Expression of recombinant SARS-CoV-2 papain-like protease (SARS-CoV-2 PLpro) in Escherichia coli RIPL

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Abstract. The SARS-CoV-2 virus that caused pandemic COVID-19 has spread rapidly to humans and causes a very serious health and social problem around the world. In order to develop vaccine and therapeutic approaches, a comprehensive study of viral structure and viral biological infection needs to be understood. In addition to four structural proteins; spike glycoprotein (S), envelope (E), membrane protein (M), and nucleocapsid protein (N), SARS-CoV-2 viral genome, consist of two open reading frame (ORF) which is located in N-terminus, ORF1a, and ORF1b. The ORF1a consist of Main protease (3CLpro) and together with papain-like protease (PLpro) play a role in the cleavage of polyproteins and translated from viral RNA to mature protein. Here we describe a method of production of 6xHis-tagged Papain-like protease fragment in E. coli RIPL, including gene expression, solubilization of inclusion bodies by different methods, and detection of gene product target by Western Blot Analyses.

Keywords: Papain-like protease, E.coli RIPL, gene expression, inclusion body.

1. Introduction

COVID-19 (coronavirus disease) caused by the new coronavirus SARS-CoV-2 has infected at least 235 million people, and more than 4.8 million people have died. The rapidly spreading SARS-CoV-2 virus has created a pandemic and become a very serious health and social problem all over the world (https://www.who.int/emergencies/diseases/novel-coronavirus-2019. Data was taken October 7th, 2021). Indonesia is also one of the countries affected by this pandemic crisis, there were more than 4.2 million infected which caused 142,000 people to die (https://covid19.go.id/. Data was taken October 7th, 2021).

To be able to break the chain of COVID19, various efforts have been made, especially the development of vaccines and therapeutic therapies. To develop drugs, vaccines, therapeutic approach or early detection devices, a comprehensive knowledge of viral structure and an understanding of the biological processes of viral infection are required.

The whole sequences of a single-stranded RNA SARS-CoV-2 has been identified and notified. High throughput sequencing generated a completed genome sequences which reported nearly 30,000 bp in length [1] and encoded 9860 amino acids [2]. RNA SARS-CoV-2 virus genome contain of structural and non-structural proteins. Nucleocapsid (N), membrane (M) envelope (E) and Spike (S) were encoded the structural proteins, whereas non-structural encoded by two ORF regions located at...
the N-terminus, such as 3-chymotrypsin like protease (3CLpro), papain-like proteases (PLpro), and RNA-dependent RNA polymerase (RdRp) [3-5].

The non-structural papain-like protease (PLpro) of SARS-CoV-2 potentially essential in virus replication [6] and play a role in processing polyprotein to mature protein [7]. The viral life cycle begins with proteolysis processing that initiated by 3CLpro and PLpro cut the polyprotein into the slice fragments for folding and packaging new viruses, thereby increasing the spread of infection. Together with RdRp, these major enzymes 3CLpro and PLpro are essential for the proteolysis and replication of new virus [8-9]. Since its essential role, PLpro presents interesting drug target.

In our previous research, we have summarized the bioinformatic analysis whole sequences of SARS-CoV-2 from Indonesia. Data were taken from May to September 2020. It was reported that the D614G mutation was the dominant mutation in 59% [10]. But nowadays from the current data, the D614G is no longer a mutation, but a reference. The development of this virus is very dynamic, research on drugs to overcome this virus outbreak continues. Based on pivotal role of PLpro as a drug target, in silico experimental has been done parallelly.

This preliminary research was conducted to supply recombinant enzyme papain-like protease (PLpro). Furthermore, to design an in vitro reaction that can be used to determine the interaction between several potential herbal compounds from Indonesia that can inhibit PLpro activity. However, in this study, the recombinant PLpro SARS-CoV-2 was not expressed as soluble protein, so recovery methods need to be optimized by extracting insoluble protein or inclusion body (IB). In this study we highlighted to optimize the expression of recombinant protein PLpro SARS-CoV-2 in E. coli RIPL and its recovery methods.

2. Materials and Methods

2.1 Bacterial strain and Plasmids

Bacterial strains were used in this study were E. coli DH5α for recombinant plasmid propagation and E. coli RIPL (provided from ITB, Bandung) for host expression. Recombinant DNA Synthesis pET21d (+) - PLpro SARS-CoV-2 (Ncol/Xhol) purchased from GenScript USA Inc. All strains were routinely grown on Luria Bertani (LB) medium (w/v) (1% Tryptone: 0.5% Yeast extract; 1% NaCl; 1.5% agar) containing ampicillin (100 µg mL⁻¹). Agar plates were incubated at 37 °C for 16 - 18 h and then stored at 4 °C. For the long-term storage, 5.0 mL of overnight culture of each recombinant strain was harvested by centrifugation and replaced with 1 mL of fresh LB then mixed with 0.25 mL of 75% glycerol stock and storage at -80 °C.

2.2 Preparation of competent cell and transformation

Luria Bertani broth was used for cultivation medium. TB (Transformation Buffer) containing 10 mM PIPES, 15 mM CaCl₂·2H₂O and 250 mM KCl were mixed, pH adjusted to 6.7. Then 55 mM MnCl₂·2H₂O was dissolved to a mixture then sterilized by 0.22 µ syringe filtration and stored at 4 °C (Inoue et. al. 1990) [11]. A single colony of each E. coli DH5α and E. coli RIPL was grown in LB broth at 37 °C, 250 rpm, for overnight. As much as 1% of overnight culture was inoculated to LB 200 mL then incubated at 37 °C to OD₆₀₀ 0.4-0.6. After reaching the desired OD, the culture is cooled on ice for 30 min. The culture then harvesting by centrifugation using HITACHI high-speed refrigerated centrifuge at 3000 rpm, 4 °C for 15 min. The pellet washed in 16.75 mL cold TB and incubated on ice for 10 min. The next step pellet suspension was centrifuged at 3000 rpm, 4 °C for 10 min. pellet harvested dissolved in 2 mL cold TB and 0.3 mL dimethylsulfoxide (DMSO). Suspension incubated on ice for 10 min then aliquoted into microtube of each 50 µL, storage at -80 °C.

For plasmid propagation and expression, 1 – 10 ng/µL of recombinant plasmid was added to thawed competent cell suspension by heat-shock method. The mixture was incubated for 30 min on ice, then heated at 42 °C for 1 min and 2 min incubation on ice for, then quickly added 0.5 mL LB broth. The mixture was treated in shaker incubator at 37 °C, 250 rpm for 1 h. The cells were collected by centrifugation at 6000 rpm for 5 min at 4 °C. As much as 100 - 200 µL mixture was resuspended by
pipetting and plating in LB agar containing 100 μg/mL ampicillin. Plate incubated at 37 °C for 16-18 hour.

A single colony of E. coli DH5α harbouring recombinant plasmid pET21d (+) - PLpro SARS-CoV-2 was cultivated in LB broth at 37 °C, for 16 -18 h. Culture then harvesting and recombinant plasmid was extracted under Zyppy Plasmid Kit protocol (Zymo Research). The extracted plasmid confirmed by restriction enzyme NcoI/XhoI.

2.3 Protein expression
The expression of gene target was conducted following pET System Manual. The E. coli Ripl harbouring recombinant plasmid pET21d (+) - PLpro SARS-CoV-2 was cultured in LB broth containing 100 ampicillin μg/ml. Shake flask fermentation was carried out in 500 ml Erlenmeyer flask containing 100 ml culture. Medium inoculated with 2.5% (v/v) of overnight seed culture. The culture was shaken at 37 °C and 250 rpm to OD600 reached 0.4 - 0.6. The culture was induced by addition of 0.3 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG). Protein expression was conducted in low temperature 20 °C, 200 rpm for 24 h. After that, cell suspension was harvested by centrifugation at 8,000 rpm, 4 °C for 15 min. Supernatant was stored at 4 °C while pellet was kept at -20 °C for further analysis.

2.4 Extraction of insoluble (Inclusion Body)
To extract Inclusion Body (IB) from cell pellet, four different protocols were performed as follow.
(1) Sample was extracted following Probond Native method. Cell pellet from 50 ml culture was dissolved in 8 ml of native binding buffer (0.05 M NaH2PO4, 0.5 M, NaCl, 0.01 M imidazole, pH 8.0) containing 8 mg of lysozyme. The mixture was sonicated for 5 minutes (2 seconds on, 3 seconds off, 27 - 30% amplitude) on ice. Then centrifuged at 3000 rpm, 4 °C for 15 min. The lysate was kept for SDS-PAGE analysis.
(2) The second method was conducted as ITB protocol (unpublished). Cell pellet was dissolved in lysis buffer (50 mM Tris pH 7.4, 100mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) in 1:4 ratio. Sample was lysed by sonication (Sonics Vibra-Cell VCX-500 Ultrasonic processor) (2 second on, 3 second off for 5 min) with amplitude 27-30% on ice. Cell lysate then centrifuged 12,000 rpm, 4 °C for 10 min. The next step, pre-debris cell dissolved in lysis buffer in 1:7 ratio, then centrifuged in 13,000 rpm, 4 °C for 20 min. Cell debris was obtained and continued to unfolding step. In this step, cell debris dilute in unfolding buffer (50 mM Tris pH 9, 100 mM NaCl, 7.3 M Urea pH 9) in 1: 7 ratios. Suspension was sonicated for 5 min, 2 second on, 3 second off, then unfolded protein was recovered by centrifugation 13,000 rpm, 4 °C for 20 min. Finally, the concentrated unfolded solution was diluted 20x unfolding buffer.
(3) This third method was performed following Palmer and Wingfield (2012) with modification [12]. Inclusion Body in this experiment was extracted by dissolving cell pellet in 4 mL lysis buffer (100 mM Tris-Cl pH 7, 5 mM Ethylenediaminetetraacetic acid (EDTA), 5 mM Dithiothreitol (DTT)) per gram wet weight of cells. The mixture be treated by adding 200 μg/mL lysozyme. Suspension was sonicated for 5 min, 2 second on, 3 second off, with 27-30% amplitude then continued to centrifugation at 12,000 rpm, 4 °C for 30 min. Pre-debris then suspended in wash buffer (100 mM Tris-Cl pH 7, 5 mM EDTA), 5 mM Dithiothreitol (DTT), 2 M urea and 2% Triton X-100) with 4 to 6 ml wash buffer per gram wet weight cells. Mixture was stirred for 15 min, at room temperature. To separate pellet and supernatant, centrifugation was carried out at 12,000 rpm, 4 °C for 30 min. The next step, pellet was re-dissolved in wash buffer (minus urea and Triton X-100) using 4 to 6 mL buffer per gram wet cells then centrifuged at 12,000 rpm, 4 °C for 30 min.
(4) Similar to the third method, the last method was adapted following Palmer and Wingfield (2012) with some modifications to the washing step. To extract insoluble protein after centrifugation, cell pellet was dissolved in 4 mL lysis buffer containing 200 μg/ml lysozyme (100 mM Tris-Cl pH 7, 5 mM Ethylenediaminetetraacetic acid (EDTA), 5 mM Dithiothreitol (DTT)) per gram wet weight of cells. Suspension was sonicated for 5 min, 2 second on, 3 second off, with 27-30% amplitude then
continued to centrifugation at 12,000 rpm, 4 °C for 30 min. Pre-debris then suspended in wash buffer (100 mM Tris-Cl pH 7, 5 mM (EDTA), 5 mM Dithiothreitol (DTT), 2 M urea and 2% Triton X-100) with 4 to 6 ml wash buffer per gram wet weight cells. Suspension then centrifuged 12,000 rpm, 4 °C for 30 min. After that, cell pellet re-dissolved in wash buffer containing urea and Triton X-100 with 4 to 6 ml wash buffer per gram wet weight cells, then continued to incubate the mixture for 15 min with gentle shaking, at room temperature. Centrifuged at 12,000 rpm, 4 °C for 30 min. Repeat washing step with wash buffer containing urea and Triton X-100 twice to centrifugation step. Finally, pellet was dissolved in wash buffer (minus urea and Triton X-100) using 4 to 6 mL buffer per gram wet cells then centrifuged at 12,000 rpm, 4 °C for 30 min.

Each step, sample was taken for SDS PAGE analysis

2.5 SDS PAGE

Recombinant proteins were analysed by SDS-PAGE according to Bollag et al., 1996 [13]. The slab gel system consisted of 12.5% by 1.5 mL 1.5 M Tris buffer pH 8.8, 60 μL 10% SDS, 3 mL 30% polyacrylamide, 30 μL 10% ammonium persulfate (APS), 7 μL TEMED and 5% stacking gel (1.5 mL ddH2O, 625 μL 0.5 M Tris-HCl buffer pH 6.8, 25 μL 10% SDS, 334 μL 30% polyacrylamide, 15 μL 10% ammonium persulfate (APS), 7 μL TEMED).

The cells pellet was resuspended in of 5X protein sample buffer (0.25 M Tris-HCl pH 6.8 0.5 M DTT 10 % SDS 50 % Glycerol 0.5 % bromphenol blue) then boiled at 100 °C for 5 min then loaded into the gel. Spectra™ Multicolor Broad Range Protein Ladder was used as the protein marker. Electrophoresis was run in 1x running buffer (1.92 M glycine, 1% (w/v) SDS, and 0.25 M Tris-HCl) and performed at room temperature for 90 min at constant voltage 120 v. After electrophoresis, the gel was stained following Xpress blue solution (Xpress Blue™ Protein Stain – Himedia) protocol.

2.6 Western blot analysis

After separating by SDS PAGE, proteins were transferred to a PVDF sheet membrane, using a semi-dry a tank system, blotting apparatus (Power Blotter System-Thermo Scientific). A sandwich of filter paper, SDS PAGE gel and membrane PVDF were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol). The PVDF membrane is immersed in methanol for 15 minutes prior to use. Furthermore, the transfer process to the PVDF membrane was carried out on 2.5 mA, 21 V for 30 minutes. Afterward, PVDF membrane with transferred protein was incubated in blocking buffer (5% skim milk blotting grade-blocker dissolved in phosphate buffer saline (PBS) containing 0.05% Tween 20) for 1 hour at 37 °C with gentle shaking. After blocking, membrane was washed in PBS-T (PBS-Tween 20) with shaking and replaced new PBS-T buffer every 10 min for 1 h. The following step blocking, membrane was probed with a primary antibody (6x His Tag Monoclonal Antibody HIS H8, Invitrogen) that dissolved in 5% skim milk in PBS-T with 1:3.000 ratio. The membrane was incubated at 4 °C for 16 - 18 h, thereafter continued at 37 °C for 1 h with gentle shaking. After that membrane washed with fresh PBS-T buffer every 10 min for 1 h.

The next probing step was secondary antibody (goat anti-mouse IgH (H+L) horseradish peroxide conjugate, Invitrogen) that dissolved in 5% skim milk in PBS-T with 1:100.000 ratio. The membrane was incubated 37 °C for 1 h with gentle shaking and washed with fresh PBS-T buffer every 10 min for 1 h. The next step was to be able to visualize the probing results by treatment a PVDF membrane to the chemiluminescent substrate (Super Signal™ West Femto, Thermo Scientific). Incubation process was carried out in a light-proof room for 5 min. To visualize the protein target, both PVDF membrane treated with Femto substrate was incubated with film (high performance chemiluminescence film, Amersham Hyperfilm™ ECL) for 1 - 15 min. Visualization was conducted by staining film with developer and fixer solution (Carestream Kodak autoradiography GBX fixer and developer), staining of developer - water - fixer - water - each for 30 seconds - 1 minute.
3. Result and Discussion

3.1 Re-transformation plasmid into E. coli DH5 and RIPL and plasmid verification

The recombinant DNA synthesis was constructed using the DNA sequence of PLpro SARS-CoV-2 and expression plasmid pET21d(+) with 6xhis tag sequence at C-terminal (Figure 1). To propagate plasmid, DNA synthesis pET21d (+) - PLpro SARS-CoV-2 (NcoI/XhoI) was re-transformed into E. coli DH5. Ampicillin was used as selectable marker for pET21d (+). After extraction, to verify that DNA synthesis was correctly constructed, digestion by enzyme was conducted. The digestion of pET21d (+) - PLpro SARS-CoV-2 with NcoI/XhoI resulted two fragments of 945 bp and 5.4 kb (Figure 2). For further analysis, plasmid was sent to sequencing (data not shown). This result showed that the recombinant DNA synthesis PLpro SARS-CoV-2 was successfully constructed.

Figure 1. The map of recombinant pET21d (+) - PLpro SARS-CoV-2 (NcoI/XhoI).

Figure 2. Restriction pattern of pET21d (+) - PLpro SARS-CoV-2 digested by NcoI and XhoI (A) Control undigested plasmid (B) Plasmid digested by NcoI and XhoI.
3.2 SDS PAGE Analysis
SDS-PAGE is an electrophoretic method that separates proteins based on their molecular weight. The expression of PLpro under T7promoter was visualized after induced by 0.3 mM IPTG. Protein extract was determined from four different methods. Theoretically molecular weight of PLpro at approximately 35.6 kDa (https://web.expasy.org/compute_pi/). Figure 3 showed the recombinant protein extracted as soluble protein, performed from Probond Native method and insoluble protein performed by ITB method. Line 1 showed the supernatant produced by harvesting with centrifugation. In the first line, there was no band detected. It showed as control that there is no secretory system in E. coli. Line 2 was clear lysate, produced as soluble (Probond). In this method, imidazole was used as a lysis buffer. The protein band that was produced as insoluble performed in line 3-6 (ITB). Protein was extracted by using urea as denature agent. The line 5, concentrated unfolded sample showed the band intensity as a target protein. Figure 4 showed the comparison of third and fourth method in washing step. Using the same buffer, the fourth method was carried out in three washing steps in a buffer containing urea and TritonX-100. Protein band of PLpro could be observed with high intensity at line 2, 5 and 7.

Figure 3. SDS PAGE of Inclusion Body Extraction pET21d(+) PLpro in E. coli DE3. (M) Spectra™ Multicolor Broad Range Protein Ladder 170 – 10 kDa (1) supernatant (2) sonicated clear lysate (Probond) (3) sonicated clear lysate (ITB) (4) pre-debris lysate (ITB) (5) concentrated unfolded solution (ITB) (6) unfolded solution 20x diluted.

Figure 4. SDS PAGE analysis of Inclusion Body Extraction pET21d(+) PLpro in E. coli DE3 (Palmer and Wingfield). (M) Spectra™ Multicolor Broad Range Protein Ladder 170 – 10 kDa (1) sonicated clear lysate (third method) (2) clear lysate pre-debris (with urea and TritonX-100, third method) (3) clear lysate debrish (without urea and TritonX-100, third method) (4) sonicated clear lysate (fourth method) (5) clear lysate pre-debris W1 (with urea and TritonX-100, fourth method) (6) clear lysate pre-debris W2 (with urea and TritonX-100, fourth method) (7) clear lysate pre-debris W3 (with urea and TritonX-100, fourth method) (8) unfolded solution (without urea and TritonX-100, fourth method).
In *E. coli*, the formation of protein aggregate as inclusion body can be contributed by several factors. One of them is high level of protein expression [12,14]. Inclusion body could be recovered from cell lysate by selective extraction with detergent [12] or using high concentration of chaotropes like urea [14], but extraction of inclusion body will be more effective with high concentration of guanidine chloride [15].

### 3.3 Western blot analysis

Western Blot, a bioanalytic immunoassay methods, is an important technique to detect specific protein molecule from mixture protein [16]. In this technique, combinations of different types of proteins are separated by molecular weight through the gel electrophoresis. The results of this separation are then transferred to a PVDF sheet or nitrocellulose membrane, followed by detection of immunoproteins using fluorescent or chemiluminescent detectable antibodies. In general, the western blot process can be divided into 5 parts; (1) running SDS PAGE, (2) transfer to the PVDF membrane, (3) blocking, (4) probing using 2 types of antibodies; primary and secondary (5) visualization of protein bands [17].

After transferring, the PVDF membrane was then blocked using a 5% Skim milk Blotting Grade-Blocker solution. The blocking process aims to prevent the antibody from binding to the non-specific membrane, consequently, lead to high background. In the probing process, two specific antibodies are used, primary antibodies (6x-His Tag Monoclonal Antibody) and secondary antibodies (AntiMouse IgG). Primary antibodies are used to identify specific proteins or epitopes in group of protein. Secondary antibodies could recognize and bind to specific parts of the primary antibody. The concentration of the primary antibody is very important. If it is too low, the target protein could not be detected. If it was too high, a non-specific protein reaction could occur and detect the non-target protein [17].

In SDS PAGE analysis, we detect protein target which approximately 35.6 kDa. Furthermore, western blot was conducted to confirm that the protein detected in SDS PAGE was the target protein of interest. Protein target detected in SDS PAGE was then selected and western blot analysis was performed (Figure 5). The western blot analysis resulted the protein target was detected in all lines (2-7). Line 1, sonicated clear lysates as a soluble method, detected a certain band but with low intensity. The concentrated unfolded solution (ITB) showed highest intensity of all. This result showed that protein PLpro was successfully expressed under T7 promoter.

![Figure 5](image.png)

**Figure 5.** Western blot analysis of insoluble recombinant protein pET21d(+) PLpro in *E. coli* DE3. (M) Spectra™ Multicolor Broad Range Protein Ladder 170 – 10 kDa (1) positive control (2) sonicated clear lysate (Probond) (3) concentrated unfolded solution (ITB) (4) clear lysate pre-debrish (with urea and TritonX-100, third method) (5) clear lysate debrish (without urea and TritonX-100, third method (6) clear lysate pre-debrish W1 (with urea and TritonX-100, fourth method) (7) unfolded solution (without urea and TritonX-100, fourth method).
4. Conclusion

Insoluble protein PLpro SARS-CoV-2 was expressed under T7 promoter of pET21d(+) system, after 0.3 mM IPTG induction, the protein was produced in low temperature 20 °C for 24 h. The recovery of inclusion body using specific detergent and urea as denaturation agent, could produce protein band target that was detected in SDS PAGE. From western blot analysis, specific protein target could be well observed. Furthermore, PLpro will be further purified to be used as a model of recombinant enzyme.

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References

[1] Kumar S, Nyodu, R, Maurya V K and Saxena S K 2020 Coronavirus Disease 2019 (COVID-19) 23–31.
[2] Chen L, Liu W, Zhang Q, Xu K, Ye G, Wu W, et al 2020 RNA Microbes. Infect. 9 313–319.
[3] Huang Y, Yang C, Xu X 2020 Acta Pharmacol. Sin 41 1141–1149.
[4] Naqvi AAT, Fatima K, Mohammad T, Fatima U, Singh IK, Singh A, Atif SM, Hariprasad G, Hasan GM, Hassan MI 2020 Acta Mol. Basis Dis. 1 1866
[5] Chan JF, Kok KH, Zhu Z, Chu H, To KK, Yuan S 2020 Microbes. Infect. 9 221-236.
[6] Gao X, Qin B, Chen Pu, Zhu K, Hou P, Wojdyla JA, Wang M, Cui S 2021 Acta Pharm Sin. B 11 237-245
[7] Wu C, Liu Y, Yang Y, Zhang P, Zhong W, Wang Y 2020 Acta. Pharm. Sin. B 10 766-788.
[8] Shang J, Ye G, Shi K, Wan Y, Luo C, Aihara H, Geng Q, Auerbach A, Li F 2020 Nature 581 221–224.
[9] Tong TR 2009 Infect. Disord. Drug Targets 9(2) 223-45.
[10] Ulfah M, Helianti I 2021 Iran J. Microbiol 13(2) 145-155
[11] Inoue H, Nijima H and Okayama H 1990 Gene 96 23–28.
[12] Palmer I. and Wingfield PT 2012 Curr. Protoc. Protein Sci. Chapter 6 Unit 6.3.
[13] Bollag DM, Rozyczki MD and Edelstein SJ 1997 Protein methods (2nd ed.). (New York, USA: John Wiley-Liss, Inc).
[14] Singh A, Upadhayv Y, Upadhayv AK 2015 Microb Cell Fact 14 41.
[15] Fitzgerald GA, Komarov A, Kaznadzey A, Mazo I, Kireeva ML 2021 Protein Expr. Purif. 183 105861.
[16] Hnasko TS, Hnasko RM 2015 Methods Mol Biol 1318 87-96.
[17] Mahmood T, Yang PC 2012 Am J Med Sci 4(9) 429-434.