Introduction

The concept that mosquito immunity could be an important determinant of the infectivity of malaria parasites for different mosquito species was put forward 85 years ago [1]. Even in permissive parasite-vector species combinations, the parasite population undergoes substantial stage-specific losses during its development in the mosquito [2,3]. Manipulation of the mosquito immune system has been acknowledged as a mechanism to kill parasite numbers and transmission and has been postulated to control melanization and complement function in mosquitoes. This study aimed to characterize AgSRPN6 biophysically and determine its biochemical mode of action. The structure model of AgSRPN6, as predicted by I-Tasser showed the protein in the native serpin fold, with three central \( \beta \)-sheets, nine surrounding \( \alpha \)-helices, and a protruding reactive center loop. This structure is in agreement with biophysical and functional data obtained from recombinant (r) AgSRPN6, produced in Escherichia coli. The physical properties of purified rAgSRPN6 were investigated by means of analytical ultracentrifugation, circular dichroism, and differential scanning calorimetry tools. The recombinant protein exists predominantly as a monomer in solution, is composed of a mixture of \( \alpha \)-helices and \( \beta \)-sheets, and has a mid-point unfolding temperature of 56 \(^\circ\)C. Recombinant AgSRPN6 strongly inhibited porcine pancreatic kallikrein and to a lesser extent bovine pancreatic trypsin in vitro. Furthermore, rAgSRPN6 formed inhibitory, SDS-stable, higher molecular weight complexes with prophenoloxidase-activating proteinase (PAP)1, PAP3, and Hemolymph protein (HP)6, which are required for melanization in the lepidopteran model organism, Manduca sexta. Taken together, our results strongly suggest that AgSRPN6 takes on a native serpin fold and is an inhibitor of trypsin-like serine proteinases.

Abstract

Serine proteinase inhibitors of the serpin family are well known as negative regulators of hemostasis, thrombolysis and innate immune responses. Additionally, non-inhibitory serpins serve functions as chaperones, hormone transporters, or angiogenic factors. In the African malaria mosquito, Anopheles gambiae s.s., at least three serpins (SRPNs) are implicated in the innate immune response against malaria parasites. Based on reverse genetic and cell biological analyses, AgSRPN6 limits parasite numbers and transmission and has been postulated to control melanization and complement function in mosquitoes. This study aimed to characterize AgSRPN6 biophysically and determine its biochemical mode of action. The structure model of AgSRPN6, as predicted by I-Tasser showed the protein in the native serpin fold, with three central \( \beta \)-sheets, nine surrounding \( \alpha \)-helices, and a protruding reactive center loop. This structure is in agreement with biophysical and functional data obtained from recombinant (r) AgSRPN6, produced in Escherichia coli. The physical properties of purified rAgSRPN6 were investigated by means of analytical ultracentrifugation, circular dichroism, and differential scanning calorimetry tools. The recombinant protein exists predominantly as a monomer in solution, is composed of a mixture of \( \alpha \)-helices and \( \beta \)-sheets, and has a mid-point unfolding temperature of 56 \(^\circ\)C. Recombinant AgSRPN6 strongly inhibited porcine pancreatic kallikrein and to a lesser extent bovine pancreatic trypsin in vitro. Furthermore, rAgSRPN6 formed inhibitory, SDS-stable, higher molecular weight complexes with prophenoloxidase-activating proteinase (PAP)1, PAP3, and Hemolymph protein (HP)6, which are required for melanization in the lepidopteran model organism, Manduca sexta. Taken together, our results strongly suggest that AgSRPN6 takes on a native serpin fold and is an inhibitor of trypsin-like serine proteinases.
specific target proteinase similar to the binding of a substrate. Upon cleavage of the serpin at its so-called scissile bond (designated $P_1-P_1'$), the serpin undergoes a substantial conformational “stressed-relaxed” transition, which covalently traps the target proteinase [33,39]. Crystal structures of serpins provide additional information into their mechanism of inhibition. So far, more than 80 serpin crystal structures in five distinct conformational states have been solved [39]. To date, protein structures of only a few insect serpins are available. The crystal structure of AgSRPN2 was solved recently in its native conformation [40], and only a few insect serpins are available. The crystal structure of AgSRPN2 was solved recently in its native conformation [40], and only a few insect serpins are available.

A small number of SRPNs have been analyzed for their potential involvement in malaria parasite transmission. AgSRPN6, which is a biomarker for malaria parasite invasion, limits the number of rodent malaria parasites that progress through the midgut and salivary gland epithelium [29,30]. Depletion of AsSRPN6 by RNAi in susceptible An. stephensi leads to a significant increase in the number of developing P. berghei oocysts, whereas AgSRPN6 depletion in susceptible An. gambiae has no effect on the number of developing parasites but delays the progression of parasite lysis by the complement system and may additionally limit melanization. These observed phenotypic differences are possibly due to differences in the respective target serine proteinases in the two mosquito species as both SRPN6 proteins contain identical RCLs. Additionally, knock-down of AgSRPN6 significantly increases the number of sporozoites reaching the salivary glands. Its endogenous target proteinase(s) await identification, and any additional information into their mechanism of inhibition. So far, no additional information into their mechanism of inhibition. So far, no additional information into their mechanism of inhibition.
atic trypsin (5 ng) and N-acetyl-Ile-Glu-Ala-Arg-p-nitroanilide (Biochemistry Core Facility, Kansas State University). All proteinases and substrates were purchased from Sigma-Aldrich (St. Louis, MO, USA), with the exception of proteinase K (Promega, Madison, WI, USA) and N-acetyl-Ile-Glu-Ala-Arg-p-nitroanilide, which was synthesized in-house.

Sedimentation velocity

Purified intact rAgSRPN6 was dialyzed against 20 mM Tris and 250 mM NaCl, pH 8.0 at 4°C for 24 h with one buffer exchange to reach a concentration of 1.0 mg/ml. Same dialysis buffer was used as a blank. Sedimentation velocity runs were performed in an Optima XL-I analytical ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 49,000 rpm, 25°C in an An-60 Ti rotor. Sedimentation was monitored at 280 nm using double sector cells with a final loading of 400 µl per well. Acquired data were analyzed using DCDT+ software version 1.16, and sedimentation coefficients were calculated using g(s*) fitting function in the DCDT+ software [48].

Circular dichroism

To investigate the effect of pH on the CD spectrum of rAgSRPN6, purified protein was dialyzed as above and the pH was adjusted to 3.0 or 11.0 by stepwise addition of either 0.1 M HCl or 1.0 M NaOH. Samples were centrifuged at 13,000 rpm for 5 min to remove any pellet resulting from pH change. Far-UV CD spectra (190-260 nm) were recorded at 25°C with a Jasco J-815 CD Spectrometer (Jasco Corporation, Tokyo, Japan). A cell of 0.2 mm path length was used in the scanning from 260 nm to 190 nm with a scan rate of 50 nm/min. Spectra were the averaged over ten scans. All CD spectra were smoothed and corrected by background subtraction for the spectrum obtained with buffer alone. Data were expressed as molar ellipticity per mean residue ([θ] = degree • cm²•dmol⁻¹) with a calculated mean residue molecular weight of 111.78, based on a molecular weight of 53,877 Da and 482 amino acid residues.

Differential scanning calorimetry

To check the thermal stability of rAgSRPN6, purified protein (1.0 mg/ml) was dialyzed as above, deaggregated, and loaded into VP-DSC MicroCalorimeter (MicroCalTM Inc.). Temperature was scanned at 1°C/min from 20°C to 85°C. Heat capacity, Cₚ (kcal/mol/°C), was plotted after background subtraction of a blank experiment without protein.

Formation of SDS-stable complexes between rSRPN6 and proteinase

Active M. sexta PAP1 (15 ng) or PAP3 (20 ng) was incubated with 200 ng of purified rAgSRPN6 (molar ratio of 1:10 for proteinase/serpin) at room temperature for 10 min and then treated with SDS reducing sample buffer at 95°C for 5 min. The reaction mixtures were resolved by 10% SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis using 1:2,000 diluted antiserum against M. sexta HP6 [50] and AgSRPN6.

ProPO activation assays

To investigate the effect of rAgSRPN6 on proPO activation, cell-free hemolymph was collected from day-2 fifth instar M. sexta larvae as described previously [19]. 200 ng of recombinant rAgSRPN6 (or rAgSRPN7 as negative control) was mixed with 1 µl of hemolymph for 10 min at room temperature. Subsequently, 1 µl of Micrococcus luteus (10 µg/µl in sterile water, Sigma) was added to trigger proPO activation in the hemolymph. After incubation for 10 min at room temperature, PO activity was measured using dopamine as a substrate. One unit of PO activity was defined as the amount of enzyme producing an increase in absorbance (ΔA₄₇₀) of 0.001 per min.

Results and Discussion

AgSRPN6 mediates mosquito innate immune response against malaria parasites with unknown mechanisms [29,30]. To gain further insight into AgSRPN6’s mode of action, we investigated its structural, biophysical, and biochemical properties using modeling, circular dichroism, differential scanning calorimetry, and amidase activity assays.

Protein Structure Prediction of SRPN6

Since no structure is currently available for AgSRPN6, a predicted 3-dimensional model was computed using the I-TASSER server [44,45], which yielded a C-score of −0.77 and TM score of 0.62 (0.14) for the top model. The predicted structure is similar to the native fold of other serpins consisting of three β-sheet domains, 8 α-helices and a βCL (Figure 1). A comparison with other structures suggested that SRPN6 overall shares highest similarity with human antithrombin III (PDB: 1E93, chain I) with a calculated RMSD of 1.47 Å. Similarity to AgSRPN2, the only mosquito serpin for which the protein structure was solved by X-ray crystallography, is lower, with a calculated RMSD of 1.69 Å (PDB: 3PZF, [40]). Similarity to M. sexta Serpin 1K (PDB: 1SEK, [51]) is comparable with an RMSD of 1.67 Å, and likewise to T. molitor Serpin48 (PDB: 3OZQ, [52]) with an RMSD of 1.63 Å.

Sequence comparison between AgSRPN6 and other arthropod serpins had previously revealed an insertion of 27 amino acids (aa 146–174) between helix D and strand 2A. This insertion is restricted to the members of a mosquito-specific expansion cluster including SRPN4, 5, 6, 16 and 19 [22,53]. The structure of this insertion is predicted to form two one turn α-helices above and perpendicular to helices D and E (Fig. 1A, maroon). An additional feature of SRPN6 is a 41 amino acid long C-terminal extension. This extension is predicted to form a short β-sheet, which runs parallel to helix D (Figure 1A, pink). Several hydrogen bonds are predicted between the insertion and N-terminal extension, specifically between amino acid residues 19/162, 20/164, 20/146, 21/151, and 23/149, suggesting that these two structural features interact and stabilize each other. Additionally, the insertion contains several aspartic acid residues that form a negatively charged surface area which could be an important binding site. Relatively few serpins with either insertions within the reactive site or N-terminal extensions, can be found in the literature. An interesting example is the D. melanogaster Necrotic serpin, which has an N-terminal extension that is cleaved on immune-challenge. This cleavage modifies the affinity of the Nec serpin for Elastase, and presumably it’s in vivo target proteinase [54]. If the N-terminal extension in AgSRPN6 is cleaved in vivo is currently unknown.
Incubation of full-length rSRPN6 with lysates resulted in the appearance of this truncated band (Fig. 2B).

Production of recombinant An. gambiae SRPN6

To further characterize AgSRPN6, we expressed recombinant protein, excluding signal peptide, in E. coli (Fig. 2A). Primary trials indicated almost all rAgSRPN6 was insoluble after expression at 37°C. Therefore, large scale production of soluble, rAgSRPN6 was expressed at a lower temperature of 20°C with low speed shaking at 150 rpm. The N-terminally His-tagged recombinant SRPN6 was purified by Ni-NTA affinity chromatography, followed by SP-sepharose cation exchange chromatography. Approximately 20 mg of rAgSRPN6 could be obtained from 1.5 L of bacterial culture. SDS-PAGE analysis of recombinant SRPN6 indicated purified SRPN6 was of high purity (Fig. 2B) with the apparent molecular weight of 54 kDa. In addition to this major band, another band at 53 kDa position appeared in some fractions. N-terminal sequencing results confirmed that this band was an N-terminally truncated form of rAgSRPN6 (SRPN6Ag), covering the last 201 amino acid residues of full-length SRPN6. This polypeptide was insoluble, and purified under denaturing conditions by Ni-NTA and preparative SDS-PAGE. SRPN6Ag was used successfully to produce polyclonal antibodies against AgSRPN6 (Fig. S1).

Biophysical and structural parameters of recombinant SRPN6

To gain information on the structural parameters of rAgSRPN6 in solution, we performed sedimentation velocity experiments by analytical ultracentrifugation (Fig. 3A). The sample contained a single molecule species with the sedimentation coefficient of 3.74 Svedberg units and the molecular mass was calculated as 53.6 kDa. This is approximately equal to the molecular weight (53.9 kDa) calculated from amino acid sequences of rAgSRPN6. This result indicates that rAgSRPN6 exists predominantly as a monomer in solution and is not prone to form dimers or polymers as previously reported for mutant forms of α1 antitrypsin [55] or neuroserpin [56].

Native and rAgSRPN6 have a calculated pI of 9.6, which is substantially more basic compared to most Anopheles serpins with calculated pIs between 5 and 6. To test whether pH influences the conformation of the protein we determined the far ultraviolet (UV) circular dichroism spectra of recombinant SRPN6 at different pH.

The canonical native serpin fold is composed of a mixture of α-helices, β-sheets, β-turns, and loops, presented as a minimum peak at 222 nm preceded by a shoulder at 208 nm in the far-UV CD spectra [57–59]. The far UV CD spectrum of SRPN6 is highly similar to that of reported serpins (Fig. 3B). All spectra demonstrated broad negative bands in the wavelength range of 208–222 nm, with [theta] = −9900 deg. cm². dmol⁻¹ for SRPN6 at pH 8.0 and 11.0, and [theta] = −10500 deg. cm². dmol⁻¹ for SRPN6 at pH 5.0. The general shape of the CD spectra at pH 5.0, 8.0 and 11.0 was approximately indistinguishable, suggesting that recombinant SRPN6 kept the same high degree of secondary structure under either acid or basic conditions.

The native conformation of serpins, which generally represents the active conformation of the protein, is the least stable conformation. This is reflected in an average midpoint unfolding temperature (Tm) of the native form of about 58°C, while serpin molecules in the latent conformation usually display melting temperatures of 85–90°C ([60], and references within). The thermal stability of recombinant SRPN6 was analyzed by Differential scanning calorimetry (DSC). As shown in Fig. 3C, rAgSRPN6 displayed a Tm of 56°C. The DSC thermogram was distorted at temperatures higher than the Tm, most likely due to aggregation of rAgSRPN6 at higher temperatures. No heat capacity peak was observed during the second DSC scan, suggesting that the SRPN6 structure was destroyed completely when the temperature increased above 56–58°C and denaturation was irreversible even when the temperature decreased below Tm. Accordingly, rAgSRPN6 protein concentration in solution decreased around 55°C (Fig. 3D), due to protein aggregation and precipitation. Taken together, the DSC analysis results are consistent with rAgSRPN6 protein taking on a native conformation in solution, with little or no propensity for a latent conformation.
Inhibitory activity of recombinant rSRPN6

The functionality of purified rAgSRPN6 was assayed in commercial proteinase inhibition tests, in which the enzymatic activity of proteinases was monitored using chromogenic substrates. The tested proteinases included mammalian digestive enzymes (trypsin, chymotrypsin), mammalian tissue proteinases with distinct functions (elastase, kallikrein), several serine proteinases from human blood (cathepsin G, plasmin, thrombin) and subtilisin-like proteinases (proteinase K, subtilisin Carlsberg).

SRPN6 has the basic residue Arginine at the predicted P1 site, suggesting it as a potential inhibitor of trypsin-like serine proteinases, which prefer Arginine or Lysine at the N-terminal residues of the target peptide bond. The results are summarized in Table 1. As expected based on the substrate specificity of the proteinase, rAgSRPN6 had no inhibitory activity against cathepsin G, chymotrypsin, proteinase K, or elastase. Plasmin, subtilisin Carlsberg, thrombin, trypsin, and kallikrein prefer an Arg, or Lys at the P1 site, and therefore are potential inhibitory targets of SRPN6. However, rAgSRPN6 did not inhibit Plasmin, subtilisin Carlsberg, thrombin, and only weakly inhibited trypsin by 13% at a serpin/trypsin molar ratio of 10:1. rSRPN6 inhibited kallikrein most efficiently among all tested commercial proteinases, with 44% and 75% inhibition at rAgSRPN6/kallikrein ratio of 1:1 and

Figure 2. Production of rAgSRPN6. (A) Schematic representation of rAgSRPN6. Letters above the boxes represent the amino acid residues and the numbers underneath indicate their positions in wild type AgSRPN6. Predicted P1-P1' cleavage sites are indicated by arrows. (B) SDS-PAGE analysis of purified rAgSRPN6. Fraction #16, #18, #20, and #22 from SP-sepharose cation exchange chromatography (1 μg/sample, Lane 1–4 respectively) were separated by 10% SDS-PAGE followed by commassie blue staining. Lane 5: 1 μg of rAgSRPN6 (fraction #22) was incubated with 3×10⁹ E. coli XL1-blue cells at room temperature for 15 min and subjected to SDS-PAGE. Lane 6: 3×10⁹ E. coli XL1-blue cells.
doi:10.1371/journal.pone.0048689.g002

Figure 3. Biophysical and structural parameters of rAgSRPN6. (A) Sedimentation velocity analysis of rAgSRPN6. Dialyzed rAgSRPN6 (1 mg/ml) was evaluated by analytical ultracentrifugation. Data were analyzed using DCDT+ software version 1.16 [48]. (B) Far-UV CD spectra of rAgSRPN6. The protein was dissolved in 20 mM Tris-HCl buffer containing 250 mM NaCl with pH of 8.0 (——) or 5.0 (---) or 11.0 (----). [θ] represents mean residue ellipticity defined in "Material and methods". (C) Calorimetric scans of rAgSRPN6. Samples were prepared by dialysis against 20 mM Tris and 250 mM NaCl, pH 8.0 and analyzed by DSC. Traces were baseline-corrected by subtraction of a scan of buffer. (D) SDS-PAGE analysis of purified rAgSRPN6 heated at different temperatures. One μg of purified rAgSRPN6 was heated at 30, 35, 40, 45, 50, 55, 60, and 65°C for 5 min, respectively, and centrifuged at 13,000 rpm for 5 min. The supernatants were separated by 10% SDS-PAGE followed by commassie blue staining (Lane 1–8 respectively). Lane 9: Supernatant from 1 μg of rAgSRPN6 analyzed after DSC scan.
doi:10.1371/journal.pone.0048689.g003

Characterization of Anopheles gambiae SRPN6
201 amino acid residues of AgSRPN6 (Fig. 5A, left panel), also detected by AgSRPN6 antibodies produced against the last 201 amino acid residues of AgSRPN6 that were migrated to the catalytic domain became fainter and a new immunoreactive band against the last 201 amino acid residues of AgSRPN6 that were also detected by AgSRPN6 antibodies produced against the last 201 amino acid residues of AgSRPN6 (Fig. 5A, left panel). The residual amidase activity was measured using FPRpNa as substrate. doi:10.1371/journal.pone.0048689.g004

**Figure 4. Stoichiometry of inhibition of kallikrein by rAgSRPN6.** Recombinant AgSRPN6 was incubated with porcine kallikrein at different molar ratios for 10 min at room temperature. The residual amidase activity was measured using FPRpNa as substrate. doi:10.1371/journal.pone.0048689.g004

10:1, respectively. Kallikrein activity decreased linearly as rAgSRPN6 concentration increased (Fig. 4). Therefore, rAgSRPN6 (and most likely the native protein) is a functional serine proteinase inhibitor. Its inhibitory specificity is distinct from that of aFXa [61] and SRPN2 [24], the only other mosquito serine proteinase inhibitor. AgSRPN6 is regulating melanization as these three proteinases are critical components of the proPO activation pathway in M. sexta [18,47,49]. To further test this hypothesis, we investigated AgSRPN6’s effect on proPO activation ex vivo. Naïve hemolymph from day 2 fifth instar M. sexta larvae has low basal PO activity, and exposure of M. luteus significantly increased the PO activity of hemolymph (Fig. 5D and [63]). Pre-incubation of hemolymph with rAgSRPN6 significantly decreased PO activity (Fig. 3D). Therefore, rAgSRPN6 had inhibitory effect on the proPO activation in M. sexta hemolymph, most likely by inhibiting PAP1, PAP3 and HP6 in the hemolymph. These findings are consistent with our working hypothesis that one function of AgSRPN6 is regulating melanization in vivo. Recently, the first PAP from An. gambiae was identified and characterized biochemically. AgCLIPB9 is part of a regulatory unit that controls melanization in adult females, by functioning as a PAP. However, active recombinant CLIPB9Xa did not form inhibitory complexes with rAgSRPN6 (Fig. 3), and its amidase activity was unaltered in the presence of rSRPN6. However, additional PAPs are likely to be present in the hemolymph of mosquitoes, which may be inhibited by SRPN6. Antibodies against the last 201 amino acid residues of AgSRPN6 that were

**Table 1.** Proteinase inhibition assays with rAgSRPN6.

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Human cathepsin G (Cath G), bovine chymotrypsin (CHY), porcine elastase (ELA), porcine kallikrein (KAL), Human plasmin (PLAS), Fungus protease K (ProK), Bacillus licheniformis Subtilisin Carlsberg (SubC), human thrombin (THRMB), and bovine trypsin (TRYP) were incubated with purified rAgSRPN6 and assayed for residual enzyme activity. "–", inhibition <10%. Values represent mean percent inhibition ± S.D. (n = 3). doi:10.1371/journal.pone.0048689.t001

Characterization of Anopheles gambiae SRPN6

The residual amidase activity was unaltered in the presence of rSRPN6. However, rSRPN6 had inhibitory effect on the proPO activation in M. sexta hemolymph, most likely by inhibiting PAP1, PAP3 and HP6 in the hemolymph. These findings are consistent with our working hypothesis that one function of AgSRPN6 is regulating melanization in vivo.

AgSRPN6 forms SDS-stable complexes with insect serine proteinases

Knockdown of AgSRPN6 increases the number of melanized ookinetes in the refractory L3–5 strain as well as in CTLA-depleted mosquitoes [29]. One potential explanation for these observations is that AgSRPN6 under normal physiological conditions suppresses melanization of parasites, by inhibiting one or more PAPs and thus preventing activation of proPO and melanization. To test this hypothesis, we made use of the M. sexta melanization cascade, which is established in vitro and ex vivo. A characteristic feature of a serpin-proteinase reaction is the formation of a high molecular weight, SDS-stable complex [62]. We made use of M. sexta serine proteinase PAP1, PAP3, and HP6 to investigate whether rAgSRPN6 can form such complex in vitro. M. sexta PAP1 antibodies recognized a 34 kDa band corresponding to the catalytic domain of PAP1 but not rAgSRPN6 (Fig. 5A, right panel). After the two proteins were incubated together, the 34 kDa catalytic domain became fainter and a new immunoreactive band migrated to the ~80 kDa position anticipated for a complex of rAgSRPN6 and PAP1 catalytic domain. This ~80 kDa band was also detected by AgSRPN6 antibodies produced against the last 201 amino acid residues of AgSRPN6 (Fig. 5A, left panel), indicating that it was composed of these two proteins. Mass-spect analysis confirmed the presence of rAgSRPN6 and PAP1 in the ~80 kDa band (data not shown). Surprisingly, the previously published antiserum raised against soluble full-length rAgSRPN6 [29] did not recognize this complex (Fig. S2).

Similar results were obtained for the interaction between rAgSRPN6 and PAP3 (Fig. 5B). The interaction of rAgSRPN6 and PAP3 resulted in the formation of an ~80 kDa complex under reducing conditions, which was absent from the control lanes with only PAP3 or SRPN6 alone. This complex was recognized by both rAgSRPN6 antibodies and M. sexta PAP3 antibodies (Fig. 3B) indicating that SRPN6 can also form a covalent complex with PAP3.

Consistent with previous report [47], anti-HP6 antiserum recognized the 39 kDa proHP6 zymogen and a 33 kDa truncated HP6 in the absence of factor Xa, and after activation with factor Xa, a 29 kDa band corresponding to the catalytic domain of HP6Xa was detected by the HP6 antiserum (Fig. 5C, right panel). When rAgSRPN6 was mixed with active HP6Xa, a new immunoreactive band at ~72 kDa position (the expected size for a SRPN6:HP6Xa complex) was observed. This band was also recognized by AgSRPN6 antiserum (Fig. 5C, left panel). The incubation of truncated AgSRPN6 (SRPN6ΔN) and active HP6Xa also resulted in the appearance of 72 kDa band recognized by both antibodies (Fig. 3C), indicating truncated SRPN6 retains the ability to form SDS-stable inhibitory complexes with proteinases.

The high M, covalent complex formation between rAgSRPN6 and PAP1, PAP3, and HP6 supports the hypothesis that AgSRPN6 can regulate melanization as these three proteinases are critical components of the proPO activation pathway in M. sexta [18,47,49]. To further test this hypothesis, we investigated AgSRPN6’s effect on proPO activation ex vivo. Naïve hemolymph from day 2 fifth instar M. sexta larvae has low basal PO activity, and exposure of M. luteus significantly increased the PO activity of hemolymph (Fig. 5D and [63]). Pre-incubation of hemolymph with rAgSRPN6 significantly decreased PO activity (Fig. 3D). Therefore, rAgSRPN6 had inhibitory effect on the proPO activation in M. sexta hemolymph, most likely by inhibiting PAP1, PAP3 and HP6 in the hemolymph. These findings are consistent with our working hypothesis that one function of AgSRPN6 is regulating melanization in vivo.

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**Figure 4. Stoichiometry of inhibition of kallikrein by rAgSRPN6.** Recombinant AgSRPN6 was incubated with porcine kallikrein at different molar ratios for 10 min at room temperature. The residual amidase activity was measured using FPRpNa as substrate. doi:10.1371/journal.pone.0048689.g004

**Figure 5.** Immunoreactive band at 80 kDa position (the expected size for a SRPN6:HP6Xa complex) was observed. This band was also recognized by AgSRPN6 antiserum (Fig. 5C, left panel). The incubation of truncated AgSRPN6 (SRPN6ΔN) and active HP6Xa also resulted in the appearance of 72 kDa band recognized by both antibodies (Fig. 3C), indicating truncated SRPN6 retains the ability to form SDS-stable inhibitory complexes with proteinases.

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created for this project do recognize the native state of the serpin and also bind to serpin-proteinase complexes formed with heterologous proteinases in vitro. However, no SRPN6-proteinase complexes were identified in hemolymph, supernatants of the hemocyte-like 4a3B An. gambiae cell line [64], and P. berghei-infected epithelia (data not shown), suggesting that the endogenous target(s) of AgSRPN6 are either absent or present in undetectable concentrations under physiological conditions.

Conclusions

Structural modeling of AgSRPN6 suggested that this serpin can take on the metastable classical serpin fold, which is supported by the biophysical data presented in this study. The biochemical characterization confirmed that rAgSRPN6 is indeed an inhibitor of trypsin-like serine proteinases. Additionally, this study provides the first biochemical evidence that AgSRPN6 controls the melanization response by inhibiting proteinases of the prophenoloxidase activation pathway. It is noteworthy that we found
differences between the abilities of different antiserum to recognize higher molecular weight complexes between rAgSRPN6 and target proteinases. The new antiserum raised against a C-terminal polypeptide of AgSRPN6 is a key step towards future identification of the cognate proteinase targets and will allow the identification of novel components of the proPO and or complement pathway in mosquitoes.

Supporting Information

Figure S1 SDS-PAGE of recombinant SRPN6Ag. The last 201 amino acid residues of SRPN6 (SRPN6Ag) were expressed in E. coli and purified under denaturing conditions. 10 μl of elution fraction #1–#5 (Lane 1–5, respectively) were separated by 10% SDS-PAGE followed by Coomassie Blue staining. (TIF)

Figure S2 Detection of SDS-stable complex formation between An. gambiae SRPN6 and M. sexta PAP1. Purified recombinant SRPN6 (200 ng) was incubated with M. sexta PAP1 (15 ng) at room temperature for 10 min. The mixtures were subjected to SDS-PAGE and immunoblot analysis using previously published antiserum against either full-length rSRPN6 (left panel) or MsPAP1 (right panel). The band with apparent molecular weight of 80 kDa (the expected size for a SRPN6:PAP1 complex) was recognized by MsPAP1 antibodies (right panel), but not by antibodies against recombinant full length SRPN6 (left panel). However, this band was detected by the antibodies against SRPN6Ag (Fig. 5A–5C). Asterisks, SRPN6-PAP1 complex; triangles, non-complexed rSRPN6; squares, active PAP1. (TIF)

Figure S3 No covalent complex formed between rSRPN6 and serine proteinase CLIPB8 and CLIPB9. Recombinant SRPN2 was incubated with Factor Xa-activated CLIPB8Xa. Western blot analysis was performed using rabbit anti-SRPN6 antibodies against recombinant SRPN6Ag. No complex band except recombinant rSRPN6 was detected. A smaller band was detected after incubation with FactorXa, suggesting that rSRPN6 constitutes a substrate for this proteinase. Triangles, non-complexed full-length rSRPN6; diamond, cleaved rSRPN6. (TIF)

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Author Contributions

Conceived and designed the experiments: KM MZ CA. Performed the experiments: CA YH XZ SL. Analyzed the data: CA YH XZ SL MZ KM. Contributed reagents/materials/analysis tools: JMT YH XZ MZ SL. Wrote the paper: CA SL MZ KM.

References

8. PAP3 and HP6.