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Purification and characterization of naturally occurring HIV-1 (South African subtype C) protease mutants from inclusion bodies

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ABSTRACT

Human immunodeficiency virus (HIV) infections in sub-Saharan Africa represent about 56% of global infections. Many studies have targeted HIV-1 protease for the development of drugs against AIDS. Recombinant HIV-1 protease is used to screen new drugs from synthetic compounds or natural substances. Along with the wild type (C-SA) we also over-expressed and characterized two mutant forms from patients that had shown resistance to protease inhibitors. Using recombinant DNA technology, we constructed three recombinant plasmids in pGEX-6P-1 and expressed them containing a sequence encoding wild type HIV protease and two mutants (I36T↑T contains 100 amino acids and L38L↑N↑L contains 101 amino acids). These recombinant proteins were isolated from inclusion bodies by using QFF anion exchange and GST trap columns. In SDS–PAGE, we obtained these HIV proteases as single bands of approximately 11.5, 11.6 and 11.7 kDa for the wild type, I36T↑Tand L38L↑N↑L mutants, respectively. The enzyme was recovered efficiently (0.25 mg protein/L of *Escherichia coli* culture) and had high specific activity of 2.02, 2.20 and 1.33 μmol min^{-1} mg^{-1} at an optimal pH of 5 and temperature of 37 °C for the wild type, I36T↑T and L38L↑N↑L, respectively. The method employed here provides an easy and rapid purification of the HIV-1(C-SA) protease from the inclusion bodies, with high yield and high specific activities.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) and human immunodeficiency virus (HIV) is a crucial health issue in sub-Saharan Africa [1,2]. It affected nearly 25 million people in 2013, and more than 17% of infected individuals live in South Africa [3,4]. The HIV is classified into two types; HIV-1 and HIV-2, with HIV-1 the most prevalent. The latter is subsequently divided into four groups [5]. Group M or major, is the most common strain of the virus accounting for almost 90% of all HIV-1 infections. This group is subdivided into ten subtypes based on their geographic distribution. This classification enabled researchers with an opportunity to concentrate on one specific genetic variation [6]. In Europe and America they focused mainly on the HIV-1 subtype B virus [7,8].

Within sub-Saharan Africa, HIV-1 subtype C (C-SA) is the most prevalent and since its discovery in 1980, and was considered to be the first subtype identified in north-east Africa [9,10]. It has spread all over Africa and now comprises 70% of the global HIV infection to date. Despite the dominant infection rate, very little experimental work on C-SA protease has been performed [11,12].

Human immunodeficiency virus type 1 (HIV-l) [13], encodes an 11-kDa monomeric protease open reading frame. This protease is responsible for an autoproteolytic release of the protease from the gag-pol fusion precursor protein and a subsequent processing of the gag and pol proteins to yield the viral structural proteins [14–16]. Since the protease plays a crucial role in the life cycle in HIV maturation [17], immense effort has been made in the past few decades to design specific and potent inhibitors for this protease which can be used for the therapy of acquired immunodeficiency

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syndrome [18]. Otto el al. [19]. and others [20] have demonstrated a number of alterations in the HIV protease in viruses. The true significance of many of the observed sequence changes is unclear since these mutations often co-exist with additional mutations in the protease and elsewhere in the viral genome. Assessing the effect of alteration in the viral infectivity, growth properties and drug susceptibly will expand our understanding of the role of protease gene in HIV biology and pathogenesis.

However, protocols for producing recombinant HIV-1 protease are usually complex and time-consuming, require modification and additional purification steps because of the low purity of the final product [21], and are expensive [22]. Therefore, even after two decades of years of research, production of HIV-1 protease is considered an important obstacle in developing new drugs for treating AIDS [23].

In this study, we report an efficient procedure for the over expression and purification of three strains of HIV PR utilizing recombinant biosynthesis in *Escherichia coli.* These are South African wild type C (C-SA) HIV protease (PR) and two mutants. The first mutant has one extra amino acid (I36T↑T) and the second mutant has two additional amino acids (L38L↑N↑L) in each monomer. They were obtained from patients in South Africa that did not respond to commercially available protease inhibitors but responded to the following reverse transcriptase inhibitors (RTIs): efavirenz, d4t (stavudine) and 3TC (lamivudine).

2. Materials and methods

2.1. Cloning of the gene coding the HIV-1 C-SA protease and its mutants

HIV-1 C-SA PR sequence data were obtained from the AIDS Virus Research Unit (NICD, South Africa). The pET-11b expression plasmid with the PR insert (wild type, I36T↑T and L38L↑N↑L) was obtained from Professor Yasien Sayed (University of the Witwatersrand, South Africa). The plasmid was designed to minimize autocatalysis by introducing the Q7K substitution. HIV-PR genes were amplified by performing PCR with primers containing BamHI and XhoI restriction sites in the forward and reverse primers respectively (forward: 5′- ATA GGA TCC CCG CAG ATC ACT CTG TGG AA – 3′ and reverse 5′-CTA CTC GAG TCA GAA GTT CAA AGT GCA ACC CAG-3′). PCR products were digested using BamHI and XhoI, and were inserted to pGEX-6P-1 incorporating GST-tag at the N-terminus of the HIV-PR (GE Healthcare, USA).

2.1.1. Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a 1.2% (w/v) agarose gel in Tris-acetate-EDTA buffer (TAE); prepared as a 50 times dilution from a stock solution containing 242 g Tris, 57.1 mL acetic acid and 100 mL 0.5 M EDTA at pH 8 [24]. The gel was prepared with 0.5 μg/ml ethidium bromide to visualize the nucleic acid fragments, and was run at 100 V, 64 mA for approximately 45 min. The gel was removed from the electrophoresis tank and viewed using Syngene Gel documentation system (Vacutec South Africa).

2.2. Overexpression and isolation from inclusion bodies

For protein expression the *E. coli* strain BL21 (DE3), and BL21 (DE3) pLysS, Novagen USA) was transformed with plasmids containing GST-HIV-PR wild type and mutant forms using the heat shock method [25]. A single colony of transformed *E. coli* cells was inoculated in Luria-Bertani (LB) medium containing 50 μg/mL of ampicillin and 34 μg/mL of chloramphenicol (LBAC medium) and allowed to grow in a shaker incubator at 37 °C overnight. One ml of overnight cultures was transferred into 500 mL flasks containing 100 mL of fresh LBAC (initial $OD_{600} = 0.05$) [26]. Cells were grown at 37 °C and protein over expression was induced by adding 1.0 mM IPTG at early exponential phase (OD_{600} between 0.5 and 0.7). Cells were harvested 3 h after induction by centrifugation at 5000 × *g* at 4 °C for 10 min. The expressed HIV-PR wild type and mutants were found in the inclusion bodies and the expressed proteins were recovered according to the method of Volontè et al. [27]. The pellet was re-suspended in 10 mL of buffer A (10 mM Tris–HCl, pH 7.9 containing 1 mM PMSF). Crude cell extracts were prepared by sonication (Omni International Sonic Ruptor 400 Ultrasonic homogenizer) with an operating frequency of 20 kHz. The homogenized sample was subjected to 30 s on/30 s off pulses for 10 min at 20% amplitude. Thermal effects were minimized by placing the sonication sample on ice. The sonicated samples was centrifuged at 13,000× *g* at 4 °C for 20 min. The insoluble fraction was re-suspended in buffer A with 1% [v/v] Triton X-100, and gently homogenized until the pellet was fully dissolved and was again centrifuged (13,000× *g* for 20 min). The final pellet was solubilized in buffer B (10 mM Tris–HCl, pH 8, containing 5 mM DTT and 8 M urea) with gentle shaking for 60 min at room temperature. Finally, the samples were centrifuged at 34,000× *g* at 10 °C for 20 min, and the supernatant was collected.

2.3. Purification and re-naturation of GST-HIV-PR from inclusion bodies

Purification was carried out using an AKTA purifier 100-950 system (GE Healthcare, USA). The crude extract from inclusion bodies was passed through a HiTrap QFF column (GE Healthcare 5.0 mL) regenerated with buffer [28]. The column was washed with 5 column volumes of buffer B (10 mM Tris, 8 M urea and 5 mM DTT). Bound proteins were eluted using a NaCl gradient from 0 to 1 M in the same buffer (2 mL/min). The protein fractions, containing GST-HIV PR with the expected molecular weight, were desalted using a HPrep™ 26/10 desalting column (GE Healthcare) to remove excess urea [29]. The proteins were eluted (2 mL/min) in PBS buffer pH7.3. Eluted proteins were then loaded onto a GSTrap FF affinity column (GE Healthcare 1.0 mL). The GSTrap column was washed extensively using PBS pH 7.4 and the proteins were eluted (1 mL/min) with 50 mM Tris-HCl pH 8.0 containing 10 mM reduced L-glutathione (Sigma Aldrich) [27]. The purified fusion protein was dialyzed against PreScission buffer at 4 °C, The GST domain of fusion protein was removed by adding PreScission protease (20 U per mg of fusion protein as recommended by the manufacturer) [30] for 24 h at 5 °C followed by 2 h at room temperature. The proteolyzed sample was re-loaded on a GSTrap column equilibrated in PreScission buffer. The cloned HIV protease was recovered in the flow-through fractions. Part of the purified protein was sent for primary sequence determination (Section 2.6). The rest of the eluted protein was refolded by dialysis against 10 mM sodium acetate, 2 mM NaCl, and 1 mM DTT pH 5 at 4 °C. The concentration of the protease was determined by using Beer-Lamberts law, with a molar extinction coefficient of 25,480 M^{-1} cm^{-1} from absorbance spectra obtained on a Jasco V-630 spectrophotometer [26]. The protein profile after each purification step was monitored using SDS-PAGE and western blot analyses (Section 2.4). The specific activities of purified renatured HIV PR were determined as described in Section 2.5.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses

Samples (25 μg were electrophoresed in 15% SDS-PAGE [31]. The gel was stained using Coomassie blue G250 and de-stained in 40% methanol and 10% acetic acid mixture. The SDS-PAGE gels were also

transferred to nitrocellulose membranes. Membranes were blocked with 3% bovine serum albumin (BSA) in Tris buffered saline (20 mMTris-HCl, pH 7.4, 500 mM NaCl and 0.01% Tween 20 (TBST)) for 1 h, and incubated with primary antibody (GST-antibody) in 1% BSA in TBST at 4 °C overnight. Membranes were then washed thrice (10 mL TBST, 10 min) and treated with horseradish peroxidase-conjugated secondary antibody (mouse, ab97046; 1:2000) (1 h, room temperature). Membranes were washed again TBST three times (, 10 min each). The membrane was the incubated with an1-Step™ TMB-Blotting Substrate Solution (Thermo scientific) [32]. The protein and western blot profiles of the gel were captured using Syngene Gel documentation system (Vacutec South Africa).

2.5. Sequencing and characterization of C-SA HIV-PR and its mutants

Purified HIV-PR and its mutants samples were recovered from the SDS-PAGE gel were sent to the Central Analytical Facility at the Stellenbosch University for sequencing analysis. The gel containing expected HIVPR was de-stained and dehydrated, followed by trypsin digest. The fragments were then purified using a C18 column. Purification of fragments (peptides) was followed by LC-MS analysis. The raw files generated by the MS were imported into Proteome Discoverer v1.3 (Thermo Scientific) and processed using the Mascot 2.3.1 algorithm (Matrix Science) to obtain primary sequence.

The specific activity of the C-SA PR was determined using a method adapted from Naicker et al.(2013). It is based on the hydrolysis of the HIV-1 PR fluorogenic substrate (Abz-Arg-Val-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2) which mimics the capsid/p2 cleavage site in the HIV-1 Gag polyprotein. An increase in fluorescence emission from the 2-aminobenzoyl group was detected at an excitation wavelength of 337 nm and emission wavelength of 425 nm, resulting from cleavage of the Nle-Phe(NO_2) peptide bond [33,34]. A substrate concentration of 250 μM and an enzyme concentration of 10–50 nM, with an excitation bandwidth of 2.5 nm and emission bandwidth of 5 nm were used for the 1 min measurements during steady state [26]. The intensity signal associated with complete cleavage of 1 nM of substrate was measured and used to convert intensity to activity.

2.6. Non-reducing SDS-PAGE

A non-reducing SDS-PAGE was performed to prove the dimeric form after refolding. It uses the same discontinuous chloride and chloride fronts as normal SDS to form moving boundaries that stack and separate polypetides by mass charge ratio. Briefly the denatured HIV protease was refolded by dialyzing against 10 mM sodium acetate 1 mM DTT and 0.1 M NaCl, first in a 6 kDa cut-off for 6 h later a 12 kDa cut-off to remove remainder of unfolded HIV PR overnight. Refolded (dimeric) and denatured (monomeric) protein samples were then used in the non-reducing SDS-PAGE and electrophoresed at 80 V for 90 min. The gel was then stained with Coomassie brilliant blue R250 for 2 h followed by destaining this 10% acetic acid, 40% methanol solution then viewed using Syngene Gel documentation system (Vacutec South Africa).

3. Results

3.1. Construction and over expression of HIV-1 protease (wild type and mutants)

Wild type (WT) HIV-1 C-SA and two mutants HIV-1-I36T↑T, HIV-1-L38L↑N↑L were amplified from pET-11b resulting in 297 bp, 300 bp and 303 bp as shown in the PCR products, respectively as shown in Fig. 1. HIV-1 subtype C- SA wild type and its mutants of PCR products were cloned into the expression vector pGEX-6P-1 (as the other vectors gave low expression yields) and expressed in *E. coli* BL21 (DE3)pLysS.

The results showed that optimal laboratory-scale production of HIV-1 protease could be achieved when *E. coli* BL21 (DE3)pLysS cells transformed with the pGEX-6P-1 with HIV PR and grown at 37 °C in LB medium and were harvested at 3 h after induction with 1.0 mM IPTG. All IPTG-induced proteins accumulated as inclusion bodies. Recovered insoluble fractions of HIV-1 protease from each construct were assessed by performing SDS–PAGE and western blotting. GST-HIV PR expressed from this construct had a molecular weight of approximately 37.5, 37.6 and 37.7 kDa for the wild type, I36T↑T and L38L↑N↑L, respectively as shown in Fig. 2. In Fig. 2B only wild type HIV PR is shown and the mutants were also similar to the wild type. This protein reacted specifically with an anti-GST specific antibody

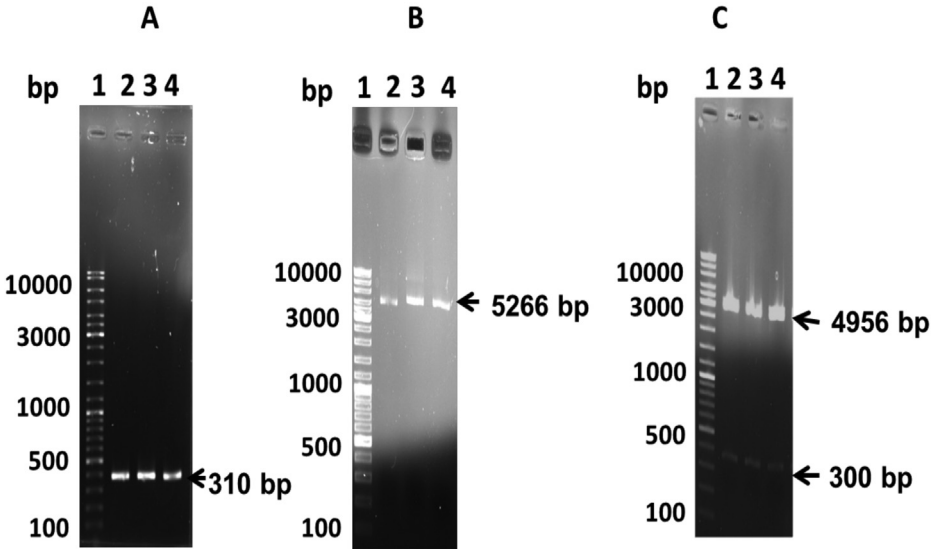

Fig. 1. Cloning of C-SA HIV protease and two mutants genes to pGex 6p1. (A) PCR amplification of three protease genes from pET-11b (B) pGEX-6P-1 with inserts. (C) Confirmation of the presence of protease gene by restriction digestion using XhoI and BamHI. 1: Molecular markers; 2: HIV PR wild type; 3: HIV PR I36T↑T; and 4: L38L↑N↑L.

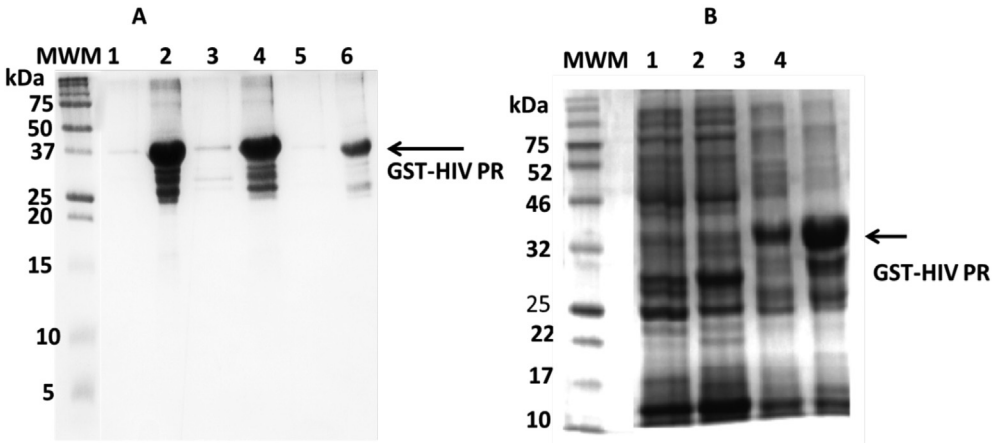

Fig. 2. Over expression of GST-HIV PR wild type and its two mutants. These were grown at 37 °C after 3 h IPTG induction and proteins were isolated from inclusion bodies. A) MWM, molecular weight marker, Lane 1:Un-induced WT GST-HIV PR, Lane 2: induced WT GST-HIV PR, Lane 3: Un-induced GST-HIV PR I36T↑T mutant, Lane 4: induced GST-HIV PRI36 mutant, Lane 5: Un-induced GST-HIV PR L38L↑N↑L mutant, Lane 6: induced GST-HIV PR L38L↑N↑L mutant.The arrow indicates the induced GST-HIV PR. B) SDS-PAGE of the wild type HIV-protease showing soluble (supernatant) and insoluble fractions (pellet). MWM, molecular weight marker, Lane 1:uninduced soluble (supernatant) fraction, Lane 2: induced soluble fraction, Lane 3:uninduced insoluble (inclusion bodies) fraction, Lane 4: induced insoluble (inclusion bodies) fraction.

(Fig. S1). Western blotting confirmed that approximately all of the expressed fusion protein was present in the insoluble form of inclusive bodies. This fraction was used for further purification.

3.2. Purification and refolding of GST-HIV-PR

Affinity binding in GST columns depends on the nature of the GST, and since denatured GST will not bind, a renaturation step is required before purification is performed. Putting the crude mixture directly into the desalting column resulted in severe protein loss due to precipitation. To improve the yield, an extra polishing step was required which is why the QFF column was used before desalting to remove majority of contaminating proteins. The amount of protein loss due precipitation was significantly reduced.

Insoluble GST-HIV-PR proteins were resuspended in Buffer B (8 M urea, 10 mM Tris and 5 mM DTT). GST-HIV-PR was then purified from the crude extract on a HiTrap QFF column (Fig. 3A) followed by a HiPrep desalting column as shown in Fig. 3B, and then a GSTrap column as shown in Fig. 3C, as described in the Methods Section.

The desalted GST-HIVPR was subsequently cleaved with a HRV 3C enzyme and has shown up to 70% cleavage as seen from SDS-PAGE when comparing undigested fusion protein with digested fusion protein as shown in Fig. 4A. The mixture was then loaded once again onto the GSTrap in order to obtain pure HIV-PR which was eluted in peak 1 (Fig. 3C) of the GSTrap column. The results of SDS-PAGE showed a high degree of purity ≥90% as judged by SDS-PAGE (Fig. 4B), while the sample in peak 2 partly co-eluted with the fusion partner GST and the HRV 3C protease (Data not shown). An overall yield of ≥0.25 mg of pure HIV-1Pr/L of fermentation broth was achieved (starting from 5 mg of the fused protein/L).

3.3. Characterization of the recombinant HIV-1 protease and its mutants

A summary of the LCMS data for sequencing shown in Table 1 below. The protein sequencing results confirmed that the isolated

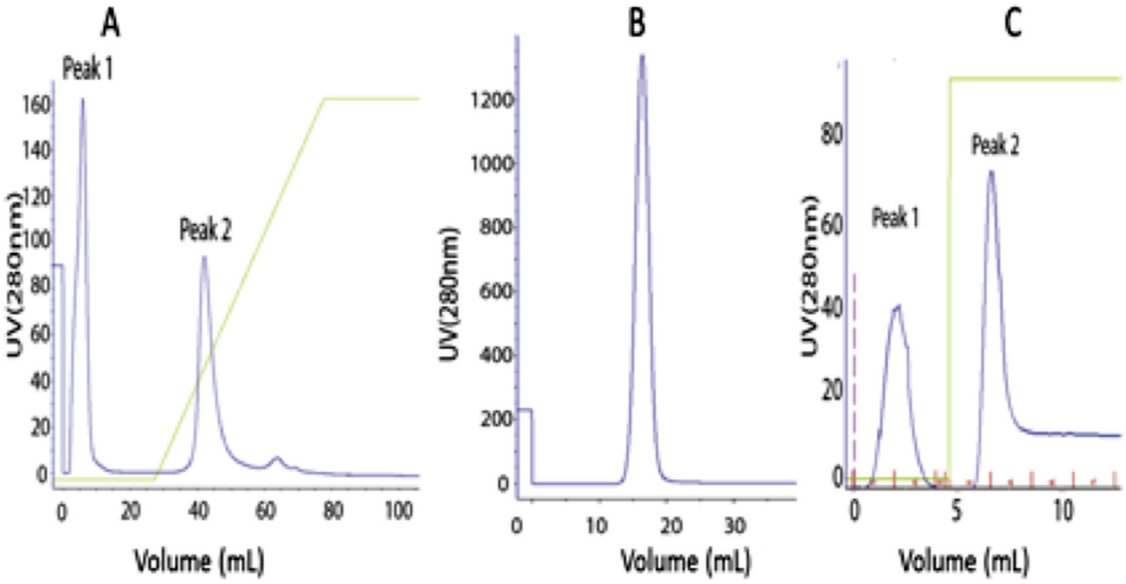

Fig. 3. Purification of GST-HIV-PR from inclusion bodies using AKTA purifier 100-950 system. A). Purification through QFF column, protein was eluted in peak 2 with linear NaCl gradient (0–1 M), B) Desalting of eluted GST-HIV PR with HiPrep 26/10 desalting column. C). Purification of GST-HIV PR using GSTrap column, protein was eluted in peak 2 after a segmented glutathione (10 mM) gradient.

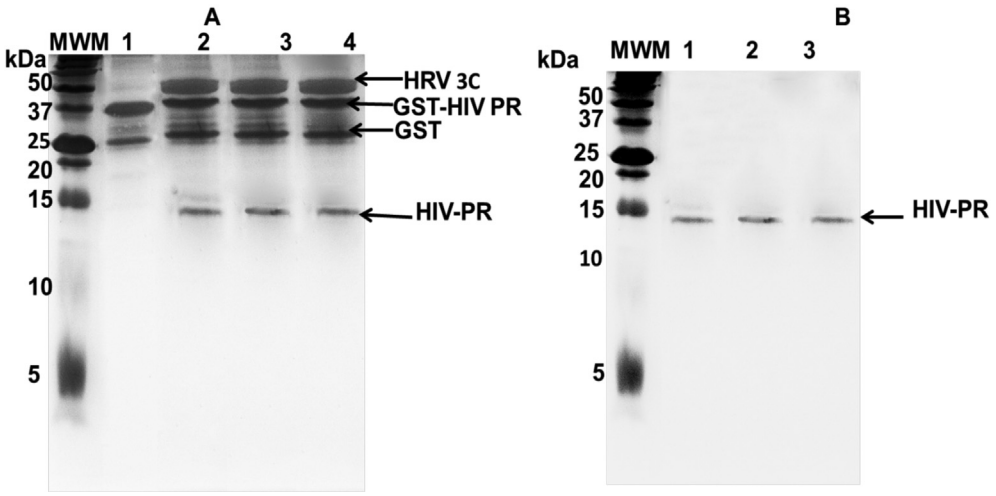

Fig. 4. Cleavage of HIV-PR from GST-HIV-PR fusion protein by HRV 3C protease and subsequent purification. A) MWM, molecular weight marker, Lane 1: GST-HIV PR only, Lane 2: cleaved wild type, Lane 3: cleaved I36T↑T mutant, Lane4: cleaved L38L↑N↑L mutant.B) Purified HIV-PR and mutants, Lane 1: wild type HIV-PR, Lane 2: I36T↑T and Lane 3: L38L↑N↑L.

Table 1
Summary of LCMS data for protein sequencing.

Proteases	Proteins identified			
	Accession	Description	Score	MW [kDa]
Wild type	CAR92387	Pol protein (Fragment)	553.83	65.4
	CAP07925	Protease and reverse transcriptase (Fragment)	587.15	66.9
I36T↑T	P05962	Gag-Pol polyproteins (Fragment)	542.26	58.8
	CAP07925	Protease and reverse transcriptase (Fragment	493.93	67.3
L38L↑N↑L	CAQ63378	Pol protein (Fragment)	553.83	65.4
	CAR92387	Protease and reverse transcriptase (Fragment	331.2	66.9

proteins were indeed HIV-PR. All the proteins were found to contain HIV protease and reverse transcriptase fragments with a high score. The wild type HIV-PR had the highest score followed by the I36T↑T then lastly the L38L↑N↑L mutant.

The cloned HIV-1 South African subtype C protease and two mutants were assessed using chromogenic HIV-1 substrate, mimicking the protease cleavage site in the *gag-pol* protein precursor. Fig. 5 shows that the enzyme activities of all three cloned HIV PRs demonstrate a concentration-dependent manner.

Catalytic activity of the protease enzyme was calculated by dividing the spectrophotometrically measured absorbance per minute of the hydrolyzed substrate by the molar extinction coefficient. The specific activity of the protease was calculated from the slope of the plot and was found to be 2.02, 2.20 and 1.33 μmol/min/mg, for the wild type and the mutants, I36T↑T and L38L↑N↑L, respectively.

All the other kinetic parameters of the C-SA HIV PR were calculated and the data is summarised in Table 2 below. The data shows that the I36T↑T requires less substrate to achieve maximum velocity meaning it has the lowest K_m value compared to the wild type and the L38L↑N↑L mutant. Again, the I36T↑T has the highest reaction rate (k_{cat}) when compared to the other two proteases. Of all the proteases, the I36T↑T had the highest affinity toward the substrate (k_{cat}/K_m) when compared to the other proteases.

3.4. Non-reducing SDS-PAGE

The dimeric form of the HIV-PR (wild type) was confirmed by running a non-reducing SDS PAGE. When denatured, the HIV PR exists as a monomer (11 kDa), and when refolded it dimerises to form a 22 kDa functional enzyme as seen in Fig. 6 below.

4. Discussion

HIV-1 encodes a protease essential for viral replication and pathogenesis based on homology with other retroviruses [35]. The active form has an open reading frame to generate a 99 residue dimeric protein. The expression described in this study of that sequence, resulted in an active dimeric protease, confirming a recent observation [36].

The bacterial expression system is universally used, as it is

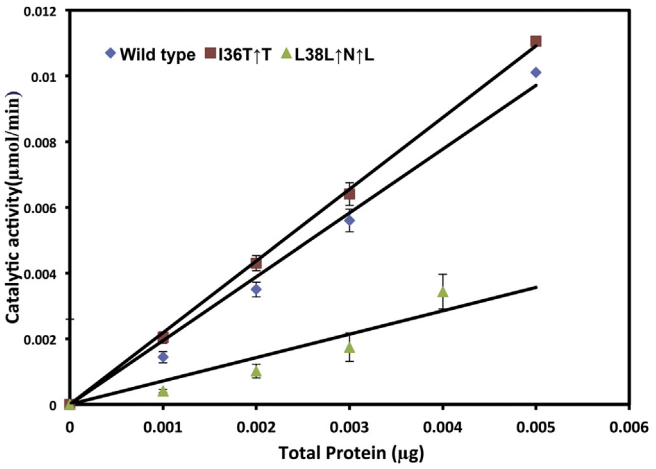

Fig. 5. Specific activities of the C-SA PR and the two mutants. The activity was determined following hydrolysis of the HIV-1 protease substrate (Abz-Arg-Val-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2) in 50 mM sodium acetate and 1 M NaCl (pH 5.0).

Table 2
A summary of the kinetic parameters of the three proteases with the substrate (Abz-Arg-Val-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2).

Parameter	Wild type	I36T↑T	L38L↑N↑L
Specific activity (μmol min^{-1} mg^{-1})	2.02 ± 0.01	2.20 ± 0.01	1.33 ± 0.02
K_M (μM)	139.70 ± 3.01	88.44 ± 3.00	213.30 ± 4.00
V_{max} (μmol min^{-1})	0.0018 ± 0.0000	0.0015 ± 0.0002	0.0026 ± 0.0003
k_{cat} (s^{-1})	0.106 ± 0.001	0.131 ± 0.009	0.044 ± 0.005
k_{cat}/K_M (μM^{-1} s^{-1})	0.042 ± 0.002	0.052 ± 0.003	0.016 ± 0.002

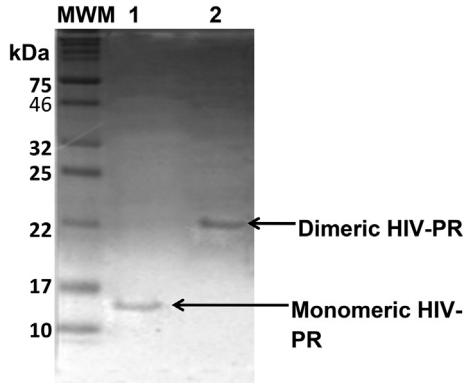

Fig. 6. Non reducing SDS- PAGE showing monomeric HIV PR and dimeric HIV PR. The denatured HIV protease was refolded by dialyzing against 10 mM sodium acetate, 1 mM DTT and 0.1 M NaCl, first in a 6 kDa cut-off for 6 h later a 12 kDa cut-off to remove remainder of unfolded HIV PR overnight. Refolded (dimeric) and denatured (monomeric) protein samples were the used in non-reducing SDS-PAGE. MWM, Molecular weight marker, Lane1: Monemeric, Lane 2: Dimeric HIV PR.

relatively inexpensive, and with ease of manipulation and a rapid growth rate [14]. Among the challenges that arise with the use of bacteria as host systems in recombinant protein expression are protein stability, conformation, structural flexibility, insolubility, host cell toxicity and low production yields [37]. Expressing proteins as fusion proteins effectively resolves these challenges [37]. This involves the use of fusion tags such as glutathione transferase (GST), maltose-binding protein (MBP), protein disulfide isomerase I (DsbA), thioredoxin A, small ubiquitin-related modifier (SUMO), mistic, and N-utilization substance A (NusA) [38]. In this study, we expressed HIV-proteases as GST-fusion proteins. A higher expression yield (>0.25 mg/L) than previously reported [39]was achieved. The specific activity (2.02 μmol/min/mg) of the wild type was also higher than the previous report. Costa et al. showed that the use of GST maintains enzyme conformation and enhances structural stability [40]. The higher specific activity observed can also be attributed to the high purity achieved by the GST affinity column [41].

In this study, the HIV -1 subtype C proteases (both wild type and its mutants) were highly expressed at a low IPTG concentration (1.0 mM). To further increase the recombinant protein solubility, we chose *E. coli* BL21 (DE3) pLysS over *E. coli* BL21 (DE3) as the host strain, which in previous studies demonstrated appropriate protein expression [15]. The use of *E. coli* BL21 (DE3) pLysS reduced basal expression which is observed in *E. coli* BL21 (DE3) which did not induce sufficient protein for purification. By adopting these strategies, the recombinant proteins were expressed as inclusion bodies and were easily purified and identified using western blot analysis.

We also assayed both the wild type and the mutant proteins for specific proteolytic activity after re-naturation. In the re-natured preparations of mutants, I36T↑T showed somewhat higher activity (2.20 μmol/min/mg) and L38L↑N↑L showed less activity (1.33 μmol/min/mg) when compared to the wildtype (2.02 μmol/min/mg). Our results suggest that, irrespective of the size of the flanking sequences within the protease, these mutant proteins possess similar activities as that of the wild type. Comparing the mutants with the wild type, it is seen that the one amino acid insertion mutation in I36T↑T has a slightly increased catalytic efficiency. A significant decrease in catalytic efficiency was observed in L38L↑N↑L. This means that the double insertion has a prominent effect on the enzymes active site and thereby reducing the affinity for substrate. The production of these mutants will enable us to test newly designed specific protease inhibitors [11] and will potentially assist to combat the mutant forms of HIV.

From the overlay of the mutant I36T↑T and the wild type protein (Fig. 7) there was no significant change in the enzyme structure,

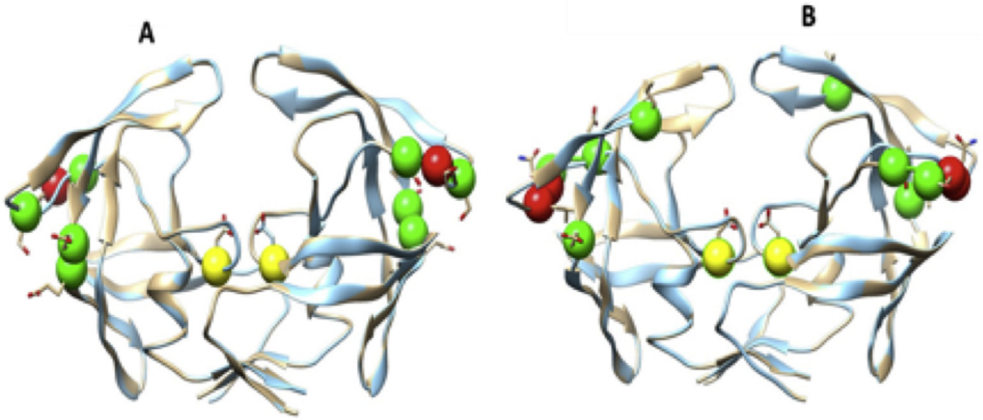

Fig. 7. Ribbon representation of overlay of mutant proteases (brown) and wild type HIV-1 subtype C protease (blue). Amino acid mutations are depicted in green, amino acid insertions in red and the catalytic Asp residue in yellow. A is I36T↑T,B is L38L↑N↑L (Created using UCSF Chimera version 1.9 [42]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

whereas there was a conformational change in the double insertion (L38L↑N↑L). This could have resulted in the reduction of substrate affinity in the L38L↑N↑L mutant.

In summary, the expressed and purified two mutant proteases exhibited similar catalytic and physical properties than the well-characterized C-SA wild type protease [2,26]. This will enable us to verify the theoretical activities obtained from our computational model. (Husain 2015 Accepted for publication in Chem Biol& Drug design). Future studies will involve the biosynthesis of ^{15}N enriched H-V PR for NMR studies.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2016.02.013.

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