of serine protease with molecular weight of 36 kDa.

After IPTG induction, the crude lysate was subjected to SDS-PAGE analysis before and after cell lysis to observe the effects of transformation and induction on the production of serine protease. Based on Figure 4.3, the soluble protein of transformed *E.coli* BL21 (DE3) without induction showed no bands corresponding to the full length of serine protease which has a theoretical molecular weight of 46 kDa. In lane 5, the soluble protein transformed *E.coli* BL21 (DE3) with 0.4 mM of IPTG induction displayed a thick band representing the serine protease which also indicate that recombinant serine protease was mainly expressed as soluble protein instead of inclusion bodies.

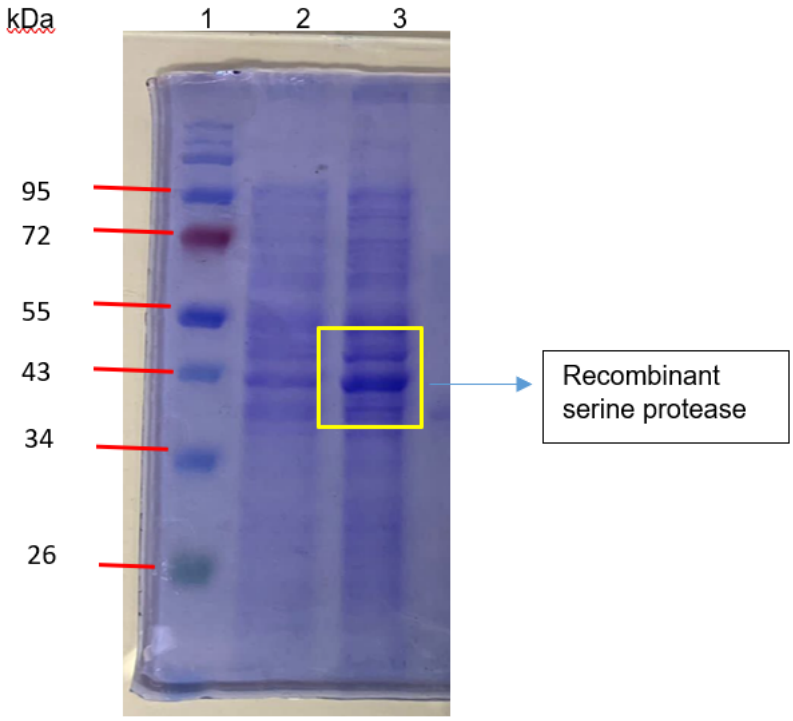


Figure 4 SDS-PAGE analysis of protein from supernatant of transformed *E.coli* BL21 (DE3) upon induction at 37°C for 3 hours. The protein marker used was Color Prestained Protein Standard, Broad Range (10-250 kDa) from New England Biolabs Inc. Lane 1: protein marker; Lane 2: Soluble protein of transformed *E.coli* BL21 (DE3) with OD_{600nm} at 0.6 and uninduced cells after before lysis; Lane 3: Soluble protein of transformed *E.coli* BL21 (DE3) with OD_{600nm} at 0.6 and induced with 0.4 mM of IPTG after cell lysis.

Purification of Recombinant Protease by chromatography technique

As serine protease is an intracellular protein which it should be inside pellet, cell lysis was made to extract the protein inside pellet. Cell lysis also known as cellular disruption is a method of breaking down or destroying the outer boundary of cell releasing intracellular DNA, RNA, protein or organelles from a cell [14]. In this study, probe sonicator was used to lyse the homogenized pellet for 5 minutes with amplitude set to 50%.

The lysing process was initiated by homogenizing the pellet in lysis buffer which aid in disrupting the cell membrane by altering the pH. The tip of probe sonicator was applied directly to the homogenized pellet in lysis buffer allowing lysing occur efficiently due to the high shear force resulted from high pressure of sonicator. It is crucial to keep the sample on ice during the prolonged sonication

Figure 5 Probe sonicator used to lyse the cell pellet.

process as the heat generated by the vibration can cause protein denaturation [15]. Thus, sonication was paused every 2 minutes to avoid overheating. In this study, the cell lysis was done successfully and proven by the presence of thick band in the third lane of SDS-PAGE with the size of 46kDa as illustrated in Figure 6. Immobilized metal-affinity chromatography (IMAC) or specifically HisTrap column was used for the first step of purification of recombinant serine protease. IMAC is commonly used to purify recombinant protein with polyhistidine affinity tag since previously, the recombinant serine protease was equipped with polyhistidine tag at C-terminus. The presence of immobilized metal ion in the matrix such as Ni^{2+}, Cu^{2+}, Zn^{2+} and Co^{2+} interact specifically with histidine residue on the protein surface which facilitate the separation process [16]. The polyhistidine tag exhibit strong affinity with immobilized metal ion matrix allowing the purification using AKTA^{TM} Pure Machine to be successfully conducted due to the ability of histidine imidazole ring that have electron donor group and readily to form coordination bonds with immobilized metal ions. Recombinant serine protease equipped with histidine tag was efficiently retained at Ni-NTA resin.

As the recombinant serine protease equipped with histidine-tagged bind strongly to the column matrix, it was eluted by using a low pH buffer and high concentration of free imidazole was added in the preparation of elution buffer in order to completely elute the protein. Imidazole was needed because it can act as counter ligand by binding with immobilized metal ion and eventually elute protein that weakly bind to the column. Thus, high concentration of imidazole aid in interfering with the binding of recombinant protease to metal ion resulting in elution of serine protease. After sonication, the crude lysate was filtered using 0.22 µM syringe filter and then subjected to 1 mL HisTrap column. Sample application was done by using pump instead of sample loop or superloop as it can be used to load higher sample volume of up to 200 mL into the AKTA^{TM} Pure Machine.

The flow rate was set at 1.0 mL/min. Prior to sample application into the column, the column was washed with binding buffer (25 mM Tris base, 500 mM NaCl, 30 mM imidazole and 10% glycerol) which contain low concentration of imidazole that suitable for all proteins. The addition of high concentration of sodium chloride may increase the ionic strength of protein in binding with Ni-NTA and remove contaminant from Ni-NTA resin by reduce non-specifically protein [17] while low concentration of imidazole was added to binding buffer to interfere with weak binding of other protein and elute protein that weakly bind. The bound recombinant serine protease was eluted from the column by using elution buffer (500 mM imidazole, 25 mM Tris base, 500 mM NaCl, 10% glycerol).

Based on the chromatogram, the serine protease was observed to be eluted at phase 4 where the concentration of imidazole was expected to be about 20-40% as shown in Figure 6 at the highest peak. The fraction with highest peak of serine protease was observed at fraction 8 with absorbance value of 500 mAU. Fraction 6 until fraction 9 were pooled together and concentrated by buffer

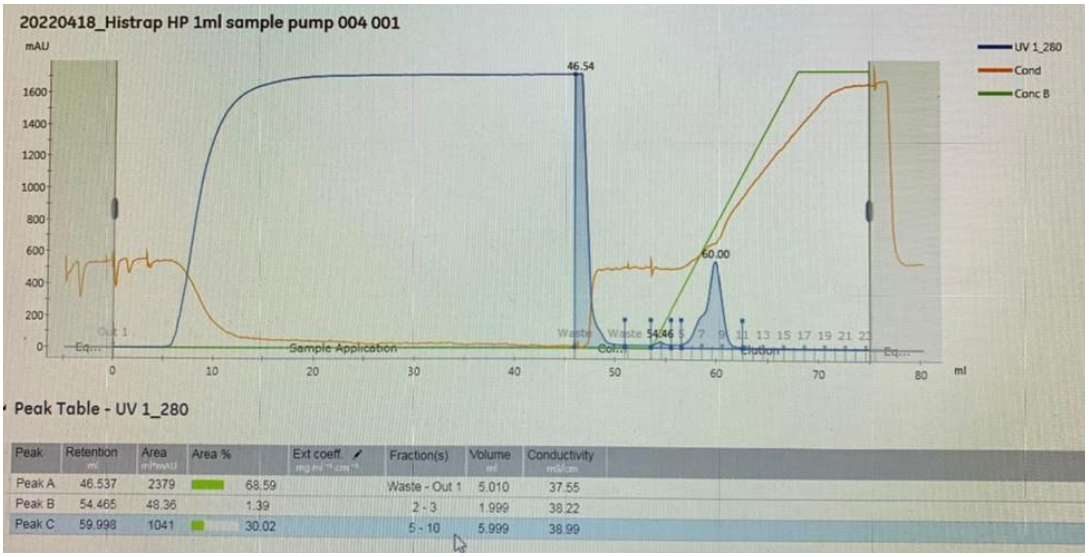


Figure 6 The chromatogram of histidine-tagged affinity purification chromatography of partially purified recombinant protease generated through purification using HisTrap column. Phase 1: equilibration; Phase 2: sample application; Phase 3: column wash; Phase 4: elution and Phase 5: Equilibration.

exchange into Tris buffer (25 mM Tris base). SDS-PAGE was conducted upon completion of concentration of serine protease from fraction 6 until fraction 9 by buffer exchange. Based on the Figure 6, all the thick band represents the presence of serine protease. As shown in lane 3, the band of serine protease from crude lysate was observed to travel lesser through the gel compared with other protease that went through subsequent purification including purified using HisTrap column and concentrated using buffer exchange. This also indicate that protease from crude lysate has higher molecular weight compared to others. The molecular weight of serine protease after cell lysis was 46 kDa, molecular weight of serine protease fraction purified using HisTrap column was 36 kDa and molecular weight of serine protease concentrated using buffer exchange was 36 kDa.

The molecular weight of serine protease was influenced by the removal of 18 amino acids present in the N-terminal extension that also known as pro-peptide which act as an inhibitor of protease activity by binding across and block the active site of intracellular subtilisin protease (ISP). Removal of pro-peptide occur after purification process. Presence of N-terminal help in regulating the activity of ISP by blocking the active site and catalytic triad rearrangement which eventually, limit the protein to migrate further in gel. ISP was active in the presence of calcium which essential in the structural rearrangement of catalytic activity that aid in structural stability, proper folding of protease and releasing pro-peptide. The presence of pro-peptide consisting conserved LIPY/F-motif also leads to inhibition of catalytic activity by disrupting the conformation of catalytic triad in shifting Ser and His residues [18]. After removing, the active site is unblocked and catalytic triad rearrange to functional confirmation.

The removal of 18 amino acids from pro-peptide during purification steps may occur due to the presence of calcium that improve the activity and lead to auto-proteolysis of protein resulting in decrement in size of serine protease. This explained why serine protease purified with HisTrap column and buffer-exchanged travelled further than serine protease in crude extract, thus having smaller molecular weight due to the removal of pro-peptide that influenced the migration through gel. The molecular weight of serine protease from three purification steps shown to meet with the expected molecular weight. The molecular weight of serine protease after cell lysis was 46 kDa, molecular weight of serine protease purified with HisTrap column was 36 kDa as well as molecular weight of

serine protease concentrated with Amicon Ultra-15 Centrifugal Filter also 36 kDa.

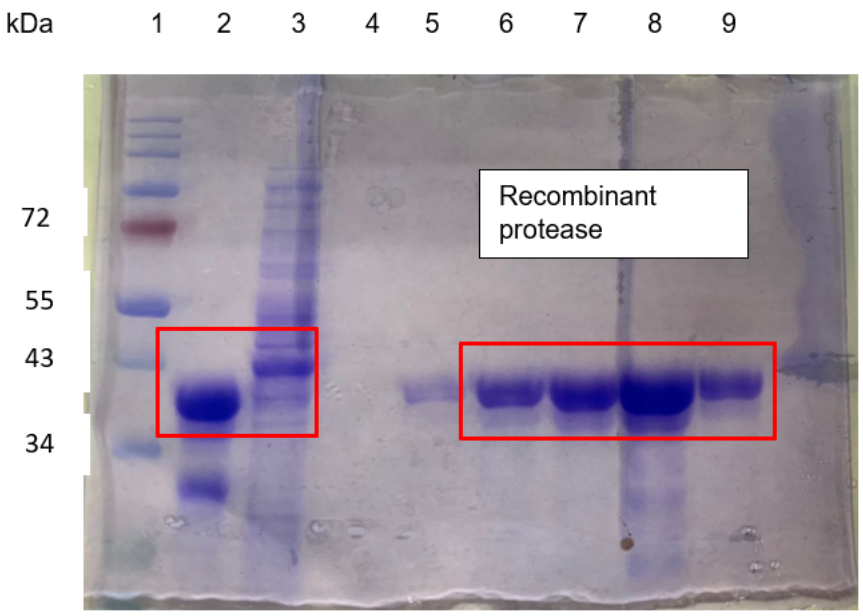


Figure 7 SDS-PAGE analysis of protein from crude lysate of transformed *E.coli* BL21 (DE3) upon induction at 37°C for 3 hours, protein purified using HisTrap column and protein concentrated using buffer exchange by Amicon 30 MWCO device. The protein marker used was Color Prestained Protein Standard, Broad Range (10-250 kDa) from New England Biolabs Inc. Lane 1: Protein marker; Lane 2: Protein concentrated using buffer exchange; Lane 3: crude lysate; Lane 5: HisTrap column wash; Lane 6-9: Protein fraction (6-9) purified using HisTrap column.

Protease Assay

The activity of serine protease was measured through protease assay method involving protease from all three purification steps; crude lysate of transformed *E.coli* BL21 (DE3) upon induction at 37°C for 3 hours, protein purified using HisTrap column and protein concentrated by buffer exchange using Amicon 30 MWCO device. Protease assay method utilized casein as a substrate. The amino acid tyrosine was released along with other amino acids and peptide fragments as the serine protease digest casein. Free tyrosine will primarily react with Folin & Ciocalteu Phenol or Folin's reagent and resulted in the formation of blue colored chromophore following the measure of absorbance using spectrophotometer at OD_{660nm}. Theoretically, the more tyrosine released from digestion of casein, the more blue chromophore will formed indicating the high activity of serine protease. In order to correlate changes in absorbance with the amount of released tyrosine, the protease activity absorbance values from each sample were compared to a tyrosine standard curve. The tyrosine standard curve was prepared by known concentration. The activity of protease can be calculated from the standard curve in units or the amount in micromoles of tyrosine equivalent released from casein per minute.

Right after protease assay was conducted, a purification table of serine protease was generated. Based on Table 2, the crude lysate with volume of 48 mL had a protein concentration of 10.01 mg/mL and total protein of 480.48. However, total activity and specific activity of crude lysate was unable to be calculated due to the nature of serine protease which in pro-peptide form resulting it to be enzymatically inactive. As already mentioned, the short N-terminal extension that controlled the activity of serine protease by rearranging the catalytic triad and blocking the active sites rendered the full length of proenzyme to become inactive [18]. It was discovered that the serine protease had carried out its particular proteolytic processing after being purified with a HisTrap column because color changes during a protease assay demonstrated its activity.

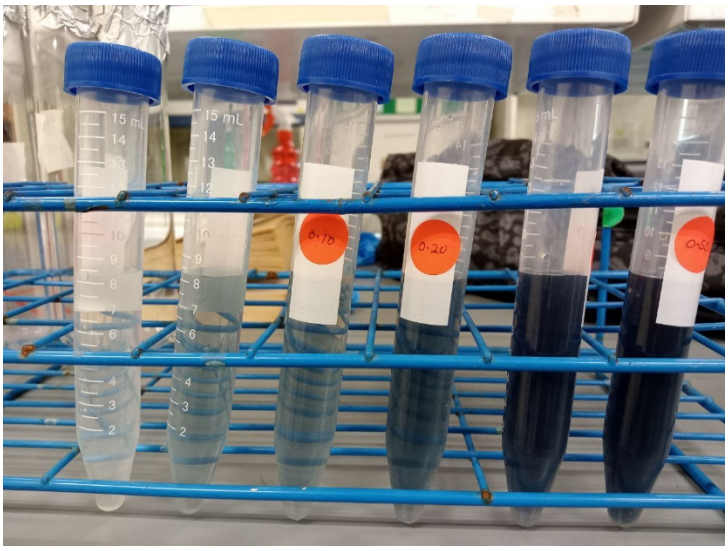


Figure 8 Gradient color changes in tyrosine standard curve added into each vials with known concentration for measurement of activity of serine protease in correlating the changes in absorbance reading from all three samples. From left; blank with no tyrosine added, 0.05 mL tyrosine added, 0.10 mL tyrosine added, 0.20 mL tyrosine added, 0.40 mL tyrosine and 0.50 mL tyrosine added.

The partially purified protease eluted through HisTrap column resulted in protein concentration of 1.35 mg/mL, enzyme activity of 0.058 Units/mL, total protein of 5.4 mg and specific activity of 0.043 U/mg. Besides, the purified serine protease concentrated using buffer exchange by Amicon Ultra-15 Centrifugal Filter resulted in protein concentration of 1.21 mg/mL, total protein of 0.605 mg, enzyme activity of 0.124 U/mL and specific activity of 0.103 U/mg. The yield recovery of the recombinant protease decreased at every step of purification due to the removal of other unwanted proteins and in this project, the overall yield that had been achieved was 26.67%.

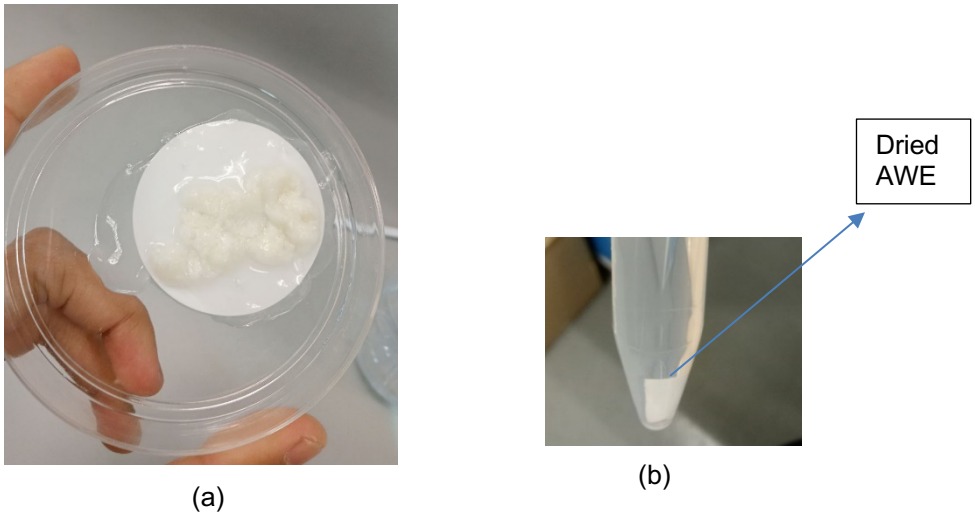
Table 2: Purification table of serine protease for crude lysate and purified protease from purification steps

Purification Step	Volume (mL)	Nanodrop (mg/mL)	Total Protein (mg)	Enzyme activity (U/mL)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude Lysate	48	10.01	480.48	NA	NA	NA	NA	NA
HisTrap HP Immobilized Metal Affinity Chromatography (IMAC)	4	1.35	5.4	0.058	0.233	0.043	1	100
Amicon Ultra-15 Centrifugal Filter	0.5	1.21	0.605	0.124	0.062	0.103	2.380	26.67

Degradation of Artificial Wound Eschar (AWE)

The degradation of AWE was a modified method which may not suitable as the published method involve the used of fluorescent tagged substrate for analyzing the activity of serine protease degrading each major components of AWE substrate through the appearance of each dye [14]. Previously, the evaluation on the breakdown of eschar by papain was conducted by placing AWE on Franz Diffusion Cells [14] and the new cost-effective method is placing AWE in a 6-well plate system [6]. Originally, the breakdown of each protein in AWE was determined by the cumulative fluorescent intensity of collagen, elastin, and fibrin (major protein in wound eschar) which each were labelled with various fluorescent dyes as illustrated in Table 3.

The analysis could provide a useful way to compare the selectivity of debridement for different enzymes. In this project, AWE substrate was generated consisting of thrombin as polymerization-inducing agent, fibrinogen is a polymerizable protein act as clotting component and insoluble fibrin. As shown in Figure 9, the thrombin-induced fibrinogen polymerization resulted in the formation of gel-like structure indicating that thrombin had cleaved the fibrinogen, converting soluble fibrinogen into insoluble fibrin and in the absence of thrombin, the ability of fibrinogen in forming fibrin was prevented [19]. Dried AWE was subjected for the degradation analysis.



(a) (b)

Figure 9: (a) Addition of thrombin resulted in the formation of gel-like structure on top of the 47mm Nylon membrane filter due to the thrombin-fibrinogen polymerization. (b) Dried AWE substrate that had been cut into smaller pieces for determination of debriding activity by serine protease

Table 3: Component of artificial wound eschar (AWE) with respective fluorescent tagged dye and measurement for emission and excitation wavelength of each dye using fluorescent plate reader. Adapted from [6;14].

Component	**Dye**	**Wavelength excitation/emission (nm)**
Collagen	FITC	485/520
Fibrin	Coumarin	440/480
Elastin	Rhodamine	540/590

In this study, both presented method was modified by using similar method as protease assay due to the limitation of substances in tagging the protein with fluorescent dye. The activity of serine protease concentrated using buffer exchange was measured in three different hours to observe its ability in efficiently digesting each component of AWE substrate (fibrinogen, collagen, fibrin). In order to measure the activity of serine protease following protease assay, AWE was utilized as a substrate

instead of casein. Theoretically, as casein being digested by protease, tyrosine will released along with other amino acid and reaction with Folin resulting in the formation of blue chromophore indicating the activity of protease digesting casein. The result in degrading the component of AWE substrate also showing the formation of blue chromophore however, the low intensity of blue chromophore resulted from the reaction which explained that protease have low activity in digesting AWE substrate.

This also could be explained that the concentration of recombinant serine protease used in degrading activity was low. Previously, the measurement of debridement process was observed in high concentration of papain about 200-1600 U/mg and in this study, 10 µM of protease was used for debriding activity which inadequate to digest AWE substrate. In addition, a similar concentration of protease was used in protease assay method allowing protease easily digest casein as casein was in solution form compared to solid form of AWE substrate. It was observed that high concentration of protease was needed to perform debriding activity against AWE substrate. Also, previously high concentration of vibriolysin (1050, 1400 and 2100 units/gm) had been employed as an effective agent for debridement of cutaneous wounds such as burn and skin ulcer. This support that the idea of using high concentration of enzyme was needed to efficiently used as debridement agent against chronic wounds. The absorbance value of protease in respective hours were measured at absorbance 660nm as shown in Table 4 which different compared to the published method for measurement of emission and excitation wavelength of each dye tagged on AWE component [6]. This also could affect the debriding activity of serine protease on AWE substrate. Thus, the modified method should revert to published method to observe the ability of recombinant protease perform its action in degrading protein of wound eschar.

Based on table 4, it was observed that after 1hr, the purified recombinant protease concentrated using Amicon Ultra-15 Centrifugal Filter resulted in enzyme activity of 0.008704 U/ml while after 6hr, recombinant protease have enzyme activity of 0.006173 U/ml and 0.002122 U/ml after 24hr. Even though the activity of recombinant protease in degrading AWE is low, protease do showed a little activity in degrading AWE as the modified method in degrading AWE is not suitable which influenced by the concentration of protease and tagging of protein with fluorescent dye.

Table 4: Absorbance value of serine protease observed at OD660nm at different hours with protease activity calculated based on tyrosine standard curve as protease assay method.

Time (hours)	Absorbance (OD660nm)	Enzyme activity (Units/ml)
1	0.041	0.008704
6	0.057	0.006173
24	0.083	0.002122

Conclusion

The purified recombinant protease may show some activity in degrading each protein in AWE substrate with the used of high concentration of recombinant protease as AWE substrate is in solid form while tagging each protein in AWE with fluorescent dye to measure the cumulative fluorescent in respective hour to observe the appearance of dye. The recombinant enzymes should have a potential in demonstrating the ability to enhance the efficiency of removal of necrotic tissue as it has better proteolytic activity.

References

[1] Isabela Avila-Rodríguez, M., Meléndez-Martínez, D., Licona-Cassani, C., Manuel Aguilar-Yañez, J., Benavides, J., & Lorena Sánchez, M. (2020). Practical context of enzymatic treatment for wound healing: A secreted protease approach (Review). *Biomedical reports*, *13*(1), 3–14. https://doi.org/10.3892/br.2020.1300

[2] Dabiri, G., Damstetter, E., & Phillips, T. (2016). Choosing a Wound Dressing Based on Common Wound Characteristics. *Advances in wound care*, *5*(1), 32–41. https://doi.org/10.1089/wound.2014.0586

[3] Hirche, C., Citterio, A., Hoeksema, H., Koller, J. L., Martina, M., José R., Monstrey, S., Murray, A., Plock, J. A., Sander, F., Schulz, A., Ziegler, B., Kneser, U., (2017). *Eschar removal by bromelain based enzymatic debridement (Nexobrid ®) in burns: An European consensus. Burns, (), S0305417917304199–.* doi:10.1016/j.burns.2017.07.025

[4] Manna B, Nahirniak P, Morrison CA. (2021). Wound Debridement In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK507882/

[5] Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A and Ashraf M (2019) Microbial Proteases Applications. Front. Bioeng. Biotechnol. 7:110. doi: 10.3389/fbioe.2019.00110

[6] Chen, R., Salisbury, A-M., & Percival, SL., (2019). A comparative study on the cellular viability and debridement efficiency of antimicrobial-based wound dressings. *Int Wound J.* 2020;17:73-82. https://doi.org/10.1111/iwj.13234

[7] Rosano, G. L.& Ceccarelli, E. A. (2014). *Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in Microbiology, 5(), –.* doi:10.3389/fmicb.2014.00172

[8] Sandomenico, A., Sivaccumar, J. P., & Ruvo, M. (2020). Evolution of Escherichia coli Expression System in Producing Antibody Recombinant Fragments. *International Journal of Molecular Sciences*, *21*(17), 6324. https://doi.org/10.3390/ijms21176324

[9] The principle and method of polyacrylamide gel electrophoresis (SDS-PAGE) | MBL Life Science -JAPAN-. (n.d.). MBL,Life Science. https://ruo.mbl.co.jp/bio/e/support/method/sds-page.html

[10] Feizollahzadeh, Sadegh; Kouhpayeh, Shirin; Rahimmansh, Ilnaz; Khanahmad, Hossein; Sabzehei, Faezeh; Ganjalikhani-hakemi, Mazdak; Andalib, Alireza; Hejazi, Zahra; Rezaei, Abbas (2017). The Increase in Protein and Plasmid Yields of E. coli with Optimized Concentration of Ampicillin as Selection Marker. Iranian Journal of Biotechnology, 15(2), 128–134. doi:10.15171/ijb.1467

[11] SDS-PAGE. (2021). BYJUS. Retrieved from June 28, from https://byjus.com/biology/sds-page/#:%7E:text=The%20principle%20of%20SDS%2DPAGE,to%20less%20resistance%20during%20electrophoresis

[12] Shi, L., Ermis, R., Lam, K., Cowart, J., Attar, P., & Aust, D. (2009). *Study on the debridement efficacy of formulated enzymatic wound debriding agents by in vitro assessment using artificial wound eschar and by an in vivo pig model. Wound Repair and Regeneration, 17(6), 853–862.* doi:10.1111/j.1524-475x.2009.00545.x

[13] Sharma, S. (2021). Re: What is the role and necessity of TEMED in SDS-PAGE?. Retrieved from: https://www.researchgate.net/post/What_is_the_role_and_necessity_of_TEMED_in_SDS-PAGE/60db557a35e889022b78bcf1/citation/download.

[14] Shehadul Islam, Mohammed; Aryasomayajula, Aditya; Selvaganapathy, Ponnambalam (2017). *A Review on Macroscale and Microscale Cell Lysis Methods. Micromachines, 8(3), 83–* . doi:10.3390/mi8030083

[15] Martínez, L. S. M. C. S. O. (2021). *Sonication cell lysis protocol*. Sepmag. https://www.sepmag.eu/blog/sonication-cell-lysis-protocol

[16] Cheung, R. C., Wong, J. H., & Ng, T. B. (2012). Immobilized metal ion affinity chromatography: a review on its applications. *Applied microbiology and biotechnology*, *96*(6), 1411–1420. https://doi.org/10.1007/s00253-012-4507-0

[17] Gaurav, C., (2014). Re: What is the role of 300 mM NaCl used to prepare buffers used for protein samples?. Retrieved from: https://www.researchgate.net/post/What-is-the-role-of-300-mM-NaCl-used-to-prepare-buffers-used-for-protein-samples/5368e08dd11b8bbd1f8b45c7/citation/download

[18] Bjerga, G. E. K., Larsen, Ø., Arsın, H., Williamson, A., García-Moyano, A., Leiros, I., & Puntervoll, P. (2018). *Mutational analysis of the pro-peptide of a marine intracellular subtilisin protease supports its role in inhibition. Proteins: Structure, Function, and Bioinformatics.* doi:10.1002/prot.25528

[19] Persinal-Medina, M., Llames, S., Chacón, M., Vázquez, N., Pevida, M., Alcalde, I., Alonso-Alonso, S., et al. (2022). Polymerizable Skin Hydrogel for Full Thickness Wound Healing. *International Journal of Molecular Sciences*, *23*(9), 4837. MDPI AG. Retrieved from http://dx.doi.org/10.3390/ijms23094837