

Optimizations of expression and purification of recombinant HIV-1 CRF01_AE p24 protein in *Escherichia coli* for development of immunodiagnostic assay

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ABSTRAK

Latar belakang: Metode konvensional untuk mengonfirmasi infeksi HIV adalah Western blot. Namun, western blot memiliki keterbatasan yaitu kontaminasi dengan antigen selular manusia dan masalah perbedaan genetik di antara sub tipe HIV-1 yang menyebabkan hasil indeterminate dan ketidakakuratan diagnosis infeksi HIV-1 dengan strain spesifik. Pemeriksaan Western blot yang tersedia di Indonesia tidak spesifik terhadap infeksi sub tipe CRF01_AE yang predominan di Indonesia. Pada penelitian ini dilakukan ekspresi, purifikasi dan Western blot terhadap protein p24 HIV-1 CRF01_AE rekombinan yang digunakan sebagai antigen.

Metode: Optimasi ekspresi protein rekombinan pada *Escherichia coli* galur BL21CP dilakukan terhadap waktu induksi, konsentrasi isopropyl-1-thio-d-galactopyranoside (IPTG) yang digunakan dalam induksi, dan suhu induksi. Purifikasi protein p24 rekombinan dilakukan dengan metode immobilized metal-affinity chromatography (IMAC) dengan sistem purifikasi Ni-NTA (Qiagen) pada kondisi native dengan optimasi pada konsentrasi imidazole dalam wash buffer.

Hasil: Konfirmasi protein rekombinan dengan Western blot menunjukkan bahwa ekspresi dan purifikasi protein p24 rekombinan telah dioptimasi dengan baik dan reaktif terhadap serum pasien HIV-1 positif di Indonesia.

Kesimpulan: Protein p24 rekombinan dari penelitian ini dapat dikembangkan untuk uji diagnostik Western blot berdasarkan sub tipe CRF01_AE yang dominan di Indonesia.

ABSTRACT

Background: Conventional method for confirmation of HIV infection is Western blot. However, it has limitations because of contamination by human cellular antigen and genetic diversity among the HIV-1 subtypes that show indeterminate result and inaccuracy for the diagnosis of different strains. Most of Western blot developed are based on HIV-1 B subtype. In Indonesia HIV-1 CRF01_AE subtype is dominantly circulated. Therefore, we optimized the expression, purification of the recombinant HIV-1 CRF01_AE p24 protein for development of immunodiagnostic assay.

Methods: Optimization of protein expression in *Escherichia coli* strain BL21CP was performed including induction time, isopropyl-1-thio-d-galactopyranoside (IPTG) and imidazole concentrations, and induction temperature. Purification of the recombinant p24 protein was used by using Ni-NTA (Qiagen) purification system in native condition.

Results: Expression and purification of HIV-1 CRF01_AE p24 protein have been performed. Confirmation of the recombinant protein by Western blot showed the expression and purification of recombinant p24 protein has been optimized well and reactive with sera of patients with HIV-1 CRF01_AE subtype positive.

Conclusion: The recombinant HIV-1 CRF01_AE p24 protein has been expressed and purified successfully, and it is potential to be used as antigen for immunodiagnostic assay.

Keywords: expression, HIV-1 CRF01_AE, p24 protein, purification, recombinant protein

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Human immunodeficiency virus type I (HIV-1) is a member of the lentivirus genus of the *Retroviridae* family.¹ This virus consists of three clades i.e. M (main), O (outlier) and N (non-M, non-N). The M group is divided into 9 subtypes (A, B, C, D, F, G, H, J, and K) and 15 circulating recombinant forms (CRFs).^{2,3} In Asia, there are three dominant HIV-1 types (C, CRF01_AE and B) distributed in different regions.⁴ In Indonesia, HIV-1 CRF01_AE is a dominant subtype, followed by subtype B.⁵⁻⁷

To date, HIV/acquired immunodeficiency syndrome (AIDS) disease is a global pandemic problem. Globally, there were 34 million people with HIV in 2010, with approximately 1,8 million deaths.⁸ In Indonesia, there were 19,973 cases of AIDS diseases with 3,846 deaths in 2009. In 2013, AIDS cases increased up a total 43,667 cases with 8,340 deaths. The most HIV/AIDS cases were recorded in Papua, followed by East Java, and Jakarta.⁹

Accurate and reliable diagnostic assays are important to control the spread of this disease.¹⁰ Screening assays for diagnostic purpose must be capable to detect the anti-HIV-1 antibody in low titer and avidity particularly in the initial phase of HIV infection. The positive results of screening assays have to be confirmed by at least one confirmatory test.¹¹ Many commercial HIV-1 p24 protein-based confirmatory assays for HIV infection have been developed.¹⁰⁻¹² Most of the assays are based on p24 protein of HIV-1 B subtype. The assays can yield the indeterminate results due to the genetic differences among HIV-1 subtypes and/or CRFs.¹³ Since the dominant HIV-1 subtype in Indonesia is CRF01_AE, the application of the commercial assays is potential to lead the diagnostic inaccuracy in HIV-1 infection.^{14,15} Therefore, in this study we optimized the expression and purification of the recombinant HIV-1 CRF01_AE p24 protein in *Escherichia coli* (*E. coli*) expression system. The optimizations can be used as preliminary data in producing recombinant HIV-1 CRF01_AE p24 protein for development of immunodiagnostic assays.

METHODS

Expression and purification of recombinant HIV-1 CRF01_AE p24 protein

The recombinant plasmid was isolated from *E. coli* Top10 culture based on the protocol of QIAprep Spin Miniprep Kit [Qiagen]. Gene encoding p24

protein was confirmed by DNA sequencing. The recombinant plasmid then transformed into *E. coli* strain BL21CP-RILP on heat shock method. The recombinant protein (6x His tag-N protein fusion) was expressed by inducing the bacterial cells with 0.2 mM, 0.5 mM, and 1 mM *isopropyl-1-thio-d-galactopyranoside* (IPTG) at 27°C and 37°C for 1-4 hours.

Bacterial pellets were solubilized in lysis buffer (300 mM NaCl, 0.1M EDTA, 50 mM NaH₂PO₄, 10 mM imidazole, pH 8.0 [Qiagen]) and frozen at -80°C then thawed before sonicated to lyse the bacterial cells. The cell membran protein solubility and lipid eradication were performed by adding Triton-X 100 to the concentration of 1.5 % (v/v). Purification of the recombinant protein was performed by following the manufacturer's instruction [Qiagen] with some optimization. Washing steps were performed 3 times. The optimization of recombinant p24 protein purification performed by different imidazole concentrations in wash buffer, namely 20 mM, 30 mM, and 40 mM.

Western blot

The purified recombinant p24 protein was analyzed on 12% sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS PAGE), The protein from the SDS PAGE gel was transferred to nitrocellulose membrane by the Trans-Blot Semi Dry Electrophoresis Transfer Cell (Bio-Rad) in accordance with manufacturer's instruction.

The recombinant protein reactivity was analyzed by observing the bands on the strips indicating the reactivity of HIV-1 CRF01_AE recombinant protein to the sera of patients with HIV/AIDS CRF01_AE positive. The positive reactivity was compared with the positive and negative sera of the western blot commercial kit [MPDiagnostics].

RESULTS

Expression of recombinant p24 HIV-1 CRF01_AE protein in *E. coli* BL21CP-RILP

The expression of recombinant protein was compared with to the wild type and non-induction controls. The migration range identified by the protein marker. According to analysis of the molecular weight, recombinant p24 protein is about 27 kDa and migrate on the range of 25-30 kDa protein marker.

Optimization of induction time

The expression of HIV-1 CRF01_AE p24 protein was induced for four hours. The induction was performed by adding 1 mM of IPTG concentration and incubated at 37°C. The expression result showed in figure 1. It showed that p24 protein migrated at range of 25-30 kDa protein marker. The band for the induced protein was thicker than the non-induced (lane 1) and the wild type proteins (lane 2-3). The thickest band can be observed on lane 8 which represented the p24 protein expression after four hours induction.

There can be observed that on migration range of 25-30 kDa were relatively thick band on lane 5-8

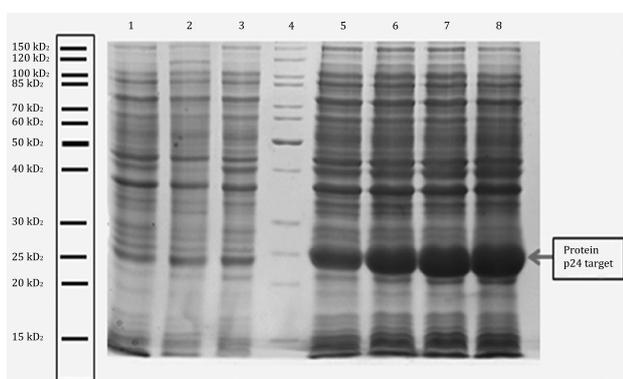


Figure 1. Electrophoresis gel of recombinant p24 protein expression by optimization on induction time. Lane 1: *E. coli* BL21CP pQE80L-p24 non-induced; lane 2: *E. coli* BL21CP wild type; lane 3: *E. coli* BL21CP pQE81L wild type; lane 4: protein marker; lane 5-8: *E. coli* BL21CP pQE80L-p24 induced at 37°C with IPTG 1 mM for 1-4 h

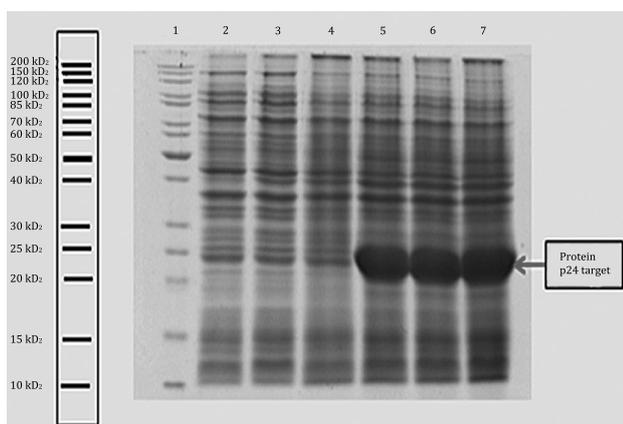


Figure 2. Electrophoresis gel of recombinant p24 protein expression by optimization in IPTG concentration. Lane 1: protein marker; lane 2: *E. coli* BL21CP wild type; lane 3: *E. coli* BL21CP pQE81L wild type; lane 4: *E. coli* BL21CP pQE80L-p24 non-induced; lane 5-7: *E. coli* BL21CP pQE80L-p24 at 37°C for four hours induced on IPTG concentration 0.2 mM, 0.5 mM, 1 mM

which represented the induction of recombinant p24 protein for one to four hours. The band for the induced protein was thicker than the non-induced (lane 1) and the wild type control protein (lane 2-3). The thickest band can be observed on lane 8 which represented the p24 protein expression after four hours induction.

Optimization of IPTG concentration

Figure 2 showed the recombinant p24 protein expression on the optimization of IPTG concentration. It showed that the thickness of bands on migration range 25-30 kDa were relatively equal on three of the IPTG concentration (lane 5-7).

Optimization of induction temperature

Figure 3 showed the recombinant p24 protein on the optimization of induction temperature. It showed that the band on migration range 25-30 kDa on lane 5 which represented the.

Purification of recombinant p24 protein in native condition

Figure 4 showed the bands on migration range 25-30 kDa for the elution 1-3 of purification for imidazole concentration 20 mM, 30 mM, and 40 mM. There can be observed that for the elution one and two of the three imidazole concentration were formed thick bands though with contaminant protein bands. However, on the elution three bands the contaminant protein bands begin to disappear. The most clean band can be observed

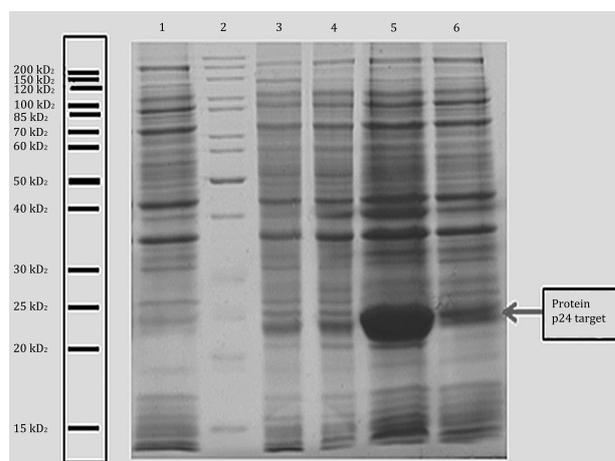


Figure 3. Electrophoresis gel of recombinant p24 protein by optimization of induction temperature. Lane 1: *E. coli* BL21CP pQE80L-p24 non-induced; lane 2: protein marker; lane 3: *E. coli* BL21CP wild type; lane 4: *E. coli* BL21CP pQE81L wild type; lane 5-6: *E. coli* BL21CP pQE80L-p24 induced with IPTG 1 mM for four hours at 37°C and 27°C

on lane 9 which represented the elution three with imidazole concentration 30 mM in wash buffer.

Recombinant protein confirmation by Western blot

Western blot was performed by using the recombinant p24 protein as the antigen reacted with the antibody from the serum of people with HIV/AIDS and serum of positive and negative controls.

Figure 5 showed that there were band on the representative of the reactivity with the HIV-1

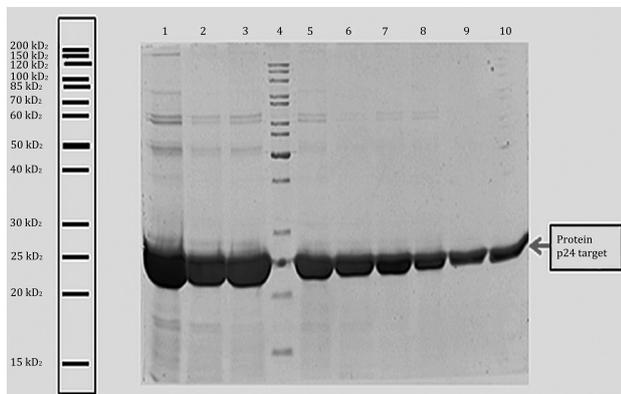


Figure 4. Electrophoresis gel of recombinant p24 protein purification in native condition by optimization of imidazole concentration in wash buffer. Lane 1-3: elution one with imidazole concentration 20 mM, 30 mM, and 40 mM; lane 4-6: elution two with imidazole concentration 20 mM, 30 mM, and 40 mM; lane 7: protein marker; lane 8-10: elution three with imidazole concentration 20 mM, 30 mM, and 40 mM

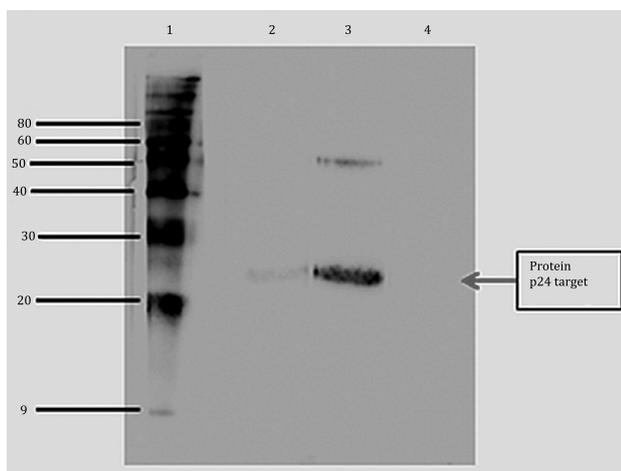


Figure 5. Western blot of p24 rekombinant protein. Lane 1: Biotin marker, lane 2: Reactivity of recombinant p24 protein with patient with HIV positive serum; lane 3-4: reactivity of recombinant p24 protein with positive and negative control serum of commercial kit

positive serum patient on migration range 20-30 kDa (lane 2). On the reactivity with the positive control serum there were two bands on migration range 20-30 kDa and 50-60 kDa.

DISCUSSION

The SDS-PAGE analyze showed that recombinant p24 protein was overexpressed. The thick band formed for recombinant p24 protein affected by the expression system used. The codon translational for recombinant p24 protein showed some codons that rarely recognize by the *E. coli*, the codons are for *proL* and *argU*. The expression system used in this research was *E. coli* BL21CP-RILP containing addition tRNA copies for *argU*, *ileY*, *leuW* and *proL* that recognize the codons for arginin AGA/AGG, isoleusin AUA, leusin CUA, and prolin CCC.¹⁶ This addition tRNA was expected to overcome the codon limitation problem for protein translational. Zang, et al¹⁷ and Singh, et al¹⁸ had overexpressed recombinant p24 protein in *E. coli* BL21(DE3). This result indicated that recombinant p24 protein was relatively high-level expressed eventhough in conventional BL21 strain without tRNA addition.

The recombinant p24 protein purification performed in native condition and from the research performed by Zhang, et al,¹⁶ based on the densitometry analyze on SDS-PAGE for the pQE30-p24 recombinant expression in BL21 signify that his-tagged recombinant p24 protein was in solubel form. For purification of recombinant p24 protein the imidazole concentration used in wash buffer was optimized. Figure 4 showed the optimal imidazole concentration in wash buffer was 30 mM in the third elution (lane 9). This imply the 30 mM imidazole was capable to reduce nonspecific binding between Ni-NTA matrices and contaminant protein containing histidine residue. This nonspecific binding frequently occur in native condition purification than in denature condition,^{19,20} since the protein has denaturated in denature kondition purification (result not show). All the purification procedures in native condition performed at 4°C to avoid protein denaturation for optimal purification. The salt concentration for binding and washing was 300 mM NaCl to ensure the ion strength of the buffer.¹⁶

Figure 5 represented the Western blot for recombinant p24 protein and the recombinant p24 protein was reactive toward the HIV-1 positive patient serum and positive control commercial kit serum. This reactivity indicated that the patient serum infected by HIV-1 CRF01_AE has contained the specific antibody for p24 HIV-1 CRF01_AE recombinant protein produced in this research. However, another protein band at migration range of about 55 kDa was found in lane 3 for the reactivity with the positive control serum. This addition band probably was the reactivity of antibody in positive control serum toward contaminant protein with molecular weight about 55 kDa. It can not be hindered that patient serum has the antibody for *E. coli*. However, in reactivity band toward the patient serum did not indicate contaminant protein of *E. coli* used as the expression system (lane 2). This result signify that recombinant p24 protein has been purified optimally with no *E. coli* contaminating protein. Another possibilities for the contaminated positive control reactivity were the improper storage of the commercial kit control serum and Western blot technique that cause contamination of unknown protein. Therefore, it is important to concern about the storage of commercially Western blot reagen including the control serum to ensure the reliability of the result. By this result can be concluded that the p24 HIV-1 CRF01_AE recombinant protein expressed in *E. coli* BL21CP-RILP can be used as the antigen in Western blot assay for HIV-1 CRF01_AE diagnostic.

In conclusion, the recombinant p24 protein optimally expressed after four hours with induction of 0.2 mM IPTG at 37°C. The protein was purified optimally at the third elution and imidazole concentration of 30 mM in wash buffer. The Western blot result indicated that the recombinant protein expressed in this study is potentially applied for immunodiagnostic assay of HIV-1 CRF01_AE infection in Indonesia.

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Conflict of interest

The authors affirm no conflict of interest in this study.

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