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Optimized Procedure for Recovering HIV-1 Protease (C-SA) from Inclusion Bodies

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Abstract
HIV-1 is an infectious virus that causes acquired immunodeficiency syndrome (AIDS) and it is one of the major causes of deaths worldwide. The production of HIV-1 protease (PR) on a large scale has been a problem for scientists due to its cytotoxicity, low yield, insolubility, and low activity. HIV-1 C-SA protease has been cloned, expressed, and purified previously, however, with low recovery (0.25 mg/L). Herein we report an optimal expression and solubilisation procedure to recover active HIV-1 C-SA protease enzyme from inclusion bodies. The HIV protease was expressed in seven different vectors (pET11b, pET15b, pET28a pET32a, pET39b, pET41b and pGEX 6P-1). The highest expression was achieved when the vector pET32a (Trx tag) was employed. A total of 19.5 mg of fusion protein was refolded of which 5.5 mg of active protease was obtained after cleavage. The free protease had a high specific activity of 2.81 µmoles/min/mg. Interestingly the Trx-fusion protein also showed activity closer (1.24 µmoles/min/mg) to that of the free protease suggesting that the pET32a vector (Trx tag) expressed in BL21(DE3) pLysS provides a more efficient way to obtain HIV-1 protease.

Keywords HIV-1 protease · Thioredoxin · Fusion tags · Inclusion bodies · Refolding

1 Introduction

HIV-1 infection is mainly transmitted through direct contact of infected bodily fluids. About 39 million people in the world are living with HIV [1]. If HIV is left untreated, the virus can progress to full blown AIDS, and has resulted in about 25.8 million deaths [2–4]. HIV/AIDS is a disease people most probably will have to live with for the rest of their lives [1, 5]. Due to the nature of the virus, new mutations develop leading to drug resistance [6]. It is therefore of key importance to know the clinical effectiveness of existing drugs against new mutants.

The HIV protease (PR) structure and its drug-resistant variants have been studied for nearly 20 years in order to combat the challenges of AIDS antiviral therapy and the evolution of HIV drug resistance [7]. Currently about 25 different antiretroviral drugs (ARV) have been approved for HIV treatment [8]. The prescribed HIV treatment incorporates a combination of drugs from different classes in highly active antiretroviral therapy (HAART) [9]. Among the approved drugs, nine of them target HIV protease [10]. Recombinant HIV-1 PR is used for screening new inhibitors. Scientists have been trying to optimize the production of this protein and to explore other hosts for the expression of the PR enzyme. These includes the use of different promoters and fusion tags, or codon optimization and various bacterial host strains and the use of different expression media [11, 12]. Volente et al. recently reported developments in the production optimization of this enzyme. They employed the use of Gluthathione S Transference fusion tag and managed to produce about 2 mg/L of culture [20]. The recovery was a bit lower than the 4 mg/L recently reported by Nguyen et al. [19].

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We have previously reported [13] a procedure where we cloned, expressed, and purified C-SA HIV protease. The vector that was used in that case was pGEX-6P-1 [13]. The protease was characterized and verified by LC-MS sequencing. Though the purity was high, recovery was low (0.25 mg/L), which then prompted us to further optimise expression and purification.

In this study were report a procedure that gives the highest recovery of HIV-1 C-SA protease from inclusion bodies. This protease was cloned with seven different vectors and the best vectors were chosen.

2 Materials and Methods

2.1 Materials

All reagents were purchased from Sigma Aldrich, unless otherwise stated. All pET vectors, enterokinase kit and *E. coli* Bl21 were purchased from Novagen (USA). PGEX-6P1 and Gstrap columns were purchased from GE Health Care (Sweden). HRV 3C protease and His-Pur cartridges were bought from Thermo Fisher Scientific.

2.2 Cloning of HIV-1 Protease and Protein Expression

The HIV-1 protease gene (having Q7K to avoid autocatalysis) from previous work [13] was amplified with specific primers and cloned to the respective vectors. For pET11b, 15b and 28b NdeI and XhoI restriction sites were used. For pET32b, 39b and 41b NcoI and XhoI sites were, and lastly BamHI and XhoI were used for pGEX 6P-1. The amplified genes and their respective vectors were then restricted and purified. These were then ligated and transformed into BL21(DE3) pLysS using a heat shock method [13, 14]. Transformed cells were plated in antibiotic selection plates and grown overnight at 37 °C. Positive clones were screened using colony PCR and further by restriction digestion and plasmid DNA sequencing. The *E. coli* cells harbouring HIV-1 plasmids were grown at 37 °C in LB medium with antibiotics (100 µg/ml ampicillin and 25 µg/ml chloramphenicol for pET11b, 32b (His and Trx-tag), 15b (His-tag) and pGEX 6P-1 (GST-tag), 34 µg/mL kanamycin and 25 µg/ml chloramphenicol for pET28b (His-tag), pET41b (His and GST-tag) and pET39b (His and DbsA-tag). The overnight culture was diluted 100-fold in Luria broth containing the respective antibiotics and grown for approximately 3 h at 37 °C. When optical density at 600 nm of the culture reached 0.4–0.5, IPTG was added to a final concentration of 1.0 mM. The cultures were grown for 4 h, cells harvested by centrifugation at 5000×*g*, 30 min, 4 °C and stored at − 20 °C.

2.3 Inclusion Body Isolation and Solubilization Optimization

The pellet was resuspended in 50 mM Tris pH 8 containing 1 mM phenlymentanesulfonlyl fluoride (PMSF), thereafter sonicated on ice to liberate the cell contents, followed by centrifugation at 14,000×*g*. The pellet comprised of the expressed proteins as inclusion bodies was washed again with the same buffer but this time with 1% triton. The final pellet was resuspended in 10 mL of three different solubilisation conditions. Two of these were mild solubilisation procedures, and they comprised of the following, procedure one had 50 mM Tris pH 12, 2 M urea, the second one contained 50 mM Tris pH 8, 3 M urea and 30% trifluoroethanol. The third solubilization procedure (denaturing) had 50 mM Tris pH 8, 8 M urea and 2 mM dithiothreitol (DTT). These three homogenized samples were then allowed to stand at room temperature for 1 h. The proteins solubilized under mild conditions were then quickly refolded by tenfold dilution with refolding buffer (50 mM Tris pH 8, 5% sucrose and 2 mM DTT).

2.4 Purification

GST (Protease expressed in pGEX 6P-1) fusion protein was purified as described previously [13]. Briefly, purification was done using an AKTA purifier 100–950 (GE Health Care). Partial purification was carried out using a Hitrap QFF (5 mL) anion exchange column (GE Health Care) and the GST-HIV-PR was eluted using a 0–1 M NaCl gradient. The bound proteins were then desalted using a Hiprep desalting column (GE Health Care, USA). Further purification was performed using a GSTrap affinity column (GE Healthcare, USA). The GST tag was then removed by overnight digestion at 4 °C with precision protease (Thermo Scientific). All contents were loaded back into the GSTrap affinity column and HIV-1 PR was collected in the unbound fraction, then refolded and stored at − 70 °C.

Fusion proteins from pET32a and pET39b were also purified by affinity chromatography, but using a His Pur Cobalt column, 5.0 mL, (Thermo Scientific). The column was first equilibrated with a 10-column volume of equilibration/wash buffer (50 mM Na_2PO_4, pH 7.5, 300 mM NaCl, 5.0 mM imidazole. Samples (20 mL) were then loaded onto the column using a sample pump. The column was washed with 5 column volumes of the same buffer. Bound proteins were then eluted using the elution buffer (50 mM Na_2PO4, pH 7.5, 300 mM NaCl, 150 mM imidazole). Purity of the eluted fraction was verified by SDS-PAGE. The samples were then refolded by removing

urea slowly by dialysis. The fusion proteins were then cleaved using enterokinase a cleavage capture kit (Novagen) according to the manufacture's protocol. Following cleavage, the enterokinase was removed from the mixture using the same kit. The remaining mixture now containing Trx and HIV-PR, was then loaded back onto the His-Pur cobalt column. Free pure HIV-PR was collected in the flow through. The purified protease was confirmed by SDS-PAGE, Western blot and LC-MS-TOF (Central Analytical Facility, University of Stellenbosch); data provided in the supplementary materials. Protein quantification for pure free HIV-PR was achieved by measuring absorbance at 280 nm and the concentration was calculated using Beer–Lamberts law. The molar attenuation coefficient used was 24 480 M^{-1} cm^{-1}. Absorbance at 340 nm was subtracted from the 280 nm absorbance to account for protein aggregation.

2.5 Enzyme Activity

The enzyme activities of the fusion protein and the free HIV-PR were measured following the breakdown of the HIV-1 fluorogenic substrate, Abz-Arg-Val-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2 as previously reported [13, 15]. Hydrolysis of the HIV-1 fluorogenic substrate was monitored by a decrease in absorbance at 300 nm. The specific activity for both the fusion protein and free PR were calculated. All enzyme catalytic activity assays were done using a Jasco V-630 spectrophotometer. The effect of pH and temperature on the purified enzyme was also determined.

3 Results

3.1 Cloning of HIV-1 Protease and Protein Expression

The HIV-PR genes were successfully cloned to the seven vectors (Figures S1, S2 and S3). The HIV-PR was then expressed in each vector as either fused or non-fused protein. All the vectors used have a T7 promoter except for pGEX 6P-1 which uses a tac promoter. A summary of the expression results is presented in Table 1. The best results were obtained when pET32b was used (Figure S4). The expression was greater for pGEX 6P-1 compared to pET39b, which was also high. Vectors pET11b,15b, 28b and 41 showed the lowest expression of the HIV-PR. Expression in these vectors was only detected through western blot as nothing was visible with SDS-PAGE.

3.2 Inclusion Body Isolation, Solubilization and Purification

Inclusion bodies from the three selected vectors were solubilized using the three procedures as described in the methods section. A summary of the solubilisation results is shown in Table 2. The fusion proteins were then purified using affinity chromatography as described in the methods section. High recovery was obtained after refolding from pET32a. There was high aggregation (seen as white precipitate) observed from the other two vectors. Again, pET32a gave the highest concentration of free PR (approx. 5.5 mg/L) after cleavage. A summary of the recovered PR is also presented in Table 2. As pET32a rendered the best results, further characterization was performed using proteins from this vector. Figure 1 shows a chromatogram of Trx fusion protein and protein profile. The protein fusion protein was purified with a single step purification procedure.

The free HIV-PR obtained after enterokinase cleavage showed a single band of 11 kDa on SDS-PAGE, Fig. 2. These was also confirmed by the LC-MS-TOF results (Table S1).

3.3 Enzyme Activity

We also assessed whether the fusion protein (Trx-HIV-PR) has activity against the synthetic substrate. These results are presented in Fig. 3. The experiment was performed using constant substrate concentration and increasing enzyme concentration. The fusion protein showed almost the same

Table 1 Optimizing expression of HIV-PR in seven different vectors

Vector	Promoter	Tag	Comment
pET11b	T7 Promoter	No tag	Low expression level. Difficult in purification
pET15b	T7 Promoter	His-N-Terminus	Very low expression, only detected through western blot
pET28b	T7 Promoter	His N and C Terminus	Very low expression, only detected through western blot (Figure S4)
pET32b	T7 Promoter	Trx, His N and C terminus	High Expression level. High purification yield. Highly recovery after refolding
pET39b	T7 Promoter	DbsA N terminus, His N and C terminus	Fair expression. High purification yield. Low recovery after refolding
pET41b	T7 Promoter	GST N terminus, His N and C terminus	Low expression level. Only detected through Western blot (Figure S6)
pGEX-6P-1	Tac Promoter	GST N Terminus	High expression level. Low recovery due to aggregation

Table 2 Protein recovery from the three solubilisation procedures

Vector	Solubilizing method	Total fusion protein from 1L	Fusion protein refolded (%)	Total HIV-PR (mg) in 1L
pET32a (His and Trx)	50 mM Tris pH 8, 8 M Urea	23.0 mg	44	4.10
	50 mM Tris pH 12, 2 M Urea	22.8 mg	49	5.50
	50 mM Tris pH 8, 3% TFE, 3 M Urea	20.2 mg	49	4.30
pET39b (His and DbsA)	50 mM Tris pH 8, 8 M Urea	4.0 mg	10	0.20
	50 mM Tris pH 12, 2 M Urea	3.4 mg	16	0.30
	50 mM Tris pH 8, 3% TFE, 3 M Urea	3.2 mg	13	0.22
pGEX 6P-1 (GST)	50 mM Tris pH 8, 8 M Urea	12.0 mg	2	0.15
	50 mM Tris pH 12, 2 M Urea	10.0 mg	5	0.20
	50 mM Tris pH 8, 3% TFE, 3 M Urea	9.0 mg	5	0.23

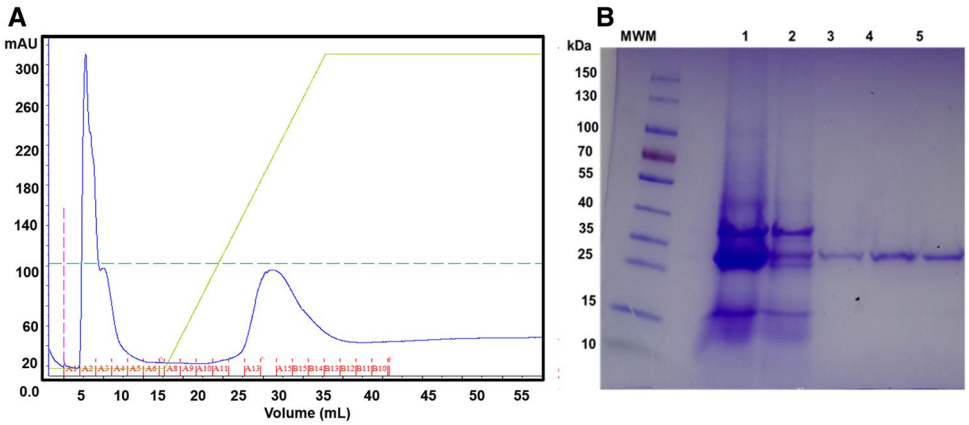

Fig. 1 Purification of Thioredoxin fusion protein using affinity chromatography. The solubilized inclusion bodies were loaded onto a His Pur cobalt column previously equilibrated with 50 mM $NaPO_4$, 300 mM, NaCl and 5 mM imidazole. Unbound proteins were washed out with 2 column volumes of the same buffer and bound proteins were eluted with same buffer but with a 0-250 mM imidazole gradient. The y-axis represents absorbance at 280 nm, the x-axis represent the fractions collected. The green line represents the imidazole gradient. Purity was verified using SDS-PAGE. **a** chromatogram showing bound and unbound fractions. **b** SDS-PAGE of the collected fractions, MMW; molecular weight marker; 1 crude protein, 2 unbound protein, 3 (A13),4 (A15), 5 (B14) are bound proteins

activity as the free PR though slightly lower. The free HIV PR had a specific activity of 2.81 µmoles/min/mg compared to 1.24 µmoles/min/mg for the fusion protein.

To confirm whether the purified protease possesses the same properties as reported in literature, the optimum pH and temperature were determined. The optimum pH and temperature pH were found to be 5 and 37 °C respectively. This is presented graphically in Fig. 4.

4 Discussion

HIV-1 protease production on a large scale is difficult because of various factors mentioned previously [16, 17]. Over the past 20 years, scientists have attempted to overcome this problem by using several expression systems containing different promoters, fusion tags with by using various hosts and media [18]. In this study, we employed several vectors and three solubilisation procedures. Four of the seven vectors used showed very low expression. This could be due to cytotoxicity of the PR which was not suppressed by the fusion tags on the vectors. The protease was reasonably well expressed as a DbsA fusion protein as seen from Table 2. Again, recovery was also low when the three solubilisation procedures in this study were used. These results are in agreement with what was reported by Nguyen et al. [19]. The expression in the pGEX 6-P1 vector was very high, the PR was expressed as a GST fusion protein. Since the protein was expressed as inclusion bodies, refolding was required before loading to the Gstrap column. In this case, 90% of the protein was lost due to aggregation. GST is reported to form disulfide bonds which makes refolding difficult. Cleavage with the precision protease was also not effective. The overall yield was 0.25 mg/L, which was low.

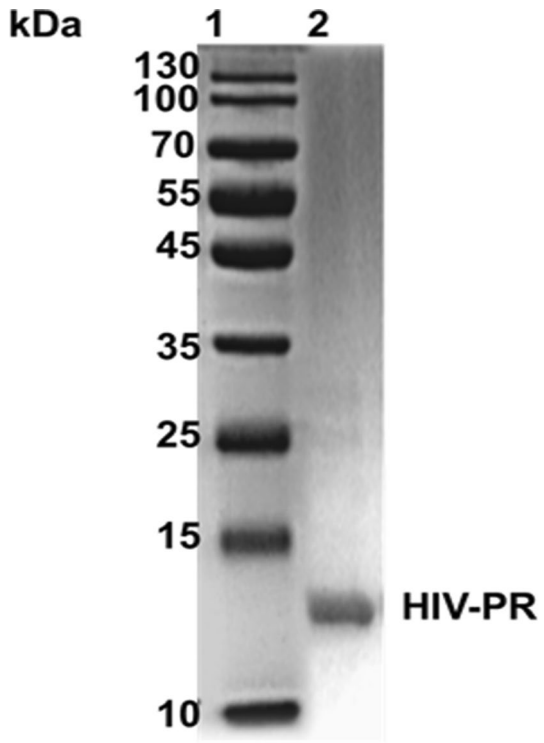

Fig. 2 SDS-PAGE of free HIV-PR. This was achieved after dialysis of the fusion protein, which was the cleaved using enterokinase. *Lane 1* molecular weight marker, *Lane 2* HIV-PR

Volente et al. also reported high expression in pGEX-6P-1 but low recovery yield [20].

Table 2 indicates that the highest expression and recovery was obtained when Trx (pET32a) fusion tag was employed. This is the highest recovery of HIV-1 protease reported from inclusion bodies. The recovery is a 22-fold increase of what we have previously reported for HIV-1 C-SA [13]. This procedure appears to solve the difficulties associated with expression, purification, and recovery of HIV-1 PR and will also be applicable to other strains. The recovery of HIV-PR we are reporting is better than the reported for subtype B (2.0 mg/L) by Volente et al. and 4.0 mg/L from Nguyen et al. [19, 20]. Again, the PR has better specific activity (2.81 µmoles/min/mg) compared to 1.19 µmoles/min/mg reported by the former [17], and even better than the method we previously reported (2.02 µmoles/min/mg) [13].

Trx is an intracellular thermostable *E .coli* protein with a molecular weight of 12 kDa and is highly soluble [21]. Trx has been reported to increase solubility in recombinant protein expression by taking advantage of its intrinsic oxido-reductase activity which aid in the reduction of disulfide bonds through thio-disulfide exchange [21]. This explains the high recovery even when any of the three solubilisation procedures were used. There was very low aggregation when the protein samples were refolded. Interestingly the Trx fusion protein had almost the same activity as the free HIV-1 PR. Trx is also useful in crystallization of certain target proteins [22]. Its rigid connection to the target protein blocks conformational heterogeneity facilitating crystallization [21]. This will provide an easy and rapid way of crystallizing new HIV-PR mutants.

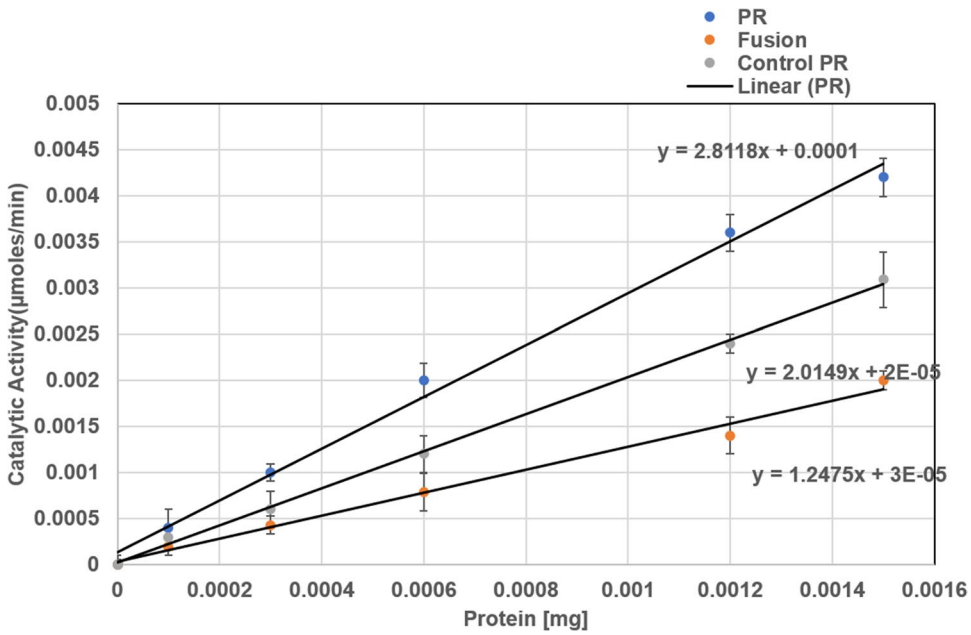

Fig. 3 Comparing activities of Trx-fusion protein, free HIV-PR and control PR purified previously [13]. This was determined following the hydrolysis of the synthetic substrate (Abz-Arg-Val-Nle-Phe-(NO_2)-Glu-Ala-Nle-NH_2) in 50 mM sodium acetate and 100 mM NaCl (pH 5) and 37 °C (n = 3)

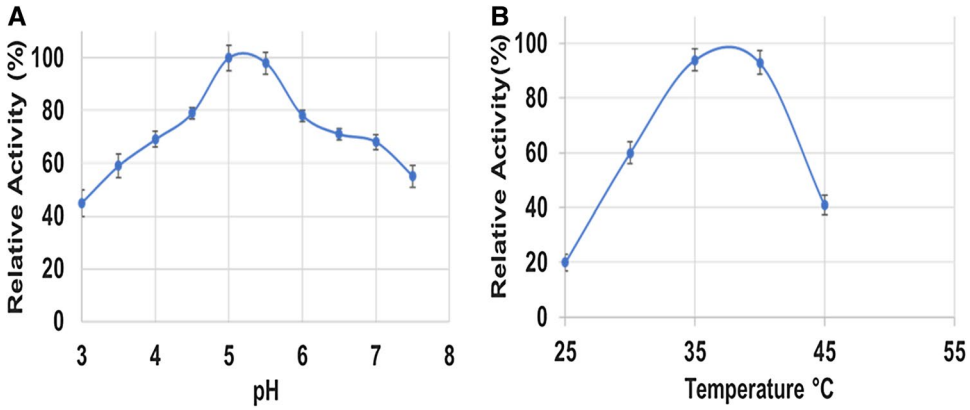

Fig. 4 Determination of optimum pH and optimum temperature for the free HIV-PR. The experiment was performed using the synthetic substrate (250 µM) and 2 µM of enzyme at varying pH and temperature. **a** optimum pH, **b** optimum temperature. The optimum pH and temperature pH were found to be 5 and 37 °C respectively (n = 3)

5 Conclusion

We have shown in this study that Trx is the best fusion tag for the expression of HIV-1 protease. This brings a solution to the issue of aggregation observed in other expression systems. Another interesting result is that the fusion protein possesses almost the same activity as the free HIV-PR. Again, the fusion, in theory, should allow for crystallization of new HIV-1 PR mutants.

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Compliance with Ethical Standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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