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Functional expression of *Escherichia coli*-derived recombinant plastocyanin from *Canna indica* L. and its anti-HIV-1 activities

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ABSTRACT: Human immunodeficiency virus (HIV) infection has been a risk to public health due to its long-life treatment. The current medications were limited due to their single therapeutic targets, drug resistance, and adverse side effects. The discovery and study of novel anti-HIV agents are necessary. From our previous study, *Canna indica* L. leaf extract showed anti-HIV activities with plastocyanin as a potential active compound. In this study, the plastocyanin gene of *Canna indica* L. was cloned using the cDNA library established, the leaf extract mRNA and expressed in pET28(+); *E. coli*.DE3(BL21). The 408-bp plastocyanin gene produced 14-kDa recombinant protein plastocyanin (Pc) including a histidine tag (his-tag). Pc was characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and liquid chromatography coupled with mass spectrometry and further purified using N-terminal 6xHis-tagged fusion binding through column affinity chromatography. As expected, Pc was determined to be approximately 14 kDa on SDS–PAGE. To study the anti-HIV activity of Pc and its primary arrangement, the his-tag was removed by thrombin cleavage. Further, Pc and its product cleaved using thrombin (Pc/T) were evaluated for their cytotoxic and anti-HIV-1 activities using the syncytium reduction and anti-HIV reverse transcriptase assays. The syncytium reduction assay revealed that Pc and Pc/T exhibited anti-HIV activity at EC_{50} of 38.54 and 128.84 µg/ml; with calculated therapeutic indices of >6.49, active and >1.94, active; respectively. Both Pc and Pc/T also exhibited anti-HIV reverse transcriptase inhibitory activity at IC_{50} of 6.55 and 4.89 µg/ml, respectively. This study demonstrated that recombinant Pc could be used as an anti-HIV agent.

KEYWORDS: anti-HIV, *Canna indica* L., plastocyanin, recombinant technology

INTRODUCTION

Over the decades, the human immunodeficiency virus (HIV) pandemic has emphasized the importance of chronic viral infection treatment. As part of the currently available treatment, patients infected with HIV must undergo anti-retroviral therapy (ART) for plasma virus suppression and reduction. At present, ART drugs are included as part of highly active retroviral therapy (HAART), which comprises two or more different antiviral categories [1, 2]. The antiviral drug effector mechanism could be considered by potential targets including the inhibition of entry, reverse transcriptase, integrase, and transcription [3]. Moreover, latent viral forms can exist in infected patients as quiescent reservoirs, which necessitates the use of ART to suppress the viral loads. Several limitations, such as long-term combination, side effects, adherence, resistance, cost, and availability, make such medication troublesome [2–4]. Hence, studies on optional antiviral compounds are necessary. Medicinal plants with ethnobotanical use can be considered a natural pool for potential anti-HIV agents. Several natural compounds reportedly provide promising opportunities for anti-HIV treatment [4–6]. Among them, the lectin family, including griffithsin derived from red algae *Griffithia* sp., is a potential candidate that has been recently developed for large-scale production using recombinant technology and studies in humans as a microbicide [7, 8]. More compounds with anti-HIV activity and different underlying effector mechanisms are expected to be found hidden in nature.

Canna indica L., a medicinal plant, is used in tropical and subtropical areas as part of traditional medicine for the treatments of many illnesses including malaria, diarrhea, dysentery, and fever. *C. indica* L. reportedly exhibits several pharmacological activities, such as antioxidant, anti-inflammatory, immunomodulatory, antitumor, antihelmintic, antibacterial, and an-

tiviral activities [9–11]. Our previous studies on anti-HIV activity of Thai medicinal plants demonstrated that *C. indica* L.-derived proteins exhibit anti-HIV reverse transcriptase activity [12]. Subsequently, a study of *C. indica* L. leaf extract activity revealed that the active protein exhibiting an anti-HIV effect was plastocyanin, which was then characterized [13]. Plastocyanin represents cupredoxin or blue copper protein and is present in plants as part of electron transfer reactions. The cupredoxin family can be found in oxygen-evolving organisms, such as cyanobacteria, algae, and plants [14]. Azurin fulfills the same role in bacteria. Interestingly, azurin reportedly exhibits parasite and HIV growth inhibition characteristics [15]. As the cupredoxin family includes plastocyanin, it would be worthwhile to study it as a potential alternative anti-HIV protein. To the best of our knowledge, no such studies have been reported so far.

This study attempted to provide further information on plastocyanin derived from *C. indica* L. For this purpose, we cloned and induced the heterologous expression of the *C. indica* L. plastocyanin gene in *E. coli*. The plastocyanin-encoding gene was identified, and the recombinant protein purified and characterized using mass spectrometry. Then, we evaluated the anti-HIV activity of active plastocyanin through the syncytium reduction assay and HIV reverse transcriptase inhibitory activity analysis.

MATERIALS AND METHODS

Cloning and identification of plastocyanin gene derived from *Canna indica* L.

RNA was extracted from *Canna indica* L. leaf samples using the RNeasy® Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions. The high-quality RNA extract (the ratio of the absorbance at 260/280 nm as 1.9 to 2.1) was subjected to 1.2% FA gel electrophoresis. First-strand cDNA was synthesized using the Omniscript® RT Kit (Qiagen, Germany). Then, the cDNA was amplified using degenerate primers designed from the deduced nucleotides encoding N-terminal sequence amino acids (5′ GCT GAA CGC GCC CGG RGA GAT CTA CAA GG 3′: R = A/G) and Anc_dT (5′ TTT TTT TTT TTT TTT TTT TTT TVB 3′: V = A/G/C; B = G/C/T). The polymerase chain reaction (PCR) product was purified using the QIAquick PCR purification kit and subjected to agarose gel electrophoresis. The expected plastocyanin gene was then sliced and extracted using the QIAquick gel extraction kit, cloned into the pGEM®-T Easy vector, and transformed into *Escherichia coli* DH5α. The recombinant plasmid was extracted and subjected for sequence analysis by automated fluorescence dye termination (Bio Basic Inc, Canada) using T7 and SP6 primers.

The plastocyanin cDNA sequence was analyzed using the BioEdit software and sequence analysis tools at the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics available at http://au.expasy.org [16]. Moreover, the plastocyanin cDNA sequence was translated into amino acid sequence to determine the presence of putative Cu ligands as conserved amino acid residues in a conserved position. A homology search was performed using Blastx tool of the National Center for Biotechnology Information at the National Institute of Health (http://www.ncbi.nlm.nih.gov).

Recombinant plastocyanin expression and purification

To induce the expression of the recombinant plastocyanin (Pc), the inserted plastocyanin gene was double-digested using the restriction enzymes *Bam*HI and *Hind*III and cloned into the expression system pET28(+); *E. coli*.DE3(BL21). Then, the *E. coli* DE3(BL21) harboring plastocyanin gene-bearing pET-28a was cultured in terrific broth (100 ml:1.2 g tryptone, 2.4 g yeast extract, 230 mg KH_2PO_4, 125 mg K_2HPO_4, and 0.4 ml glycerol) supplemented with kanamycin at 30 µg/ml and IPTG 1 mM. The cells were harvested for recombinant protein studies by centrifugation and sonicated in binding buffers (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4, and 1 mM EDTA + PMSF). The soluble protein was obtained by centrifugation at 14,000 rpm for 20 min and filtration through a 0.2 µM membrane. The protein was, then, purified by immobilized metal affinity chromatography in a HiTrap™ chelating HP column (GE Healthcare, Uppsala, Sweden). After stepwise elution using binding buffer added with a gradient elution of different imidazole concentrations of 75, 100, and 125 mM (5 ml for each concentration), each fraction was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and a molecular weight standard protein marker (Bio-Rad, CA, USA). The protein was quantified according to the method described by Bradford [17] with bovine serum albumin as the standard.

The expected plastocyanin protein was subjected to amino acid sequence analysis using liquid chromatography coupled with mass spectrometry. The protein gel was de-stained with 50% acetonitrile in 50 mM ammonium bicarbonate (Merck, Darmstadt, Germany), and proteins were reduced and alkylated by 4 mM DTT in 50 mM ammonium bicarbonate and 250 mM iodoacetamide respectively. The gel piece was dehydrated by acetonitrile and resuspended in 0.1 µg/µl of Trypsin Proteomics Grade (Sigma Aldrich, Missouri, USA) in 50 mM ammonium bicarbonate. The digestion was performed overnight at 37 °C. The digested peptides were extracted by acetonitrile and dried using a centrifugal evaporator. Peptide mixtures were resuspended in 0.1% formic acid. The sample was injected into the UltiMate 3000 nano-liquid

chromatography (nano-LC) system (Dionex, Surrey, UK) coupled with a micrOTOF-Q (Bruker Daltonics, Bremen, Germany). A mascot generic file (.mgf) was generated using DataAnalysis 3.4 version software. Mascot daemon version 2.3.2 (Matrix Science, London, UK). Moreover, the amino acid sequences of recombinant protein were analysed by the SWISS-MODEL (http://swissmodel.expasy.org/).

Pc without an N-terminal his-tag, assigned as Pc/T, was prepared. To remove the polyhistidine tag (his-tag), a thrombin protease digestion was performed with 200 µg of purified recombinant Pc and 0.1 unit thrombin (Novagen, Madison, USA) for 16 h at 20 °C and, then, purified using the HiTrap™ chelating HP column. The unbound fraction was collected, dialysed overnight into 20 mM sodium phosphate buffer pH 7.4 and concentrated to 1 mg/ml.

Far UV-CD spectra of the plastocyanin (0.5 mg/ml) were recorded using a Jasco spectrometer, model J-815, with a 1 mm pathlength. The measurements were carried out at 25 °C with a scan rate of 50 nm/min over a wavelength range of 190–250 nm. The recombinant proteins were collected after refolding in 1 mM EDTA 50 mM Tris–HCl, pH 7. Three scans were averaged for each protein sample, and the buffer was subtracted. BeStSel (Beta Structure Selection) program was used to analyze the secondary structure of proteins [18].

Anti-HIV-1 activity

Syncytium reduction assay

The syncytium reduction assay was conducted using 96-well tissue culture plates along with the cytotoxicity assay of uninfected cells. We used 1A2 cells derived from CEM-SSTART (human T-cell lymphoblastic leukemia), a T-cell line that expresses HIV-1 Tat and Rev proteins. The 1A2 cells were infected with the ∆Tat/RevMC99 virus, a defective HIV-1 that is unable to replicate in infected cells due to the lack of the tat and rev genes. The cells were multiplied to obtain a virus titer of 100–200 syncytium-forming units in a 50-µl virus stock. The infected cells were then suspended to the density of 5×10^5 cells/ml and seeded at 5×10^4 cells/100 µl per well into the tissue culture plates containing 50 µl of the medium and 50 µl of the two-fold dilutions of the protein sample prepared by dissolving the lyophilized sample into the medium solution after 1 h of incubation ∆Tat/RevMC99 (100–200 SFU per 50 µl) was added and further incubated for 3 days at 37 °C in a humidified CO_2 incubator. Azidothymidine was used as a positive control. The experimental controls included cells containing neither the protein sample nor the virus, cells with the corresponding dilutions of the protein only, and infected cells. After incubation for 3 days at 37 °C under an atmosphere of 5% CO_2, the syncytia were counted under an inverted microscope. All experiments were performed in triplicates.

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atgggcagcagccatcatcatcatcatcacagcagcggcctggtgccgcgcggcagccat   60
 M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  H    20

atggctagcatgactggtggacagcaaatgggtcgcggatccatgGCGTTCGACGTGCTG  120
 M  A  S  M  T  G  G  Q  Q  M  G  R  G  S  M  A  F  D  V  L    40

CTGGGCGGCGACGGCGGGGTGCTGGCATTCGTGCCGAGCGAGTTCTCGGTGCCCGCCGGA  180
 L  G  G  D  G  G  V  L  A  F  V  P  S  E  F  S  V  P  A  G    60

GAGACCATCGTGTTCAAGAACAACGCCGGGTTCCCCCACAACGTGGTGTTCGACGAGGAC  240
 E  T  I  V  F  K  N  N  A  G  F  P  H  N  V  V  F  D  E  D    80

GAGATACCGTCGGGGGTGGACGTGGGGTCGATCTCCATGTCTGAGGAGGACTTGCTGAAC  300
 E  I  P  S  G  V  D  V  G  S  I  S  M  S  E  E  D  L  L  N   100

GCGCCCGGGGAGATCTACAAGGTGACCTTGAAAGAGAAGGGCACCTACAGCTTCTACTGC  360
 A  P  G  E  I  Y  K  V  T  L  K  E  K  G  T  Y  S  F  Y  C   120

GCGCCGCACCAGGGAGCCGGCATGGTCGGCAAAGTCACTGTTAACTAG              408
 A  P  H  Q  G  A  G  M  V  G  K  V  T  V  N  *               136
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Fig. 1 Plastocyanin cDNA sequence and the deduced amino acids: the underlined part indicates the N-terminal sequence; * indicates the stop codon; ◯ indicates the conserved Cu-binding ligand of the plastocyanin protein; and ▲ indicates the thrombin cleavage site. The plastocyanin nucleotide sequence has been submitted to GeneBank under the accession number MZ466389.

HIV-1 reverse transcriptase inhibitory activity

We used the EnzChek™ Reverse Transcriptase Assay Kit (Molecular Probes, Eugene, OR, USA) to measure the reverse transcriptase activity as per the manufacturer's instructions. The resulting signal intensity was directly proportional to the actual RT activity. The inhibition ratio (% IR) of the protein sample was calculated as follows:

$$(\%)\ \mathrm{IR} = \left(1 - \frac{\mathrm{OD(c{+}p)} - \mathrm{OD(c{-}RT)}}{\mathrm{OD(c{+}RT)} - \mathrm{OD(c{-}RT)}}\right) \times 100$$

where OD(c+p) = OD(complete system+protein sample), OD(c−RT) = OD(complete system − RT), and OD(c+RT) = OD(complete system+RT).

IC_{50} was based on the protein concentration that can inhibit enzyme activity at 50% IR. The IC_{50} assay was determined using the GraphPad Prism 9 software with a non-linear regression model. Doxorubicin, a DNA chelator that inhibits transcription, was used as a positive control.

RESULTS

Molecular cloning of the *Canna indica* L.-derived plastocyanin gene

Fig. 1 presents the plastocyanin gene and its deduced amino acid sequence. The plastocyanin cDNA sequence was successfully amplified using the degenerated primers designed based on the N-terminal sequence and poly-A tail. The nucleotide sequence comprised a 408-bp open reading frame encoding a protein with 135 amino acid residues and with a calculated molecular weight of 14171.86 Da and pI of 5.60. The amino acid sequence analysis indicated four conserved residues as copper binding sites (Fig. 1).

Recombinant plastocyanin protein expression and purification

The heterologous *C. indica* L.-derived plastocyanin expression in *E. coli* resulted in 6 mg of crude protein per 1 g of *E. coli* cells. The affinity-purified target protein yielded 2.49 mg per 1 g of *E. coli* cells. The post-purification yield was 41.43% according to the crude protein amount. Fig. 2(a) shows the Pc protein expression analysis in *E. coli*. The SDS–PAGE analysis showed that the estimated Pc mass was 14 kDa, which was in agreement with the calculated mass from the amino acid sequences. In addition, the Pc with an N-terminal-fused histidine tag was cleaved by thrombin proteinase. The Pc/T was purified and shown as a 12-kDa protein on the SDS–PAGE analysis (Fig. 2(b)). The expressed protein was subjected to LC-MS/MS. The mass spectra analysis showed that the amino acid sequences exhibited the maximum number of hits at score 165 with the Lactuca savita plastocyanin (PLAS_LACSA) (Fig. 2(c)). This peptide sequence matched precisely with 20 amino acid sequences (KGTYSFYCAPHQGAGMVGKV) at 113–132 C-terminal (Fig. 1). Moreover, the SWISS-MODEL generated the predict results of protein matching with 5 top-ranked showing plastocyanin protein and contained four Cu-ligand binding sites, which shared highest-level 84.58% sequence identity with spinach plastocyanin. The generated secondary structure models were visualized in Supplementary Fig. S1.

The secondary structure contents of Pc and Pc/T were examined by CD spectrum in the far UV region (190–250 nm), and the analysis was performed using BeStSel program. CD spectra of the Pc pattern indicated 7.2% helix, 22.7% antiparallel, 7.9% parallel, and 14.5% for turn state with NRMSD 0.024; while the Pc/T pattern indicated 11.5% helix, 31.1% antiparallel, and 9.1% for turn state with NRMSD 0.051.

Anti-HIV activities of Pc and Pc/T

Table 1 shows the Pc and Pc/T syncytium reduction assays. Pc and Pc/T exhibited anti-HIV activities based on the syncytium reduction assay with the effective concentration that reduced syncytium formation by 50% (EC_{50}) values of 38.54 and 128.84 µg/ml, respectively. For the cytotoxicity test, both proteins exhibited the concentration that inhibited metabolic activities of the cells by 50% (IC_{50}) of > 250 µg/ml. The calculated TI showed Pc and Pc/T beyond 6.49 and 1.94, respectively. Fig. 3 indicates 1A2 cells before and after treated with Pc and Pc/T and AZT. At 100X magnification, Fig. 3a shows normal 1A2 cells. After infection with the ΔTat/RevMC99 virus, the cells showed many syncytias (Fig. 3b). Pc and Pc/T treatments showed the reduction of syncytias which indicated in Fig. 3c and Fig. 3d, respectively.

Fig. 4 shows the Pc HIV-1 RT inhibitory activity. We also revealed the anti-HIV-RT inhibitory activity with an IC_{50}. Pc and Pc/T at concentrations of 50 µg/ml yielded an HIV-1 RT inhibitory effect beyond 90%. The highest Pc and Pc/T concentrations at 100 µg/ml demonstrated the inhibition percentages of 95.94% and 98.92%, respectively; whereas the positive 1-mM Doxorubicin control of this experiment yielded an inhibition percentage beyond 98%. Furthermore, we also confirmed by thermal denaturation that the Pc was an important substance. In order to prove that the Pc's HIV-RT inhibitory activity was due to plastocyanin, the Pc was denatured by boiling at 100 °C for 15 min and tested for its effect. We found that the denatured Pc protein was significantly less potent in inhibiting the HIV-RT activity: the non-denatured Pc protein at the concentration of 50 µg/ml exhibited potent inhibition of 91.97% IR; whereas the inactivated protein at the same concentration exhibited only 7.39% IR.

DISCUSSION

Plastocyanin is a part of the cupredoxin family, and it is recognized as a blue copper protein found in higher plants. Most studies on plant plastocyanin rely on leaf chloroplast isolates from spinach and poplar focusing on the electron-transfer and metal-uptake functions. Interestingly, certain cupredoxins, such as azurin, pseudoazurin, rusticyanin, and plastocyanin, are considered therapeutic agents for cancer and viral infection diseases [19–21]. However, our literature search revealed that there have been no studies on cupredoxin-related anti-HIV activity, especially for plant plastocyanins. Viral cycle-related cupredoxin studies demonstrated that rusticyanin and azurin could bind to the viral surface protein, although their inhibitory potential was not established [15, 20].

Natural anti-HIV compounds with potential applications, such as algal griffithsin, expressed in prokaryotic systems including *E. coli* have the advantage of being low-cost and allowing large-scale production [22, 23]. The first step in recombinant technology is the study of the encoding gene and characterization of its structure [24]. Most plastocyanins in the database were derived using automated computational analysis through gene prediction methods. In this study, we cloned and successfully identified the *C. indica* L.-derived plastocyanin gene from cDNA. We searched the resulting gene against the protein database and revealed the putative conserved domain of the cupredoxin superfamily. Our LC-MS/MS analysis ensured that the Pc protein expression of *C. indica* L. in *E. coli* was the correct reading frame. The peptide sequence containing 20 amino acids at the C-terminal was identified as plastocyanin by Swiss-Prot database. On the other hand, the CD spectra showed that the secondary structure foldings of Pc and Pc/T were different. However, both Pc and Pc/T had the same functionality. In our previous study, we reported the purified native

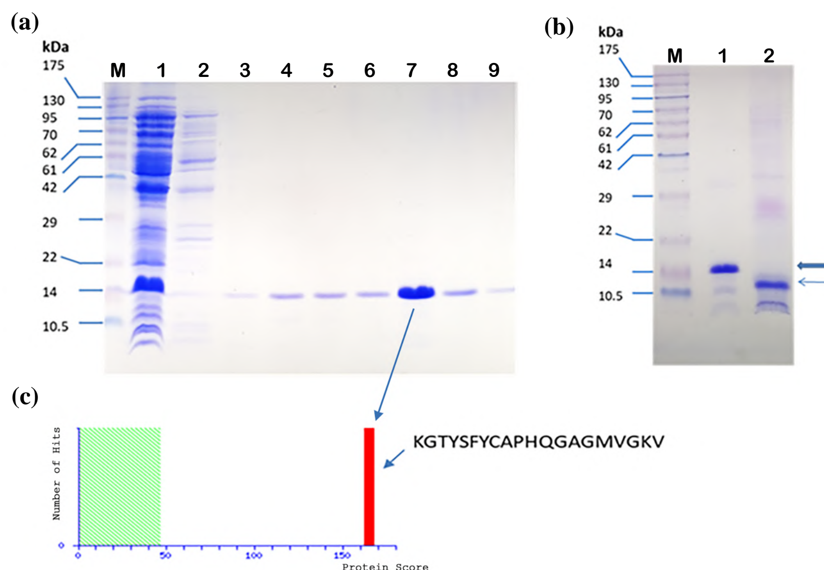

Fig. 2 Pc protein expression in pET28(+):*E. coli*.DE3(BL21). (a) Recombinant plastocyanin SDS–PAGE analysis. The crude protein was purified using the HiTrapTM chelating HP column and subsequently eluted using a gradient imidazole buffer. The protein fraction was subjected to SDS–PAGE and stained with Coomassie brilliant blue R-250. Lane M, molecular weight standard marker protein (Bio-Rad, USA); lane 1, crude recombinant protein; lane 2, protein eluted with 15% elution buffer; lanes 3–5, protein eluted with 20% elution buffer; and lanes 6–9, protein eluted with 25% elution buffer. The proteins were eluted with a stepwise imidazole gradient, respectively. (b) Thrombin protease cleavage of the recombinant fusion protein to remove his-tag and yield Pc/T. The purified recombinant fusion protein was cleaved by thrombin protease and then purified using the HiTrap™ chelating HP column. Lane 1, purified recombinant protein (Pc, thick arrow); lane 2, purified recombinant plastocyanin cleaved with thrombin protease (Pc/T, thin arrow). (c) Pc mascot score histogram. The arrow indicates the results of the recombinant plastocyanin protein score hit at 165 plastocyanin protein peptides from *Lactuca sativa*.

Table 1 Pc and Pc/T syncytium formation inhibition.

Protein sample	Syncytium reduction assay[a]			
	EC_{50} (µg/ml)	IC_{50} (µg/ml)	TI	Interpretation
Pc	38.54	> 250	> 6.49	Active
Pc/T	128.84	> 250	> 1.94	Active
AZT[b]	4.66×10^{-9} M	$> 10^{-8}$ M	> 0.46	Active

[a] The protein assay was performed at doses of 0.12–250 µg/ml.
[b] AZT was used as a positive control.

protein plastocyanin from *C. indica* L. with 10 kDa, an approximate molecular size obtained from the results of protein sequence analysis and the related protein families [13]. In this study, the heterologous *C. indica* L.-derived plastocyanin expression in *E. coli* produced the recombinant plastocyanin Pc with the expected size of 14 kDa. After cleavage with thrombin, the Pc/T was observed at a molecular weight of about 12 kDa on the SDS–PAGE. According to the protein sequence of Pc/T, containing 118 amino acids of plastocyanin with 17 additional amino acids residues from the expression vector at N-terminal, the Pc/T might be slightly larger than the native protein plastocyanin from *C. indica* L. Previous reports stated that N-terminal post-translational his-tag modifications could interfere the protein structure and might affect the protein function [25, 26]. Hence, our study also evaluated the anti-HIV activities of the thrombin-cleaved protein.

A syncytium reduction assay was developed to detect cell–cell fusions to mimic the host-cell viral infection. The assay was used to demonstrate the inhibition of HIV entry or binding to the target host cells [27]. In this study, Pc and Pc/T exhibited anti-HIV activities (based on the syncytium reduction assay) with EC_{50} values of 38.54 and 128.84 µg/ml, respectively. A previous report on a variety of Thai medicinal

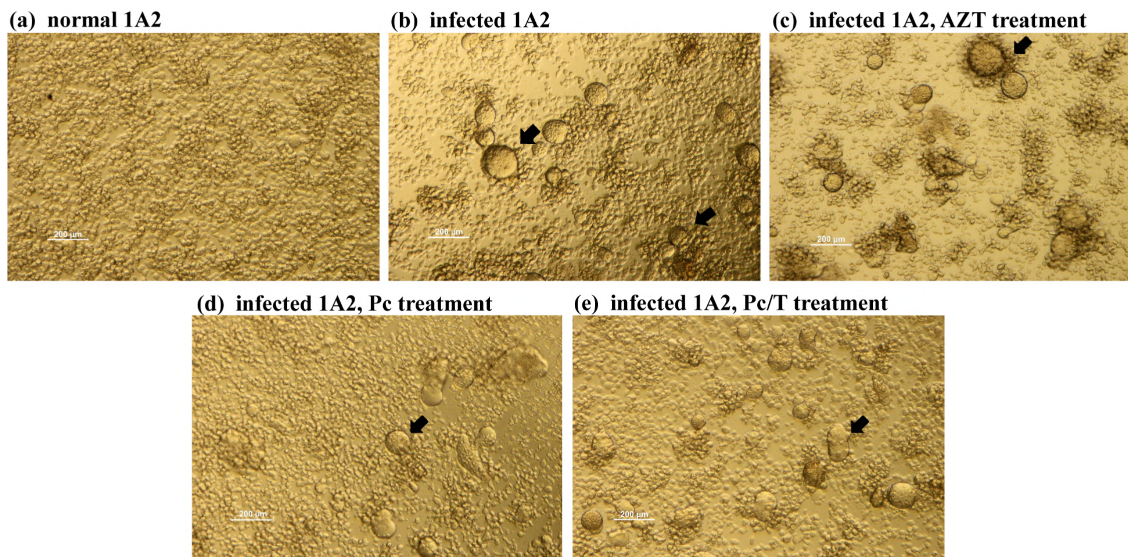

Fig. 3 Syncytium reduction assay of 1A2 cell after infection with the ΔTat/RevMC99 virus treated with different condition: (a), 1A2 normal cell; (b), syncytias of 1A2 cell after infection with the ΔTat/RevMC99 virus; (c–e), syncytias of 1A2 cell after infection with the ΔTat/RevMC99 virus and treated with AZT, Pc, and Pc/T, respectively; arrow indicates syncytium, scale bar: 200 µm.

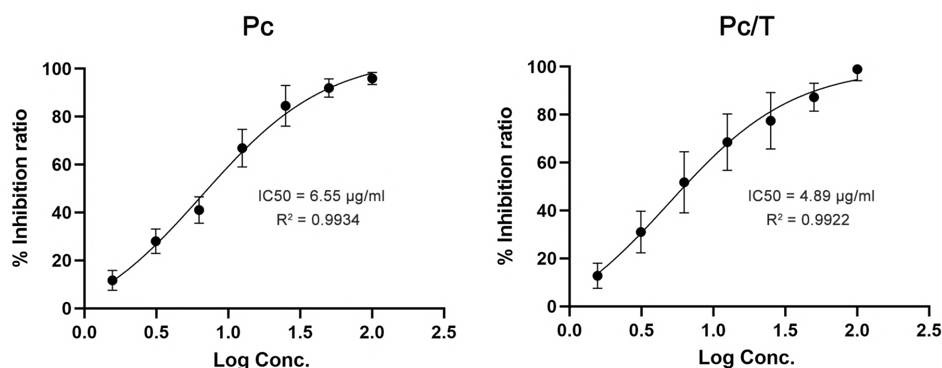

Fig. 4 Relationship between the HIV-RT inhibitory activity and log concentration of the recombinant plastocyanin protein, Pc, and Pc/T. Pc inhibitory activity against HIV-1RT was determined as a percentage of the inhibition ratio (% IR). The assay was performed in the dose range of 1.5625–100 µg/ml.

plants presented EC_{50} values ranging between 33.9 and 112.0 µg/ml [28]. Based on the EC_{50} obtained at a nontoxic concentration, the antiviral activity could be confirmed. The EC_{50} and TI values indicated potential activity that could inhibit syncytia formation in Pc and Pc/T, potentially settling the assumption for further plastocyanin and viral protein binding assays. Based on previous cupredoxin family-related anti-HIV activity studies, azurin and its derivatives demonstrated strong binding ability with gp120 resulting in HIV entry inhibition and growth suppression [15].

In addition, our previous study demonstrated additional anti-HIV-1 RT inhibitory activity-related mechanisms of the 10-kDa *C. indica* L. protein at the IC_{50} of 9.3 µg/ml [13]. In this study, Pc and Pc/T yielded IC_{50} values of 6.55 and 4.89 µg/ml, respectively. Both proteins exhibited dose-dependent inhibitory effects. At the highest concentration of 100 µg/ml, Pc and Pc/T displayed strong inhibitory effects of 95.94% and 98.92%, respectively. Several studies have screened for anti-HIV-1 RT activities among local medicinal plants. Kanyara and Njagi [29] found that the water extract of African medicinal plants exhibited anti-HIV-1 RT activities at IC_{50} of 4.0–20.0 µg/ml. Silprasit et al [30] reported that Asian medicinal plant hexane extracts displayed strong inhibitory activity with IC_{50} values of 56–146 µg/ml. Recently, Seetaha et al [31] demonstrated three potential hydrolysate peptides with high

HIV-1 RT inhibitory effect. Based on the results of this study, plastocyanin might possess both the entry and transcription inhibition anti-HIV activities. Therefore, we propose that the future aspects of artificial plastocyanin production in prokaryotic expression systems and recombinant protein efficacy must be examined.

In this experiment, we successfully expressed a recombinant protein with anti-HIV activity. However, it is important to note that our analysis, relying on the far-UV CD spectra and BeStSel, lacks the precision required to elucidate the intricacies of protein folding relative to functionality. Moreover, model analysis supports real-time CD spectroscopy studies and protein dynamics in different factors. Although we optimized the parameters of circular dichroism, further analysis, particularity the determination of the crystallization 3D structure, is essential to enhance our understanding.

CONCLUSION

We presented and heterologously expressed the *C. indica* L.-derived plastocyanin gene with anti-HIV activities in a prokaryotic system. As a result, the two anti-HIV property-related experiments showed that the Pc could play a role in syncytium reduction and HIV-1 RT inhibition. Further studies on the structural characteristics and their assumed underlying mechanisms should be performed. More experiments are necessary to demonstrate the effector mechanisms as this plastocyanin gained its interest as a multi-functional inhibitor, particularly HIV entry, protease and integrase strand transfer inhibitory activity

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.101.

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Appendix A. Supplementary data

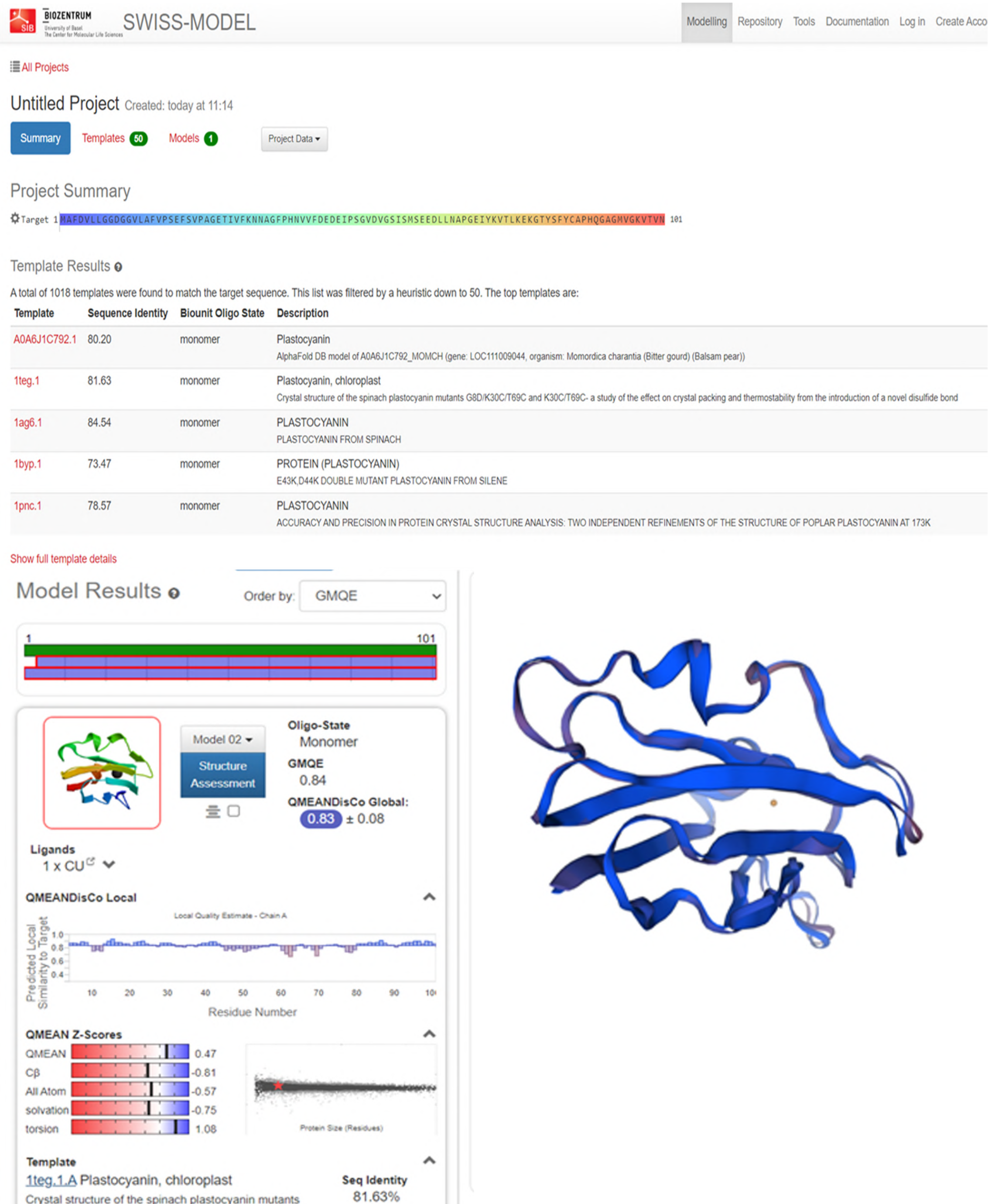

Fig. S1 Current evaluation for this version of SWISS-MODEL collected from 12 September 2023 output template search results of 101 amino acids recombinant alignment. The predict results of protein matching with 5 top-ranked show plastocyanin protein.