Molecular & Biochemical Parasitology 204 (2015) 51–63

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Biochemical characterization of plasmepsin V from *Plasmodium vivax* Thailand isolates: Substrate specificity and enzyme inhibition

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ARTICLE INFO

Article history:
Received 12 July 2015
Received in revised form 5 January 2016
Accepted 8 January 2016
Available online 12 January 2016

Keywords:
Export
Malaria
PEXEL
Plasmepsin V
P. vivax

ABSTRACT

Plasmepsin V (PMV) is a *Plasmodium* aspartic protease responsible for the cleavage of the *Plasmodium* export element (PEXEL) motif, which is an essential step for export of PEXEL containing proteins and crucial for parasite viability. Here we describe the genetic polymorphism of *Plasmodium vivax* PMV (*Pv*PMV) Thailand isolates, followed by cloning, expression, purification and characterization of *Pv*PMV-Thai, presenting the pro- and mature-form of *Pv*PMV-Thai. With our refolding and purification method, approximately 1 mg of *Pv*PMV-Thai was obtained from 1 g of washed inclusion bodies. Unlike *Pv*PMV-Ind and *Pv*PMV-Sal-1, *Pv*PMV-Thai contains a four-amino acid insertion (SVSE) at residues 246–249. We have confirmed that this insertion did not interfere with the catalytic activity as it is located in the long loop (R241-E272) pointing away from the substrate-binding pocket. *Pv*PMV-Thai exhibited similar activity to *Pf*PMV counterparts in which *Pf*EMP2 could be hydrolyzed more efficiently than HRPII. Substrate specificity studies at P_1' showed that replacing Ser by Val or Glu of the *Pf*EMP2 peptide markedly reduced the enzyme activity of *Pv*PMV similar to that of *Pf*PMV whereas replacing His by Val or Ser of the HRPII peptide increased the cleavage activity. However, the substitution of amino acids at the P_2 position with Glu dramatically reduced the cleavage efficiency by 80% in *Pv*PMV in contrast to 30% in *Pf*PMV, indicating subtle differences around the S_2 binding pocket of both *Pf*PMV and *Pv*PMV. Four inhibitors were also evaluated for *Pv*PMV-Thai activity including PMSF, pepstatin A, nelfinavir, and menisporopsin A—a macrocyclic polylactone. We are the first to show that menisporopsin A partially inhibits the *Pv*PMV-Thai activity at high concentration. Taken together, these findings provide insights into recombinant production, substrate specificity and inhibition of *Pv*PMV-Thai.

1. Introduction

More than 3 billion people worldwide are at risk of being infected with malaria and the disease led to 584,000 deaths in 2013 [1]. Five species of a protozoan parasite of the genus *Plasmodium* can cause malaria. Four of these, including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*, are human malaria species that are transmitted from person to person through mosquitoe bites of the genus *Anopheles*. Additionally, *Plasmodium knowlesi*, a species of simian malaria parasites, can also cause a significant number of cases of malaria in humans [2]. *P. falciparum* and *P. vivax* are considered as the greatest public health challenge because they are responsible for the most deaths from malaria [1]. In accordance with *Plasmodium* life cycle,

Abbreviations: DABCYL, 4-((4-(dimethylamino)phenyl)azo)benzoic acid; DMSO, dimethyl sulfoxide; EDANS, 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid; FRET, fluorescence resonance energy transfer; HAP, histo aspartic protease; HIV-1, human immunodeficiency virus-1; HRPII, histidine-rich protein II; *Pf*EMP2, *Plasmodium falciparum* erythrocyte membrane protein 2; *Pf*PMV, *Plasmodium falciparum* plasmepsin V; PMSF, phenyl-methylsulphonyl fluoride; PTEX, *Plasmodium falciparum* translocon of exported proteins; *Pv*PMV, *Plasmodium vivax* plasmepsin V.

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http://dx.doi.org/10.1016/j.molbiopara.2016.01.003
0166-6851/

P. vivax reveals unique biological features in that its sporozoites can develop into dormant hypnozoites in the liver. These hypnozoites can be activated months later and cause re-infections of the host's erythrocytes, leading to a clinical relapse [3]. Most of the *P. vivax* cases have been originated in Southeast Asia and the Western Pacific where several cases of chloroquine resistance in *P. vivax* have also been reported [4,5].

In the intra-erythrocytic stage, malaria parasites reside in a permanent parasitophorous vacuole (PV) closely surrounded by two membranes, the parasitophorous vacuole membrane (PVM) and the host erythrocyte membrane (EM) [6–8]. For parasite survival and proliferation, over 300 proteins are exported from the parasite to the host erythrocyte via the PTEX complex translocation [9,10]. These secreted proteins alter the mechanical and adhesive properties of infected erythrocytes, resulting in erythrocyte remodeling and ultimately leading to the destruction of the host erythrocyte [11]. The destruction of red blood cells are then responsible for the symptoms and death from malarial disease [12].

Erythrocyte remodeling and parasite export machinery have been recently reviewed [13], and two classes of the exported proteins have been identified [14]. The first class is known as *Plasmodium* export element (PEXEL), host-targeting signal (HT) or vacuolar transport signal (VTS), containing a pentameric motif, R-X-L-X-E/Q/D, typically found after ~35 amino acids of the N-terminal signal sequence for ER entry [15,16]. Based on the *P. falciparum* parasite genome, 463 proteins are predicted as PEXEL exportomes [17]. The malaria parasite employs the PEXEL motif to export proteins into the host erythrocyte by binding to phosphatidylinositol-3-phosphate (PI3P) in the inner leaflet of the ER membrane [18] and the PEXEL motif is subsequently cleaved by the ER-resident aspartic protease plasmepsin V (PMV) [19,20]. This enzyme recognizes the PEXEL motif and cleaves it after Leu, generating a -X-E/Q/D which is then acetylated by an unidentified enzyme, leading to the formation of Ac-X-E/Q/D [19–22]. In addition, the characterization of the PEXEL sequence reveals that the P_3-Arg and P_1-Leu residues are important for PMV cleavage and ER exit whereas the mutation of E/Q/D to Ala does not affect the N-terminal processing of the PEXEL motif but decreases the level of exported proteins to PV [17,23]. PMV also cleaves several exported proteins encoded by multigene families such as repetitive interspersed family (RIFIN), subtelomeric variable open reading frame family (STEVOR) and right-infected erythrocyte surface antigen (RESA). Interestingly, RESA proteins possess a relax PEXEL motif (R-X-L-X-X-E/Q/D) [24]. Unlike the PEXEL proteins, the second class of the exported proteins is known as PEXEL-negative exported proteins (PNEPs) [25]. PNEPs lack a signal sequence so they do not undergo the N-terminal processing by PMV. However, the first 20 amino acids of PNEPs without a classical N-terminal signal peptide are sufficient to promote the export and the exported protein requires unfolding to get across PV into the host cell [26]. In accordance with PNEPs, a surface adhesion and major virulence determinant erythrocyte membrane protein 1 (*Pf*EMP1) contains PEXEL-like sequence, K-X-L-X-E/Q/D while other proteins of PNEPs such as a ring-exported protein 1 (RXP1) [27] and skeleton binding protein 1 (SBP1) do not have the PEXEL motif entirely; therefore, these proteins cannot be cleaved by PMV [17]. It has been shown recently that the exported protein of PNEPs can drive export by other means than PMV cleavage of a PEXEL motif [24,28]. In light of its importance for parasite survival, PMV is considered as a new target for antimalarial therapy. Some HIV-1 protease inhibitors can partly inhibit the activity of *Pf*PMV on PEXEL cleavage of *Pf*EMP3 and KAHRP [20], while some small molecules of PEXEL mimetic inhibitors can potently block the activity of PMV from *P. falciparum* and *P. vivax* [29]. Recently, the crystal structure of *Pv*PMV in complex with the inhibitor WEHI-842 was published at 2.4 Å resolution [30]. WEHI-842 was developed based on the previous inhibitor, WEHI-916 [29,31]. WEHI-842 exhibited the IC_{50} values of 0.2 nM and 0.4 nM against *Pf*PMV and *Pv*PMV, respectively. *Pv*PMV contains three unusual features including (i) the helix-turn-helix motif, which is conserved only within orthologs of *Plasmodium* species, (ii) the nepenthesin loop, and (iii) an unpaired cysteine residue (C140) located in the β-flap [30,32]. With the crystal structure of *Pv*PMV in hand, medicinal chemistry efforts were made to improve the physiochemical properties of PMV inhibitors, aiming to generate compounds with enhanced permeability and greater inhibition effects [31,33]. Therefore, the identification of promising drug targets, which are shared between multiple *Plasmodium* spp., is urgently needed to control malaria parasites.

Here, we examined the genetic polymorphism of the *P. vivax* PMV-gene isolated from Thailand. With its unique sequence, we produced the soluble recombinant *Pv*PMV-Thai and developed a protocol for unfolding and refolding of *Pv*PMV-Thai in order to obtain a large amount of the active refolded-enzyme. In addition, studies on the kinetic parameters of *Pv*PMV-Thai as well as substrate specificity and inhibition study were conducted. Taken together, these findings provide useful information especially on S_2 and S_1' subsites for further structural studies and inhibitor design for *Pv*PMV-Thai.

2. Materials and methods

2.1. Cloning and sequencing of Pv*PMV genes*

Ten samples of *P. vivax* genomic DNA of Thai isolates (named MTM1578– MTM1587) were obtained from Associate Professor Mallika Imwong, Faculty of Tropical Medicine, Mahidol University, Thailand. The full-length of *pvpmv* gene was amplified from the *P. vivax* genomic DNA using polymerase chain reaction (PCR) with primers *Pv*PMV-For (5′-AATTGGATCCGCTAGCATGGTCGGAGCGAGCTTG-3′) and *Pv*PMV-Rev (5′-AATTCTCGAGCTACGCATCCGCGGGCGC-3′). PCR thermocycling conditions were as follows: 95 °C for 5 min; 25 cycles of 95 °C for 1 min, 55 °C for 40 s and 72 °C for 3.3 min; and a final heating at 72 °C for 5 min. *Pv*PMV-Thai PCR products were gel-purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany), ligated into pGEM-T Easy vector (Promega, USA). Subsequently, *Escherichia coli* DH5α chemical competent cells were transformed with the resulting constructs (pGEM-T-MTM1580, pGEM-T-MTM1584, and pGEM-T-MTM1587) using the heat shock method. The sequences of the *Pv*PMV-Thai isolates were verified by DNA sequencing using T7 and SP6 primers (Macrogen, South Korea).

Three nucleotide sequences of *Pv*PMV-Thai isolates were submitted to NCBI GenBank (accession numbers KT000395 to KT000397) for public domain use. Subsequently, the encoded amino acid sequences from each *Pv*PMV-Thai construct were aligned with other known amino acid sequences of *Pv*PMV from NCBI GenBank, including *Pv*PMV-Salvador (Sal-1) (GenBank, XM_001615583), *Pv*PMV-Korea (KOR-13) (GenBank, AY584111), and *Pv*PMV-India (Ind) (GenBank, GU569935) using Clustal Omega.

2.2. Construction of recombinant Pv*PMV*

According to the sequence analysis of *Pv*PMVs, a plasmid, pGEM-*Pv*PMV-Thai (MTM1587) was used as a template for the amplification of *pvpmvp-thai* and *pvpmvm-thai* genes using either *Pv*PMVp-Thai-For (5′-AATTGGATCCGCTAGCAGAAGCGAGTCAACGGAG-3′) or *Pv*PMVm-Thai-For (5′-GGAAGGATCCGCTAGCCTCCTCTACAAGTACAAG-3′), together with *Pv*PMVp,m-Thai-Rev (5′-GGCCCTCGAGCTATTTGTACTCATTATAAGTCCTCGG-3′) primers to produce *PvPMVp-Thai* and *PvPMVm-Thai*, respectively. The