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Synthetic peptides derived from the C-terminal 6 kDa region of *Plasmodium falciparum* SERA5 inhibit the enzyme activity and malaria parasite development

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ABSTRACT

Background: Plasmodium falciparum serine repeat antigen 5 (PfSERA5) is an abundant blood stage protein that plays an essential role in merozoite egress and invasion. The native protein undergoes extensive proteolytic cleavage that appears to be tightly regulated. PfSERA5 N-terminal fragment is being developed as vaccine candidate antigen. Although PfSERA5 belongs to papain-like cysteine protease family, its catalytic domain has a serine in place of cysteine at the active site.

Methods: In the present study, we synthesized a number of peptides from the N- and C-terminal regions of PfSERA5 active domain and evaluated their inhibitory potential.

Results: The final proteolytic step of PfSERA5 involves removal of a C-terminal ~6 kDa fragment that results in the generation of a catalytically active ~50 kDa enzyme. In the present study, we demonstrate that two of the peptides derived from the C-terminal ~6 kDa region inhibit the parasite growth and also cause a delay in the parasite development. These peptides reduced the enzyme activity of the recombinant protein and co-localized with the PfSERA5 protein within the parasite, thereby indicating the specific inhibition of PfSERA5 activity. Molecular docking studies revealed that the inhibitory peptides interact with the active site of the protein. Interestingly, the peptides did not have an effect on the processing of PfSERA5.

Conclusions: Our observations indicate the temporal regulation of the final proteolytic cleavage step that occurs just prior to egress.

General significance: These results reinforce the role of PfSERA5 for the intra-erythrocytic development of malaria parasite and show the role of carboxy terminal ~6 kDa fragments in the regulation of PfSERA5 activity. The results also suggest that final cleavage step of PfSERA5 can be targeted for the development of new anti-malarials.

1. Introduction

Recent advances in the malaria control efforts have been promising and have helped in preventing around 1 million deaths, in the past decade, according to the recent data [1]. World malaria report estimated 216 million malaria cases and approximately 660,000 malaria deaths in year 2010, suggesting that malaria is still a serious disease prevalent in the tropics [1]. The emergence of drug resistant parasites from time to time poses a constant challenge to combat the disease. Continuous efforts are thus required to understand the biology of the parasite at the molecular level, which may help in identifying novel vaccine candidates or new drug targets.

Egress of merozoites from the blood stage schizont is an important event in the asexual stages of the parasite life cycle. Merozoites thus released from the infected RBCs invade fresh erythrocytes resulting in propagation of the disease. In *Plasmodium falciparum*, a number of proteases have been suggested to play a key role in the process of egress by degradation of parasite and RBC membranes [2]. One of the major families of proteases known to be involved in this process, in *P. falciparum*, is a large multigene family of serine repeat antigens (SERAs). The *falciparum* SERA family consists of nine members, out of which *sera*1–8 genes are arranged on chromosome 2 in a tandem array and *sera*9 gene is arranged on chromosome 9 [3]. All the members of the family have a papain protease like central domain and are transcribed at trophozoite as well as schizont stages [4–7]. Two members of this family, PfSERA5 and 6, are

Abbreviations: SERA, serine repeat antigen; SUB1, subtilisin1; hpi, hours post invasion; kDa, kilodalton

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refractory to deletion and appear to be essential for parasite growth and viability [7–9].

PfSERA5 is one of the most abundant proteins in the schizont stage of the parasite [6,10] and a well studied member of this family. It is one of the promising asexual blood stage vaccine candidates and therapeutic drug target [11]. PfSERA5 could induce antibodies that either protected against blood stage infection in vivo [12] or inhibited parasite replication in vitro [13]. Recombinant proteins made from the N-terminal 47-kDa domains of PfSERA5 have been shown to be immunogenic and elicited antibodies that inhibited erythrocyte invasion and parasite replication in vitro as well as in vivo in rodent and primate models [14–17]. In addition, serum antibody titer against PfSERA5 in Ugandan adult sera from a malaria endemic area correlated with the protection against malaria symptoms and sera from these patients were effective in in vitro parasite growth inhibition [4,18,19]. Recently, BK-SE36 malaria vaccine was shown to confer malaria symptomatic protection over 70% for one year in Ugandan children [19]. Also, inhibitors of PfSUB1, an enzyme involved in processing of PfSERA5 have been shown to stall the process of merozoite egress and parasite maturation [20,21]. Together, all these studies emphasize the role of PfSERA5 in parasite growth, maturation and its potential as a drug target.

Phylogenetic and evolutionary studies of SERA genes from eight *Plasmodium* species have revealed that SERA genes can be categorized into four groups [22,23]. SERA proteins from groups I to III possess cysteine residue in their catalytic site, while members of group IV posses serine residue at that position [22]. In *P. falciparum* SERA1–5 and 9 have serine and SERA 6–8 have cysteine at the key catalytic position. PfSERA5 is approximately a 120 kDa protein, which undergoes proteolytic processing. It is cleaved into a 47 kDa N terminal domain, a central 56 kDa and 18 kDa C terminal fragments. 47 kDa and 18 kDa domains remain linked via a disulfide bond [24]. In certain alleles of SERA5, the 47 kDa domain is further processed into two 25 kDa fragments [25], both of which remain linked to the 18 kDa fragment. The 56 kDa domain is further cleaved to a 50 kDa catalytically active domain having a chymotrypsin like activity [26] and a 6 kDa fragment [24,27,28] which are shed into the culture supernatant after schizont rupture. This cleavage is sensitive to leupeptin and E-64 inhibition. It was recently speculated that this additional processing of P56 to P50 may serve as a proteolytic inactivation step [2].

The proteolytic activity of PfSERA5 has been an issue of continuous debate among different groups because of replacement of cysteine-to-serine within the catalytic triad of the protein. A single report has demonstrated the chymotrypsin-like activity of *Escherichia coli* expressed recombinant SERA5 central domain [26]. This final processing event, cleavage of 56 kDa domain to 50 kDa, is an enigma and significance of its cleavage just prior to egress is an important mechanism that needs to be studied. In the present study, we provide evidence for the enzymatic activity of PfSERA5 ~ 50 kDa protein fragment (PfSERA5P_{50}) and its inhibition by two peptides derived from the 6 kDa fragment. These peptides significantly inhibited the growth and development of *P. falciparum* parasites in vitro as well as co-localized with the protein in the parasitophorous vacuole. These results thus have implications in understanding the tight temporal regulation of PfSERA5 proteolytic activity and the importance of the final cleavage step just before release of the merozoites.

2. Results

2.1. Design and synthesis of peptides derived from the PfSERA5 sequence

We have previously shown that peptides derived from pro-region of falcipain-2 can block the activity of the parasite cysteine protease [29]. To identify similar sequence(s) for the SERA5 protein, we selected and synthesized nine different peptides across its entire length (Fig. 1). The peptides were designed based primarily on three criteria. First, the peptide sequences were selected either from the unique serine rich region of PfSERA5 (SE5 P4, P5 P6) or from the unique region of serine rich stretch from the full length protein (SE5 P9). Secondly, the peptides were designed from the regions flanking the various processing sites of the protein (SE5 P5, P7, P8) (Additional File 1) [2]. Finally, peptides SE5 P1, P2 and P3 were synthesized to span the entire 6 kDa region from the 56 kDa segment of PfSERA5 that gets cleaved to generate an active 50 kDa protein. Sequences of each peptide used in the present study are shown in Table 1.

2.2. Effect of PfSERA5 derived peptides on P. falciparum development and growth

We next evaluated the biological activity of PfSERA5 derived peptides by assessing their effects on parasite growth, development and invasion. Parasite cultures were incubated with various peptides at a concentration of 50 μM and parasite smears were examined by microscopy and flow cytometry ~20 hpi to assess new ring formation. As shown in Fig. 2a, peptides SE5 P1 and SE5 P2 (derived from the C-terminal 6 kDa region of the P56 domain of PfSERA5) inhibited the parasite growth and invasion (~60–70%) considerably in the first cycle. In comparison, peptide SE5 P3, also from the C-terminal 6 kDa region, did not affect the parasite invasion. Peptides from other domains especially the N-terminal domain (SE5 P5 and SE5 P7) as well as from the extreme C-terminal region (SE5 P9), reduced the parasitemia only by ~30–45%. They were half as effective as peptides SE5 P1 and SE5 P2. Peptide SE5 P8 did not show any effect on the parasite culture. Since the PfSERA5 protein has been known to express at late stage of the intra-erythrocytic cycle [5], we next assessed the effect of peptides SE5 P1 & SE5 P2 on parasite growth in culture by incubating these peptides with late trophozoite/early schizont stages. The addition of either of these peptides to doubly synchronized culture of *P. falciparum* 3D7 parasites (30–34 h) resulted in the accumulation of late stage parasites

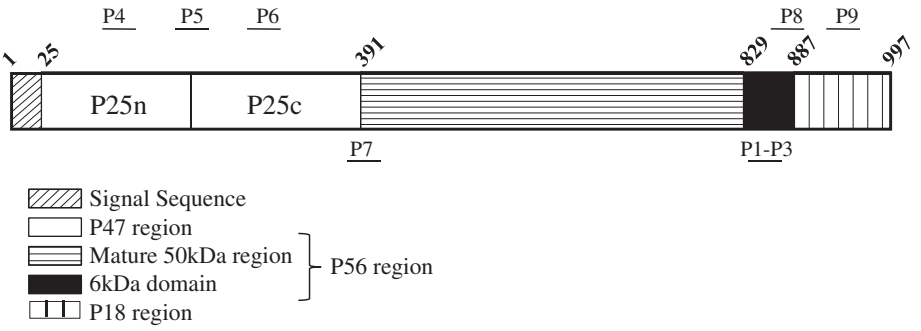

Fig. 1. Schematic representation of full length PfSERA5 protein. The numbers above indicate the amino acid positions defining the various domains of the protein. The peptides used in the study are marked across the length of the protein in the region from which they are derived.

Table 1
Amino acid sequences of the synthesized peptides, derived from PfSERA5, used in the study.

Peptides	Amino acid sequence	Position	Mol. mass (Da)
SE5 P1	Ac-TTKKESKIYDYYL-NH_2	830–842 aa	1821
SE5 P2	Ac-KASPEFYHNLYFKNF-NH_2	843–857 aa	1946
SE5 P3	Ac-NVGKKNLFSEKEDN-NH_2	858–871 aa	1663
SE5 P4	Ac-KKYVKLPSNGTTGEQ-NH_2	176–190 aa	1691
SE5 P5	Ac-GSSTGTVRGDTEPISDS-NH_2	191–207 aa	1707
SE5 P6	Ac-SESLPANGPDSPTVK-NH_2	233–247 aa	1540
SE5 P7	Ac-FKEIKAETEDDDEDDY-NH_2	384–399 aa	2003
SE5 P8	Ac-YIIFGQDTAGSGQSGK-NH_2	881–896 aa	1670
SE5 P9	Ac-TALESAGTSNEVSERV-NH_2	900–915 aa	1620

(schizonts) that had not ruptured even 6–8 h after the detection of ring stage parasites in the control cultures (Fig. 2b). We further investigated the delay in rupture of the late stage parasites by microscopically examining these treated parasites at 2 h interval up to 14 h after the rupture of control parasite cultures (i.e. those treated with SE5 P3 or PBS). Over most of the time course, the number of accumulated late-stage parasites in culture remained significantly greater than those detected in control. Fraction of ring stage and late stage parasites were calculated at each time point for control as well as peptide treated cultures. Peptide treated wells showed delay in schizont rupture and more than one third of the total number of parasites were seen in the schizont stage even 6 h post rupture, as compared to the control wells (Fig. 2c & d). Interestingly, there was no major phenotypic defect observed in these schizonts. They formed normal number of intracellular merozoites when compared with the control.

2.3. SE5 P1 & SE5 P2 peptides specifically inhibit PfSERA5P_{50} activity

To investigate, whether the delay in rupture effect of SE5 P1 or SE5 P2 peptides on parasite development was due to their ability to block the enzymatic activity of PfSERA5, we expressed the PfSERA5P_{50} clone obtained from Crabb group, Melbourne Australia [26]. The protein was expressed and purified as described by Hodder et al. with minor modifications. Fig. 3a & b shows the expression, purification and western blot analysis of the recombinant PfSERA5P_{50} domain. The activity of the recombinant protein was analyzed using synthetic substrate, Suc-LLVY-AMC. The recombinant PfSERA5P_{50} was effectively able to hydrolyze the substrate in a dose dependent manner with a V_{max} of

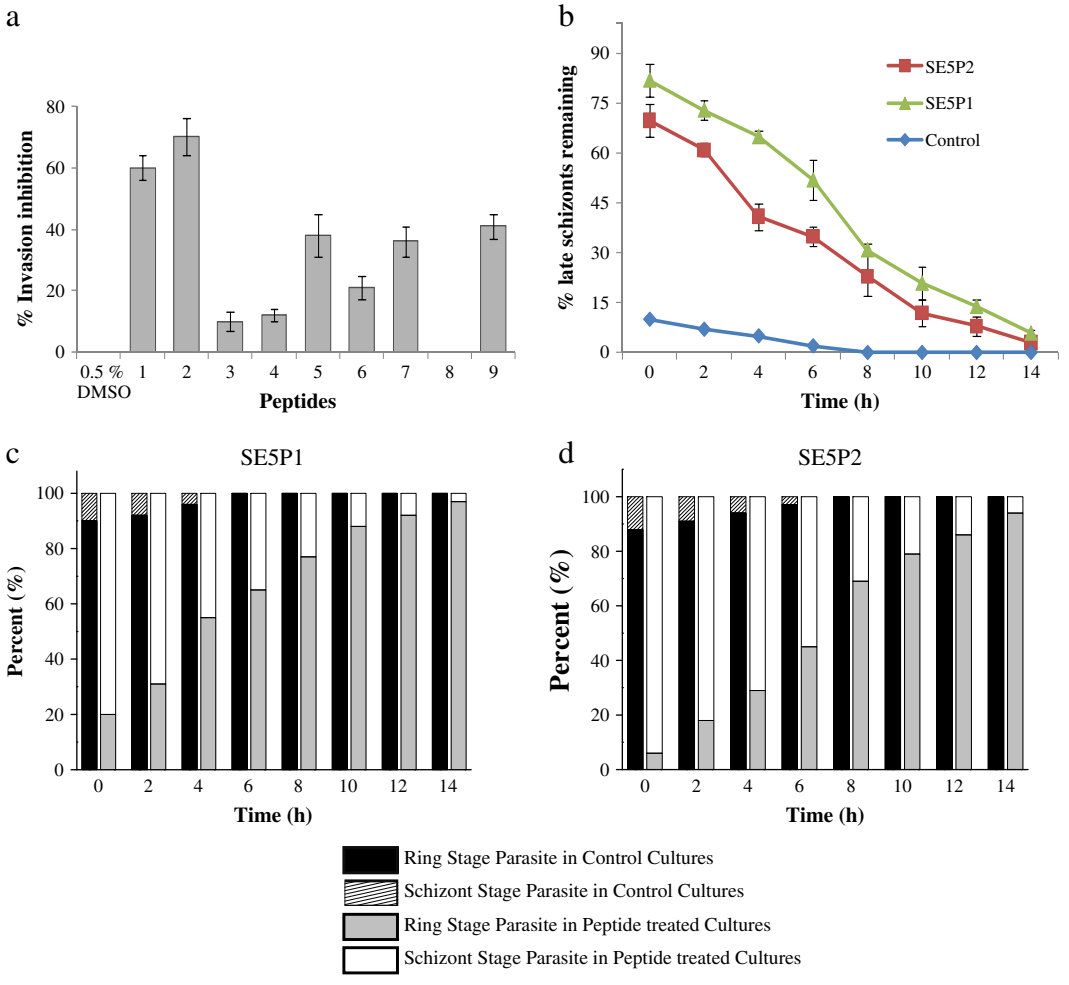

Fig. 2. Effect of SE5 P1 and SE5 P2 on *Plasmodium falciparum* growth and development. a) Growth inhibition assays to study the effect of peptides SE5 P1–SE5 P9 on parasite growth. Parasite culture at late trophozoite/early schizont stage was incubated with 50 μM of each peptide. After 48 h, parasitemia was determined for treated cultures as compared to the control samples. % invasion inhibition was calculated as described in the Materials and methods. The mean and SEM for each data point were determined from at least three triplicate experiments. b) Time course analysis of the effect of SE5 P1 and SE5 P2 on late stage parasite numbers. *Plasmodium falciparum* culture was grown in the presence of the peptides SE5 P1 and SE5 P2 and was analyzed periodically for the presence of late stage parasite. The time at which ring stage parasite first appeared in the control cultures was taken as 0 h. The cultures were analyzed every 2 h for 14 h from this time point and the number of late stage parasites and ring stage parasites was noted. The mean and SEM for each data point were determined from at least three triplicate experiments. Percent schizonts and rings present in the control vs. SE5 P1 (c) and SE5 P2 treated (d) cultures at various time points. 0 h represents the point when rings start appearing in the control cultures. ▨ and □ represent the late stage parasite in the control well and treated wells respectively, ■ and ▭ represent the ring stage parasite in the control and treated wells respectively.

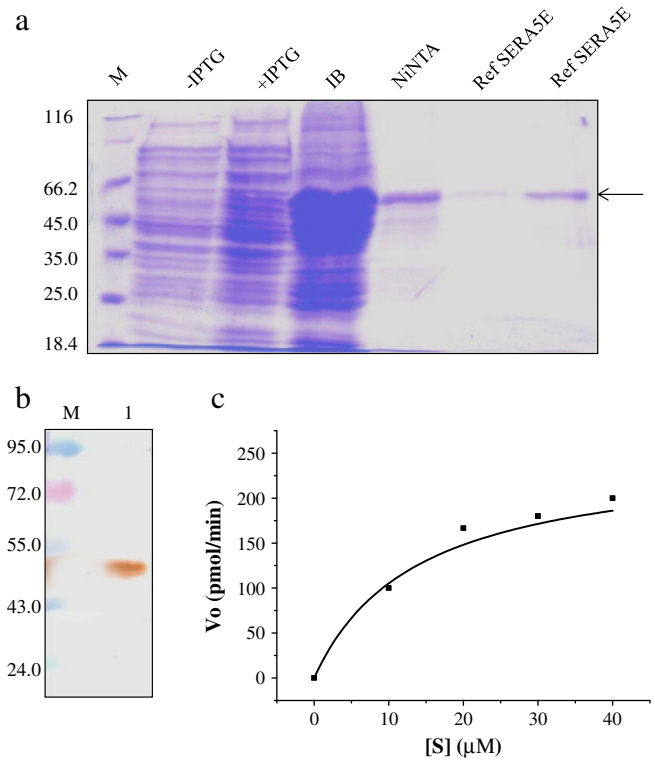

Fig. 3. Expression, purification and activity analysis of recombinant $PfSERAP_{50}$. a) Coomassie blue stained 10% SDS-PAGE analysis for Ni^{2+}–NTA purified recombinant $PfSERAP_{50}$ protein. Samples loaded are as follows: uninduced (−IPTG), induced (+IPTG), washed SERA5 inclusion bodies (IB), eluent from Ni NTA column using denaturing buffers (NiNTA), and refolded and concentrated protein (refSERA). b) Western blot analysis of recombinant purified and refolded SERA5E. c) Michaelis–Menten kinetics for the enzymatic activity of recombinant SERA5E.

250 pmol/min and K_m of 13.75 μM (Fig. 3c & Table 2). The recombinant protein lost its activity upon heat denaturation. We also cloned, expressed and purified an active site mutant of $PfSERA5P_{50}$ protein (Additional File 2a).

The effect of SE5 P1, SE5 P2 & SE5 P4 peptides on the enzymatic activity was analyzed by pre-incubating the active $PfSERA5P_{50}$ with each of these peptides, before the addition of the substrate. SE5 P1 as well as SE5 P2 peptides significantly inhibited the proteolytic activity of $PfSERA5P_{50}$, while the SE5 P4 peptide did not affect the enzymatic activity of $PfSERA5P_{50}$ at all. This inhibition was specific to the PfSERA5 protein as the enzymatic activity of recombinant falcipains 2 and 3 was unaffected by these peptides even at higher concentrations and larger incubation time (Fig. 4a & b). To rule out any contamination by the bacterial proteases during purification catalytic serine residue was replaced by an alanine using site directed mutagenesis. Mutated protein did not show any protease activity (Fig. 4a). Inhibition of the enzyme activity by SE5 P1 and SE5 P2 peptides increased with time and peptide concentration (Fig. 4c–f). To determine the mechanism of inhibition double reciprocal plot was drawn and analyzed. Steady state kinetic analysis yielded a plot with an intersection at y axis which confirms that peptides SE5 P1 and P2 act as the competitive inhibitors for the enzyme (Fig. 4g). Together, these results suggested that two C-terminal PfSERA5 peptides affect the enzymatic activity of $PfSERA5P_{50}$ protein that in turn blocks the development of the parasites in an in vitro culture.

Table 2
Kinetic constants.

V_{max} (pmol/min)	K_m (mM)
250	13.75

2.4. Molecular docking of the inhibitory peptides with the P50 domain of PfSERA5

To further provide insights into the action of SE5 P1 and P2 peptides, we carried out molecular docking studies of these peptides with the known crystal structure of PfSERA5. In spite of the large number of degrees of freedom considered to take into account the ligand flexibility, the complexes obtained by docking studies were characterized by the good sterical complementarities between PfSERA5 and peptides (Fig. 5). In order to clarify the higher inhibition activity of SE5 P1 and SE5 P2 peptides, the docking study was extended including Suc-LLVY-AMC peptide (substrate used to study the enzyme activity of the protein) to compare its pose to the results obtained for the other peptides. In particular, the Suc-LLVY-AMC–SERA5 complex (Fig. 5a) is stabilized by a network of hydrogen bonds respectively between the succinic moiety, the carbonyl of Tyr4 backbone, the hydroxyl of Tyr4 of the docked peptide and the hydroxyl of Tyr703, the hydroxyl of Ser641, and the carboxyl group of Glu638 of PfSERA5. Moreover, the complex includes a noticeable ionic interaction between the carboxyl group of the succinic moiety and ammonic group of Lys701. Analyzing the binding mode of active peptides, SE5 P1 (Fig. 5b) interacts with all four residues of Suc-LLVY-AMC, whereas SE5 P2 (Fig. 5c) involves only three residues, excluding Ser641 that is replaced by Ser640, that is very close and can be considered spatially and sterically equivalent. The inactive peptides, SE5 P4, P5, P8 and P9, don't interact with both Ser640 and Ser641 that seem very important to bind the catalytic site. Moreover, although SE5 P6 and SE5 P7 can do one H-bond respectively with Ser641 and Ser640, both are unable to interact with Glu638. From these results, it seems that, to have a good inhibition activity, the peptide must interact with Glu638 and Ser640. Ser640 and Ser641 are interchangeable due to their proximity.

In order to understand better how the proposed peptides can bind to the SERA5 active site, the volume shared between each of them and Suc-LLVY-AMC was calculated, assuming that this substrate is interacting in the best way to be hydrolyzed and keeping the same poses and conformations selected by docking studies. More in detail, Table 3 shows that the most active peptides (SE5 P1 and P2) share the higher volume with Suc-LLVY-AMC (respectively 50.1 and 57.5%) and it means that they occupy the higher space in which the substrate is placed. On the other hand, although SE5 P1 and SE5 P2 have a different binding mode, they are the peptides that share the higher number of interacting residues and the higher binding space with Suc-LLVY-AMC.

2.5. Uptake of labeled SE5 P1 and SE5 P2 peptides by P. falciparum infected RBCs and their localization

To assess the uptake of SE5 P1 and SE5 P2 peptides by the infected erythrocytes and their binding to PfSERA5 inside the cell, we performed co-localization studies in the peptide treated parasites. To do so, *P. falciparum* cultures were synchronized and treated with N-terminal biotinylated SE5 P1 and SE5 P2 peptides. Importantly, both of these peptides did enter the infected erythrocytes and co-localized nicely with the PfSERA5 staining in the parasitophorous vacuole, thereby indicating the binding of these peptides with the PfSERA5 protein (Fig. 6a). No such staining was seen in uninfected erythrocytes. In addition, cells treated with DMSO alone did not show any green fluorescence as well. To assess the effect of biotinylated peptides on SERA5 protease activity, peptides were incubated with the recombinant protein as described above. Peptides were found to inhibit the protease activity similar to the non-biotinylated peptides (Additional file 2b).

2.6. SE5 P1 and SE5 P2 peptides do not interfere with the processing of the PfSERA5 protein

It has been shown that the inhibitors of PfSUB1 or DPAP3 block SERA5 processing and rupture of late schizonts [21]. We therefore

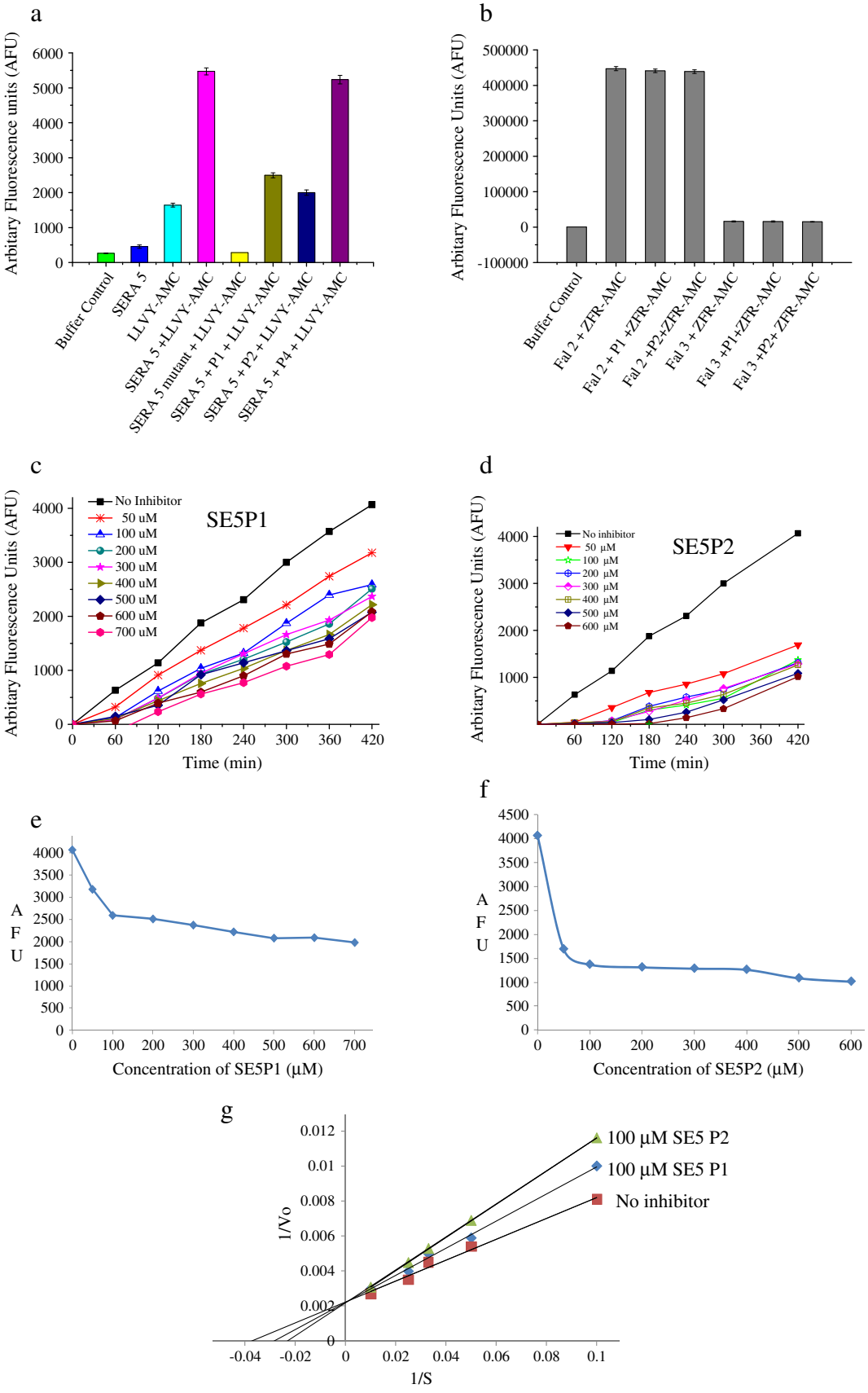

Fig. 4. Effect of the peptides; SE5 P1 and SE5 P2 on the activity of recombinant enzyme (a) SERA5 and on (b) falcipain-2 and falcipain-3. Dose dependent activity profile of recombinant SERA5 incubated with varying concentrations of (c and e) SE5 P1 and (d and f) SE5 P2. (g) Inhibition kinetics of recombinant PfSERA5P$_{50}$. Double reciprocal plot of 1/S vs. 1/Vo.

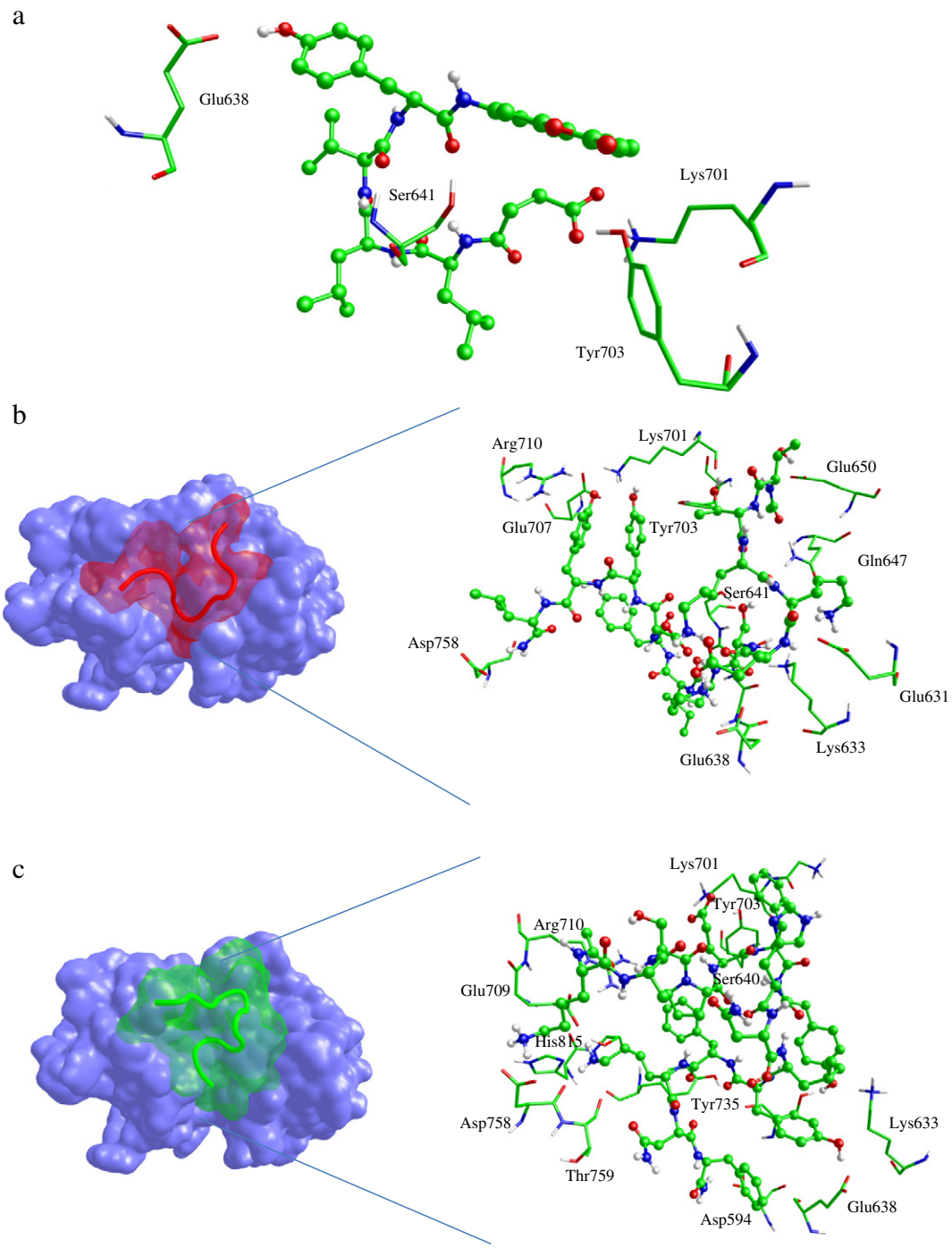

Fig. 5. Molecular docking of SE5 P1 and SE5 P2 peptides with PfSERA5. a) Main interactions of Suc-LLVY-AMC–SERA5 complex. b) & c) Complexes of PfSERA5 with the two inhibiting peptides; b) SE5 P1 c) SE5 P2 with more than 50% inhibition activity. The figures show the main interactions between the binding site and the peptide residues.

examined whether the delay in rupture by SE5 P1 or SE5 P2 peptides in the treated parasite culture is due to blockage in processing of the PfSERA5 protein. We analyzed the composition of SERA5 species in the supernatant obtained from the cultures treated with SE5 P1 and SE5 P2 as well as untreated peptides. As shown in Fig. 6b, no change in the relative abundance of SERA5 species was observed between the treated and untreated cultures. Together these results suggested that two peptides from the C-terminal end of the 56 kDa PfSERA5 protein arrest the late stage intra-erythrocytic development by blocking the enzymatic activity of the protein.

3. Discussion and conclusions

Plasmodium SERAs are a family of peripheral cysteine-like proteases that are potentially important for the host cell rupture and merozoites egress [21] and it has been a challenge to pin point the role(s) of each SERA member at various stages of parasite development. A single report has experimentally demonstrated the protease activity for PfSERA5 [26], however, the proteolytic activity of this protein has been the subject of contention because of two main reasons; 1. Experimental Cys to Ser substitution in papain [30] and Cathepsin L [31] abolishes or

Table 3
Shared volume in percentage between each considered peptide and Suc-LLVY-AMC substrate. In this calculation, all ligands are kept in the poses and conformations selected by docking studies.

Peptides	Act > 50%	Shared volume %
SE5 P1	1	50.1
SE5 P2	1	57.5
SE5 P3	0	27.9
SE5 P4	0	41.6
SE5 P5	0	19.9
SE5 P6	0	43.5
SE5 P7	0	45.1
SE5 P8	0	40.2
SE5 P9	0	39.2

reduces the protease activity of these enzymes. 2. X-ray crystallographic structure of PfSERA5 revealed aberrant features that cast doubts on its ability to interact with polypeptide substrates [32]. The role of PfSERA5 in parasite development/or egress has been highlighted in a number of studies that used inhibitors of serine proteases, cysteine proteases and selective inhibitors of PfSUB1, an enzyme known to carry-out the processing of PfSERAs [21]. However, these inhibitor studies indirectly suggested a role of PfSERA5 in parasite egress as many serine proteases as well as cysteine proteases are expressed at schizont stage.

PfSERA5 undergoes proteolytic processing with specific cleavage steps that are inhibited by distinct protease inhibitors [2]. However significance of these cleavage steps in parasite development has not been evaluated. To reaffirm the proteolytic nature of PfSERA5 and to evaluate further its role in parasite development at asexual blood stages, we designed a series of peptides across the length of the PfSERA5 protein and tested their effects on PfSERA5 protease activity as well as on parasite development/growth. Similar approaches, particularly the peptides from pro-region of a protein have been previously used to define the proteolytic activity of an enzyme [29,33]. The rationale for designing these peptides was based on the facts that these peptides may act as competitive inhibitors for PfSERA5 processing or they may block the protease activity of the enzyme. Of the nine peptides designed in the present study, the two peptides from the ~6 kDa C-terminal region of ~56 kDa segment that gets cleaved off during final processing showed a significant inhibition in the parasite growth and PfSERA5 enzyme activity. In our study the effects were specific, as these peptides did not affect the enzyme activities of falcipain-2 as well as falcipain-3 and also the peptides from the N-terminal region of PfSERA5 did not have any effect on PfSERA5 activity. These results are in line with a previous observation which suggested that cleavage of the 6 kDa region is essential for the proteolytic activity of PfSERA5 [2]. SE5 P5, P7 and P9 also showed around 50% inhibition. They belong to the P47 and P18 fragments of SERA5 which are known to inhibit parasite growth [19a]. This would explain the inhibition by these peptides. Hence, although significant, the inhibition by these peptides was not so intriguing as P1 and P2. While peptide P8 had no effect, the not so (linearly) distant P9 inhibited parasite considerably. This is due to the fact that these two peptides are

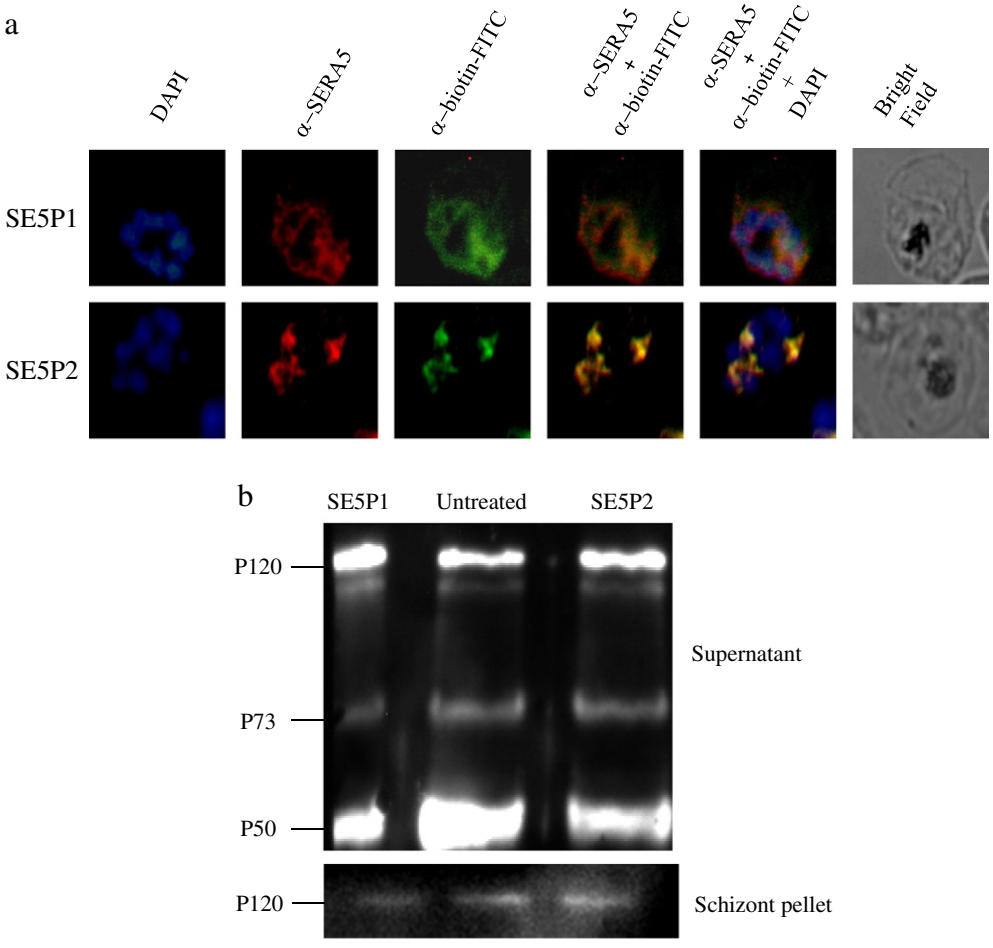

Fig. 6. Localization of SE5 P1 and SE5 P2 peptides with PfSERA5. a) The biotinylated peptides SE5 P1 and SE5 P2 were allowed to incubate with the mature blood stage parasites. The parasite was then probed for biotinylated peptides (green) and PfSERA5 (red). Parasite nuclei were stained with DAPI, a nuclear dye (blue). On superimposing, the areas of colocalization were seen as yellow. SE5 P1 and SE5 P2 were seen to be colocalized with the PfSERA5 within the parasitophorous vacuole. b) Effect of SE5 P1 and SE5 P2 peptides on SERA5 processing in the parasite. Late schizonts were treated with the peptides SE5 P1 and SE5 P2 for 9–10 h. The schizont pellet and the supernatant were collected and fractionated on the 8% SDS-PAGE. SERA5 was detected by western blot.

derived from two separate fragments of SERA5. These inhibitory peptides can act as lead compounds for designing peptidomimetic drugs against the parasite.

We next assessed the role of PfSERA5 at late stage parasite development using the two identified PfSERA5 inactivating peptides. The peptide(s) treated cultures showed lower parasitemia as well as delay in the formation of new rings. These results corroborated with a previous study, wherein a randomly selected PfSERA5 binding cyclic peptide was used and its addition to the in vitro culture delayed the development of the parasite [34]. While designing the peptides, we kept the molecular weight of various peptides in the range from 1540 Da to 2003 Da as access of peptides up to the molecular weight of ~2300 Da into the infected erythrocyte is well documented [29,34]. Results revealed that both PfSE5 P1 and P2 peptides were able to access the intracellular parasites and co-localized with the PfSERA5 protein. We also investigated the effects of these peptides on the processing of native PfSERA5 by western blot analysis of the treated vs untreated parasite extracts using anti-PfSERA5 antibodies. Together, these results suggest that peptides PfSE5 P1 and PfSE5 P2 specifically block the parasite growth in vitro by inhibiting the $PfSERA5P_{50}$ proteolytic activity.

These peptides may bind to the protein and regulate its activity via binding to the active site or binding to sites other than catalytic core. Docking studies revealed that for peptides to be inhibitory they must interact with Glu638 and Ser640/Ser641 that are interchangeable due to their proximity. Molecular docking studies further provided support to the inhibitory function of SE5 P1 and SE5 P2 peptides, as they showed best space fit around the SERA5 active site as compared to other peptides use in this study. This observation can be an important step towards designing inhibitors to block the activity of PfSERA5.

Recently SERA6 has also been implicated to play an important role in egress [9]. Delayed formation of rings and the studied role of both SERA5 and SERA6 may indicate an additive/synergistic effect of both these proteins in parasite egress. Both of these genes are refractory to deletion but it will be interesting to study the interplay of SERA5 and 6 by knocking down each of them using the latest tools available in the field of parasitology [35]. Insights into how these genes take over the function of each other can be an important finding towards understanding of *Plasmodium* biology. Polymorphism in the N terminal region of the SERA5 gene is well documented [43]. Peptides analyzed in this study bind to the active site of the protein and inhibit its activity. The active site of a protein is conserved and refractory to change, hence peptides P1 and P2 can act as successful lead compounds towards designing inhibitors against this protein.

In conclusion, the results of the present study demonstrate that PfSERA5 plays an important role in parasite development and the final proteolytic cleavage step is essential in regulating the enzymatic activity of the protein. These results have important implications as they counter the speculations about the catalytic activity of the PfSERA5 protein. Our results raise curtain on these doubts as only two out of the ten peptides designed from the PfSERA5 sequence could inhibit the enzyme activity and show a significant effect on parasite development. Our study provides a direct proof in this regard as inhibitory peptides used in this study were specifically designed from the PfSERA5 sequence. This study opens new avenues for the design of potential peptidometric drug(s) that target this mechanism and hence keeps parasite growth in check. A complete crystal structure elucidation of 56 kDa fragment will give better insights into the role of this 6 kDa domain which is cleaved off just before egress. Additional studies would be needed to piece together the role of PfSERA5 processing and molecular events and mechanisms that occur during parasite egress.

4. Experimental section

Resin, Fmoc-amino acids, other chemicals and solvents were obtained from IRIS Biotech GmbH (Marktredwitz, Germany), Carlo Erba Reagents (Rodano, Italy) and Sigma-Aldrich (Schnelldorf, Germany) and used without further purifications. Synthesis of the peptides was carried-out using the solid-phase synthesis method by standard Fmoc/t-Bu SPPS protocol using the Rink amide resin as described previously [36]. Peptide synthesis was carried out manually using a polypropylene syringe (10 ml) fitted with a polyethylene porous disk obtained from Grace. Analytical and preparative HPLC were performed by using a Varian 940-LC. MALDI TOF spectra were performed by using a Bruker Daltonics MALDI TOF-TOF AUTOFLEX III. N terminal Biotinylated peptides SE5 P1 and SE5 P2 were obtained from GL Biochem, Shanghai. The purity of each peptide was determined by HPLC and confirmed to be >95%, unless otherwise mentioned.

5. General procedures

5.1. Synthesis of peptides

Peptide synthesis was carried out in solid phase starting from 290 mg of Rink amide resin (130 μmol; loading: 0.46 mmol/g).

5.2. Coupling

The appropriate Fmoc-amino acid (5 eq.) dissolved in a solution of TBTU/HOBt (5 eq., 0.45 M in DMF) and DIPEA (10 eq.) was added to the resin and the mixture was swirled for 50 min at RT. The mixture was filtered and the resin washed with DMF (6 times).

5.3. Fmoc deprotection

A solution of piperidine (20% in DMF) was added to the resin and the mixture was swirled for 5 min at RT and filtered. Then, another amount of piperidine (20% in DMF) was added and the mixture swirled for another 15 min, filtered and the resin was washed with DMF (6 times).

5.4. Acetylation

A solution of acetic anhydride (5 eq., 1 M in DCM/DMF 1:1) and DIPEA (5 eq.) was added to the resin and the mixture was swirled for 1 h at RT. The mixture was filtered and the resin was washed with DMF (6 times).

5.5. Cleavage of peptide from the resin and deprotection of the side chains

A solution of TFA/H_2O/TIS/Thioanisole/Phenol (10:0.5:0.25:0.5:0.75, 6 ml for 300 mg of resin) was added to the resin and the mixture was stirred for 2 h at RT. At the end, the cleaved peptide was filtered in a cold solution of petroleum ether/t-Butylmethyl ether (1:1) where precipitated. The solid was centrifuged, washed with petroleum ether/t-Butylmethyl ether (4 times) and dissolved in a solution of HPLC eluents A and B (1:1).

5.6. Peptide purification

The crude peptides were purified by reverse phase HPLC using an appropriate gradient of eluents A (0.3% TFA in water/acetonitrile 97:3) and B (0.3% TFA in water/acetonitrile 30:70) with a flow rate of 14 ml/min; detection was carried out at 230 and 280 nm.

The collected fractions of pure peptides were combined and concentrated to eliminate acetonitrile. A drop of HCl 1 M was added to water solution and the peptides were lyophilized. The final product was applied to an analytical C18 column to verify its identity using the following gradient at a flow rate of 1.0 ml/min: 0–14 min from 100% eluent A to 70% eluent B. Peptides were solubilized in Dimethyl Sulfoxide (DMSO) such that the maximum concentration of DMSO in the final assay was 0.5%.

5.7. Parasite culture, peptide treatment and growth assays

P. falciparum 3D7 parasites were cultured with human erythrocytes (4% hematocrit) in RPMI media (Invitrogen, USA) containing gentamycin sulfate, $NaHCO_3$, and L-glutamine in vitro using the protocol described previously [37]. To assess the effect of the peptides derived from the SERA5 sequence on the parasite growth and development, synchronized parasite cultures at the late trophozoite/early schizont stages, at a parasitemia of 0.5%, were treated with the peptides in a 96-well culture plate in triplicate. Parasites were maintained for 36 h and were analyzed by FACS and microscopically using Giemsa stained smears. For delay in cycle studies treated cultures were compared to the emergence of the ring stage parasites in the control cultures containing PBS.

$$\%\ \text{invasion inhibition} = [(\%\ \text{parasitemia in control} - \%\ \text{parasitemia in the experimental}) / \%\text{parasitemia in control}] * 100$$

5.8. Protein purification of recombinant SERA5, immunoblotting and antibody production

The recombinant SERA5 protein was prepared by a protocol described by Hodder et al. with minor modifications. Briefly, washed inclusion bodies were solubilized in 5.5 M urea, 100 mM NaCl, 10 mM β-mercaptoethanol and 50 mM Tris pH 8.0. The solubilized inclusion bodies were centrifuged at 13,000 rpm for 30 min. Then clear solubilized supernatant was loaded on pre-equilibrated nickel–nitrilotriacetic acid-agarose (Ni^{2+}–NTA) resin at the flow rate 0.3 ml/min. The column was washed extensively with 10 times the volume of the buffer with 40 mM imidazole. Finally, the protein was eluted using the buffer containing 300 mM imidazole. The Ni–NTA purified protein was refolded using 10 times the volume dilution into 50 mM Tris pH 8.0, containing 1 mM reduced glutathione, 0.25 mM oxidized glutathione and 10% glycerol overnight on a magnetic stirrer at 4 °C at a flow rate of 0.1 ml/min. The refolded protein was purified by anion-exchange chromatography. The refolded protein was loaded on a per-equilibrated Q-Sepharose anion-exchange column in a buffer containing 10 mM NaCl, 10 mM β-mercaptoethanol, 5% glycerol, and 50 mM Tris pH 8.0. Bound protein was washed and eluted with the buffers containing 60 mM NaCl and 300 mM NaCl (10 ml), respectively. The protein was concentrated to be used for the enzyme assays. Polyclonal antibodies were raised in mice against purified SERA5P50 recombinant protein, the catalytically active domain of SERA5. Immunoblotting was performed using the above-described anti-PfSERA5 antiserum and horseradish peroxidase-labeled anti-mice IgG. Substrate DAB with H_2O_2 was used to develop the blot.

5.9. Site directed mutagenesis

Mutations were generated using a QuikChange site-directed mutagenesis kit according to the manufacturer's protocol. Mutagenized plasmids were subjected to dideoxy sequencing to confirm that the specific mutations were only incorporated at the desired positions and that no random mutation(s) occurred. All mutant proteins were purified using a Ni^{2+}–NTA affinity chromatography as described for the wild type rSERA5.

5.10. Enzyme assay and kinetic analysis

Fluorimetric assay for rSERA5 activity was carried out as described previously [26]. Briefly, in 200 μl of assay buffer (0.1 M $NaHCO_3$, pH 7.5, 5 mM $CaCl_2$, and 0.1% (v/v) Tween 20) containing 200 nM enzyme, the fluoregenic substrate Suc-LLVY-AMC was added at 30 μM concentration and the release of 7-amino-4-methyl coumarin (AMC) was monitored (excitation 355 nm; emission 460 nm) over 7 h at 37 °C using a LS50B Perkin-Elmer fluorimeter. The readings were taken every 30 min. To analyze the effect of peptide(s) on enzyme activity, rSERA5 was preincubated with the peptides for 20 min before adding the substrate. The remaining activity was determined using the fluorogenic substrate. Rate of hydrolysis at a varied concentration of the substrate Suc-LLVY-AMC was determined at a constant enzyme concentration (200 nM) in 200 μl reaction volume. Kinetic constant K_m and V_{max} values were determined using PRISM software (GraphPad, San Diego). Fluorimetric assay for falcipain-2 and falcipain-3 was carried out using the protocol described earlier [29].

5.11. Immunofluorescence microscopy

To study the uptake and binding of peptides into the infected erythrocytes, *P. falciparum* cultures were synchronized and incubated with biotinylated SE5 P1 and SE5 P2 peptides, at a concentration of 100 μm, at 34–38 h post invasion for 8 h. After the incubation, the cultures were fixed and stained as described previously [38]. Briefly, thin smears of infected erythrocytes were made on a glass slide and fixed with 4% paraformaldehyde. Slides were blocked in blocking buffer (1× PBS, 10% FBS) for 1 h at 37 °C. After blocking, slides were washed thrice with PBS and were incubated with primary antibodies diluted in blocking buffer (mice anti-PfSERA5 antibody; dilution 1:1000 and for 1 h at 37 °C). After incubation with the primary antibody, slides were washed with 1× PBS and incubated with anti-mice antibody (conjugated to Cy3, dilution 1:500) and anti-biotin antibody (conjugated to FITC, dilution 1:100) for 1 h at 37 °C. Finally, the slides were mounted on a cover slip in the presence of DAPI-antifade reagent (Molecular probes). The slides were viewed using the Nikon TE 2000-U fluorescence microscope.

5.12. Molecular docking studies

The crystal structure of the central protease-like domain of the SERA5 protein was downloaded from Protein Data Bank (PDB ID: 3CH3) and was completed adding the hydrogens, fixing the atom charges (Gasteiger–Marsili method) [39] and the potentials (CHARMM 22 for proteins) by using the features included in the VEGA ZZ package [40]. After this step, the model was optimized through a conjugate gradient minimization (30,000 steps) to reduce the high-energy sterical interactions and in order to preserve the experimental data, atom constraints were applied to the protein backbone. This step was carried out by NAMD 2.9 software [41] integrated into the graphic environment of VEGA ZZ. Finally, the co-crystallized water molecules and the two phosphate anions were removed to perform the next molecular docking phase.

All considered peptides were built by the peptide builder tool included in VEGA ZZ, choosing α-helix as secondary structure. Hence, the resulting structures were optimized by NAMD by using a minimization protocol as for PfSERA5, but, in this case, no atom constraints were applied. Each peptide was docked into refined structure of SERA5 by PLANTS software [42], keeping rigid the protein and considering flexible the peptide structures. More in detail, a sphere of 18 Å radius was selected as binding site, whose center is coincident with the center of gravity defined by Ser641 and Ser816. These two amino acids are included approximately in the middle of the large cleft of the protease domain and are close to Ser596, known to play a pivotal role in the catalytic process. Moreover, PLANTS was set to use CHEMPLP as scoring function, perform a pose search with the maximum exhaustiveness (speed1) and generate 10 clusters of structures obtained by defining the RMSD value of 2.0 as clustering threshold. All complexes were graphically inspected and the best pose of each peptide was selected by considering the docking score and the distance from the catalytic Ser596. The best structures were minimized by NAMD (conjugate gradients, 30,000 steps),

keeping fixed the residues outside from the spheroid defined by a layer of 10 Å thickness around the ligand.

In order to compare the poses of the different peptides, the volumes shared between the best pose of each inhibitor peptide and Suc-LLVY-AMC substrate, known to be hydrolyzed by SERA5, were calculated by VEGA ZZ.

5.13. Effect of peptides on the processing of SERA5

Percoll purified late schizont stage parasites were incubated with the peptides SE5 P1 and SE5 P2 and were allowed to grow in the serum free RPMI media for 8–10 h. The supernatant was collected and run on an 8% SDS PAGE. The blot was analyzed by western blot using the anti-PfSERA5 antibodies.

Author contributions

SK conceived, designed and performed the experiments. GK purified the recombinant protein. LR synthesized the peptides used in the study. AP performed the molecular docking studies. SK analyzed the data. SR, AP, ANH and PM contributed reagents/materials/analysis tools. SK and PM wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Alignment of SERA1–8. This material is available free of charge via the Internet at http://pubs.acs.org.

PDB ID: 3CH3.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.04.013.

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