Supporting Information

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SI Text

***Plasmodium falciparum* Culture.** *P. falciparum* strain 3D7 was grown in human erythrocytes (4% hematocrit) in RPMI 1640 media (Invitrogen) supplemented with 10% O^+ human serum, as described by Trager and Jensen (1). Parasite cultures were synchronized by two sorbitol treatments at 4-h intervals, according to the protocol of Lambros and Vanderberg (2).

Expression and Activity of Heme Detoxification Protein and Falcipain 2. The coding regions of heme detoxification protein (HDP) gene was amplified by RT-PCR from total RNA of *P. falciparum* (3D7 strain) erythrocytic-stage parasites. The forward and reverse primers were 5′CCCATGGGATCCATGAAAAATAGATTTTAT3′ and 5′CCTCGAGGTCGACAAAAATGATGGGCTTATC3′, respectively, and the PCR product was cloned into pQE30 vector. Protein was expressed in M15 *Escherichia coli* cells and localized to inclusion bodies, which were isolated as described (3). Briefly, cultures of bacteria-containing plasmid pHDP were grown to midlog phase, induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG, 1 mM) for 4 h at 37°C, and harvested by centrifugation at 4,000 × *g* for 20 min. The total cell pellet was resuspended in wash buffer (50 mM Tris·HCl at pH 7.5, 20 mM EDTA) containing 0.5 mg/mL lysozyme and incubated for 1 h at room temperature with intermittent shaking, and then with vigorous shaking for an additional 30 min. The washed cell pellet was lysed after adding wash buffer containing 0.5 M NaCl and 2.5% Triton X-100. The inclusion bodies were pelleted by centrifugation at 13,000 rpm for 50 min at 4°C, resuspended in wash buffer containing 1% Triton X-100 using a sonicator, pelleted again, and then washed four times in wash buffer without Triton X-100. The inclusion bodies were solubilized for 30 min in 50 mM CAPS buffer at pH 11.0 containing 1.5% *N*-lauryl sarcosine and 0.3 M NaCl and centrifuged at 10,000 × *g* for 30 min. The protein was purified from the supernatant using a His-Trap, a high-performance nickel affinity column (GE Health Care) by an imidazole gradient in 50 mM CAPS at pH 11.0 containing 0.3% *N*-lauryl sarcosine and 0.3 M NaCl. Protein-containing fractions were pooled and dialyzed against 25 mM CAPS buffer (pH 11.0) containing 135 mM NaCl. The activity of the protein was assessed by its ability to convert heme to hemozoin (Hz) (3).

Recombinant falcipain 2 was prepared as described by Shenai et al., with slight modifications (4). Briefly, bacteria containing plasmid pQE30-falcipain 2 were grown to midlog phase and induced with 0.5 mM IPTG for 5 h at 37°C. The cells were harvested, washed with ice-cold 100 mM Tris·HCl at pH 7.4 and 10 mM EDTA buffer, sonicated (12 cycles of 10 s each, with cooling for 10 s between the cycles), and centrifuged at 15,000 rpm for 45 min at 4°C. The washed pellet was solubilized in 6 M guanidine HCl, 20 mM Tris·HCl, 250 mM NaCl, and 20 mM imidazole at pH 8.0 (5 mL/g of inclusion body pellet) at room temperature (RT) for 60 min with gentle stirring. Insoluble material was separated by centrifuging at 15,000 rpm for 60 min at 4°C. Recombinant protein was purified from the supernatant using a nickel-nitrilotriacetic acid (Ni-NTA+) resin (Qiagen). The bound proteins were eluted with 1 M imidazole in a 20-mM Tris·HCl at pH 8.0 buffer containing 8 M urea, and quantified using Bradford assay (Pierce).

For protein refolding, the fractions containing falcipain 2 protein were pooled and diluted 100-fold in ice-cold refolding buffer containing 100 mM Tris·HCl at pH 8.0, 1 mM EDTA, 20% glycerol, 250 mM L-arginine, 1 mM reduced glutathione (GSH), and 1 mM oxidized glutathione (GSSG). The diluted protein was stirred moderately at 4°C for 24 h, concentrated to 25 mL using a stirred cell with a 10-kDa cutoff membrane (Pellicon XL device, Millipore) at 4°C, and then filtered using a 0.22-μm syringe filter. Falcipain 3 protein was also similarly expressed and refolded, as described by Sijwali et al. (5). Falcipain 2 and falcipain 3 activities were assessed using a fluorimetric assay in which the release of 7-amino-4-methyl coumarin (AMC) was monitored (excitation, 355 nm; emission, 460 nm) over the course of 30 min at RT, using a LS50B Perkin-Elmer fluorimeter in a buffer containing 100 mM sodium acetate at pH 5.5, 10 mM dithiothretol (DTT), 7 μM fluoregenic substrate Z-Phe-Arg 7-amino-4-methyl coumarin (Z-FR-AMC), and 200 nM enzyme (6). As an assay for the evaluation of Hb hydrolysis, the protein (30 μg) was incubated with 50 nM enzyme (recombinant active falcipain 2) in 100 mM sodium acetate at pH 5.5 and 5 mM reduced glutathione at 37°C for 3 h. Absorbance at 410 nm was monitored with a spectrophotometer. The absorbance of each sample, expressed as percentage of absorbance of Hb with buffer only, was subtracted from 100 to give the percentage hydrolysis.

Generation of Antibodies Against HDP, Falcipain 2, and Falcipain 3 Recombinant Proteins. The animals were housed and handled in accordance with the institutional and national guidelines. The animals were bred under the guidelines of the authorizing committee, and BALB/c inbred mice were used for raising the antibodies against HDP and falcipain 3 protein. Female rabbits were used for raising the antibodies against the falcipain 2 protein. Mice were immunized with 20 μg antigen (90%–95% pure) and rabbits were immunized with 300 μg protein in the presence of complete/incomplete Freund's adjuvant, using the i.p. and s.c. modes of injection, respectively. Antibody titer in serum samples were quantified by ELISA. The animals were killed after the third and/or last bleed. Antibody to plasmepsin IV was raised in mice with keyhole limpet hemocyanin (KLH)-conjugated peptide with a sequence corresponding to its protease domain (CQKANAVVDSGTSTITAPTS). Plasmepsin II Rabbit Antiserum 737, MRA-66 was obtained through the Malaria Research and Reference Reagent Resource center (MR4), American type culture collection (ATCC) as part of the BEI Resources Repository, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The specificity of the antibodies were analyzed on the recombinant proteins and the parasite lysate.

Isolation of Food Vacuole. Food vacuole isolation was carried out as described previously (7). Briefly, 100 μL of saponin-lysed trophozoites were suspended in 10 volumes of ice-cold water at pH 4.5 and immediately passed four times through a 27-G, 1.2-cm needle. This preparation was centrifuged at 13,000 rpm in a microfuge for 2 min. The pellet was resuspended in 1 mL buffer M at pH 7.4 containing 2 mM $MgSO_4$, 100 mM KCl, 10 mM NaCl, 25 mM Hepes, 25 mM $NaHCO_3$, and 5 mM sodium phosphate, to which 10 μL of 5 mg/mL DNase 1 was added and incubated at 37°C for 5 min. The suspension was microfuged for 2 min at 13,000 rpm, and the pellet was resuspended in 100 μL of ice-cold buffer M followed by addition of 1.3 mL of ice-cold 42% Percoll (Sigma) containing 0.25 M sucrose and 1.5 mM $MgSO_4$ at pH 7.4. After passing twice through a 27-G, 1.2-cm needle, the sample was microfuged at 13,000 rpm at 4°C for 10 min. The purified vacuoles were evident as a dark band in the bottom 50 μL of the gradient. These were collected, resuspended in 1 mL of

buffer M, and microfuged at 13,000 rpm for 2 min to remove the Percoll.

Microscopy. Immuno-electron microscopy was carried out on trophozoite stage of *P. falciparum* parasites. Briefly, parasites were fixed in 4% paraformaldehyde, 0.04% glutaraldehyde in 1× PBS at 4°C for 1 h and subsequently embedded in gelatin and infiltrated with a cryo-preservative and plasticizer (2.3 M sucrose/20% polyvinyl pyrrolidone). After freezing in liquid nitrogen, samples were sectioned with a Leica Microsystems Ultracut UCT cryo-ultramicrotome at −260°C. Ultrathin sections were blocked with 5% FBS and 5% normal goat serum in 1× PBS for 30 min and subsequently stained with rabbit anti-falcipain 2 antibody (1:500 dilution in blocking buffer), washed thoroughly, and incubated with 18 nm colloidal gold-conjugated anti-rabbit IgG. Sections were stained with 0.3% uranyl acetate/1.7% methyl cellulose and visualized under a JEOL 1200EX transmission electron microscope. All labeling experiments were conducted in parallel, with controls omitting the primary antibody or using preimmune sera as primary antibodies.

Analysis of Protein Interaction by ELISA. In vitro interaction between HDP and falcipain 2 was examined by an ELISA. Briefly, 96-well microtiter plates were coated overnight at 4°C with 200 ng of falcipain 2 or HDP. After blocking the wells with 3% BSA in PBS, recombinant HDP and falcipain 2 were, respectively, added in different concentrations ranging from 0 to 0.6 μM, and the plates were incubated for 2 h. Bound proteins were detected through polyclonal sera raised against that protein for 1 h. Incubation with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibodies (1:3,000) was done for 1 h and quantified after adding the substrate *o*-phenylenediamine dihydrochloride (Sigma) by measuring the resulting absorbance at 450 nm in an ELISA microplate reader.

For the binding study, Hb (200 ng) was coated on 96-well microtiter plate, and falcipain 2 treated with E-64 (2 μM) was added at various concentrations (0–0.5 μM) both without and with chloroquine, artemisinin, and pyrimethamine (0–5 μM). Bound proteins were detected by using polyclonal antibodies against falcipain 2 and quantified spectrophotometrically, as described earlier.

In Vitro Coimmunoprecipitation. Briefly, 1 μg of each protein was incubated together at room temperature for 2 h in a reaction volume of 100 μL containing 1× binding buffer (50 mM phosphate buffer at pH 7.0, 75 mM NaCl, 2.5 mM EDTA at pH 8.0, and 5 mM $MgCl_2$), 0.1% Nonidet P 40, and 10 mM DTT. The reaction mix was incubated for 2 h at 4°C with 20 μL of pre-equilibrated Protein A/G conjugated antibody beads. The beads were then centrifuged at 1,000× *g* for 5 min, washed with 200 μL of binding buffer containing 400 mM NaCl, boiled for 5 min in SDS/PAGE reducing loading buffer, electrophoresed, immunoblotted, and probed. Protein A/G beads conjugated to preimmune sera were used as a negative control.

Comparative Analysis of Various Hz Mediators in an in Vitro Assay. Various Hz mediators such as mono palmitoyl glycerol (MPG), mono oleoyl glycerol (MOG), oleic acid (OA) (1–1,200 μM), histidine rich protein-2 (HRP-2) (0.1–10 μM), and HDP (0.1–0.5 μM) were added in a 1-mL reaction buffered with sodium acetate at pH 5.2 containing a fixed concentration of freshly prepared heme (300 μM) for a reaction time of 3 h. The stocks of the lipids were prepared in chloroform, and the required amount was dispensed into microfuge tubes. The solvent was evaporated under nitrogen, and lipids were suspended in assay buffer with sonication to form a lipid emulsion, as described by Pisciotta et al. (8). The lipids were also extracted from *P. falciparum*–purified digestive vacuole by the method of Bligh and Dyer (9). Briefly, purified food vacuoles (100 μL) were suspended in PBS and freeze thawed. To this, 1:2 chloroform:methanol was added and vortexed well. Further chloroform and water was added and centrifuged at 1,000 rpm for 5 min at room temperature. The lipids were extracted in the bottom organic phase. The solvent was evaporated under nitrogen, and lipids were resuspended in assay buffer with a 30-s sonication. The Hz formation reaction was incubated at 37°C. The Hz formed was washed and quantified spectrophotometrically, as described in *Methods*. In the presence of falcipain 2 and Hb, various Hz mediators mentioned earlier were added independently and in different combinations to assess their ability to convert Hb to Hz.

CD Spectroscopy. The CD spectra of chloroquine, falcipain 2, and chloroquine added to falcipain 2 were measured at 25°C between 190 and 300 nm using a Jasco J-715 spectrapolarimeter. The path length of the cell was 1 cm. Experiments were performed in 10 mM phosphate buffer at pH 7.0. All of the spectra are the average of multiple scans (6).

Protein Identification with Liquid Chromatography Mass Spectrometry (LC-MS/MS) and MALDI-TOF/TOF. The proteins were digested in gel overnight with sequencing-grade modified trypsin (Promega); the resulting peptides were extracted with a 50% acetonitrile and 5% formic acid solution, dried in a SpeedVac (Thermo Scientific), and purified using C-18 beads (magnetic Dynabeads RPC18, Invitrogen). The peptides were fractionated by nanoflow liquid chromatography in a 10-cm-long × 75-μm–inner diameter C18 capillary column (Sigma) in line with the electrospray ionization tandem mass spectrometer and were eluted at a 200 nL/min flow rate with a 45-min linear gradient of 5%–40% acetonitrile (ACN) in 0.4% acetic acid followed by a 5-min linear gradient of 40%–80% ACN in 0.4% acetic acid.

Xcalibur 1.4 SR1 software was used to collect mass spectrometry data. The mass range for the MS survey scan was m/z 400–1,400. The MS/MS data of the five most intense ions were collected sequentially after each MS scan, using the dynamic exclusion parameter in which a specific ion was sequenced only twice and was excluded from the list for 45 s. The peak lists were generated using the SEQUEST module of Bioworks 3.1, cluster version SR1 (Thermo Electron), using the default parameters (molecular mass range, 400–3,500 Da; precursor mass tolerance, 1.4; group scan, 25; minimum ion count, 15). The acquired data were compared with the Plasmo DB using the Turbo SEQUEST program in the Bioworks Browser 3.1 software package (Thermo Electron). The dataset presented here includes only the doubly tryptic peptides that have minimum peptide identification probability of 0.9 and have a minimum SEQUEST X-correlation value of 1.5 for +1 ions, 1.8 for +2 ions, and 2.5 for +3 ions.

The MALDI TOF/TOF analysis used an Ultraflex III machine (Bruker Daltonics) (10). The proteins were digested in gel, and the pellets obtained after SpeedVac were dissolved in 0.5% TFA and loaded onto MALDI target plate by mixing 1 μl of each sample with the same volume of matrix solution (10 mg/mL α-cyanohydroxycinnamic acid in ACN/0.1% TFA 1:1) and allowed to dry. Samples and calibration standards with the same matrix composition were spotted adjacent to each other on the target plate for the optimal calibration. Spectra were externally calibrated with the calibration standard containing the following peptide mass: (M+H)+ Angiotensin_II -1046.54180, Angitensin_I-1296.684780, Substance_P-1347.735430, Bombesin 1619.822350, Renin_Substrate-1758.932610, adrenocorticotropic hormone (ACTH) (1–17)-2093.086170, ACTH(18-39)-2465.198340, ACTH (1–24)-2932.590000, and Insulin b-chain- 3494.650780. Measurements were performed in the positive ion reflector mode with the accelerating voltage of 25 kV (ion source 1) and 21.85 kV (ion source 2), respectively. The laser wavelength and frequency were 337 nm and 100 Hz, respectively. The final mass spectra were

produced by averaging 1,500 laser shots. The spectra were processed and internally calibrated with trypsin autolysis peaks. For the peptide mass fingerprinting–based identification of proteins, the tryptic peptide mass maps (monoisotopic) were searched against MSDB and PlasmoDB by using the Biotools version 3.2 (build 1.31) (Bruker Daltonics). Standard search parameters were set to allow a mass tolerance of up to 100 ppm and 1 missed tryptic cleavage with no restriction in protein molecular weight.

Immunoprecipitation and Immunoblotting. The lysate for immunoprecipitation was prepared from purified food vacuoles in buffer P (10 mM Tris·HCl, 100 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1× protease inhibitor mixture (Roche Diagnostics), and 5% (vol/vol)glycerol at pH 7.5). The lysate was centrifuged at 13,000 × g at 4°C, and the supernatant was incubated with anti-falcipain 2, anti-HDP, or preimmune antisera crosslinked to Protein A/G Sepharose beads (Pierce) by disuccinimidyl suberate (DSS) with end-to-end shaking at 4°C for 12 h. The beads were washed several times with buffer P, eluted with glycine-HCl buffer at pH 2.8, and examined by 12% SDS/PAGE followed by silver nitrate or Sypro Ruby (Invitrogen) staining. The proteins were identified by liquid chromatography mass spectrometry (LC-MS/MS) analysis using a linear trap quadrupole (LTQ) mass spectrometer connected to a Surveyor HPLC system (Thermo Electron). Western blots of the eluted immunoprecipitates were probed with anti-falcipain 2, anti-plasmepsin IV, anti-plasmepsin II, and anti-HDP antibodies.

Gel Permeation Chromatography. Food vacuoles were isolated and suspended in 1× PBS containing a protease inhibitor mixture (Roche), lysed by three freeze thaw cycles, and clarified by centrifugation at 20,000 × *g* for 30 min at 4°C. Cleared lysate (~5 mg protein) was fractionated on Superose-6 HR 10/300 column (Amersham) equilibrated with the lysis buffer (11). Fractions of 0.75 mL were collected for a total of two column volumes and analyzed by Western blotting using anti-falcipain 2, anti-plasmepsin II, anti-plasmepsin IV, and anti-HDP antibodies. The column was routinely calibrated using commercially available standards. Those used for calibration were thyroglobulin (660 kDa), ferritin (440 kDa), catalase (220 kDa), aldolase (158 kDa), and BSA (66 kDa) purchased from Amersham Bioscience. Recombinant proteins falcipain 2 and HDP were run on a Superdex-75 column (GE Healthcare Life Sciences) with the molecular mass standards including BSA (66 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and lysozyme (14 kDa).

Glycerol Gradient Centrifugation. Isolated food vacuoles from midtrophozoite parasite lysate were lysed in 0.5% Nonidet P-40/Hepes-buffered saline (10 mM Hepes at pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, and protease inhibitors). Lysate was cleared by centrifugation, and 500 μL of lysate was layered on top of a 9-mL 5–45% step glycerol gradient. Gradients were centrifuged at 38,000 rpm for 18 h at 4°C in a SW41 rotor (Beckman). The molecular mass standards BSA (66 kDa), catalase (220 kDa), ferritin (440 kDa), and thyroglobulin (660 kDa) (Amersham Biosciences) were resuspended in 0.5% Nonidet P-40/Hepes buffer and run in parallel. Twenty 0.5-mL fractions were collected from each gradient, and equal volumes of each fraction were mixed with loading dye. Protein samples were resolved by SDS/PAGE and analyzed by Western blotting using anti-falcipain 2, anti-plasmepsin II, anti-plasmepsin IV, and anti-HDP antibodies.

Magnetic Separation of Hz. The LD magnetic column (Miltenyi Biotec, Germany) was loaded onto a magnetic activated cell sorting (MACS) separator, and the column was washed with 2 mL degassed buffer A (PBS at pH 7.2, 0.5% BSA, and 2 mM EDTA). The isolated food vacuoles were resuspended in 500 μL of the same buffer and applied to the LD column. The unbound proteins were eluted by washing twice with 1 mL of degassed buffer A. The isolation of magnetically associated Hz proteins was carried out by removing the column from the separator and eluting the bound proteins with 2 mL of buffer A, using the plunger supplied with the column. Hz was recovered by centrifugation for 15 min at 12,500 × *g*, and the pellet was resuspended in 100 μL water.

Hz Formation Assay in the Presence of Antimalarial Drugs. The assay, as mentioned in *Methods*, was also performed in the presence of chloroquine, artemisinin, and pyrimethamine. Initially, falcipain 2 was treated with the drug for 10 min at room temperature and then added to Hb in the presence of HDP to allow the Hz formation. In a different reaction, falcipain 2 was initially allowed to digest Hb, and later drugs were added to see their binding to released heme. Subsequently, HDP was added to this reaction to allow Hz formation. The amount of Hz produced was then calculated as described in *Methods*. The percentage inhibition of Hz formation by drugs was fit to standard growth curve or dose–response equations in GraphPad Prism, version 5.0 (nonlinear least-squares analysis) for the calculation of IC_{50}.

Infrared Spectroscopy. To obtain IR spectra, KBr (potassium bromide) pellets were prepared from dried samples of Hz prepared from Hb, and spectra were acquired for 25 cycles with a Fourier-transform IR spectrometer (Perkin-Elmer model 1800), as described earlier (11).

X-Ray Powder Diffraction. X-ray diffraction pattern was acquired with the X-ray diffractometer X′ Pert PRO (PANalytical) in the 2 θ range 5°–30° with Cu K_a radiation (λ = 1,540562 Å), operating at 30 mA and 40 kV, with a step size of 0.0170° and scan step time of 2 s.

Scanning Electron Microscopy. SEM pictures were acquired using a Hitachi S-4700 FEG_SEM. The Hz prepared from two recombinant proteins using Hb was coated with Au/Pd of about 4 Å in thickness before visualization at 2 kV and 10 mA.

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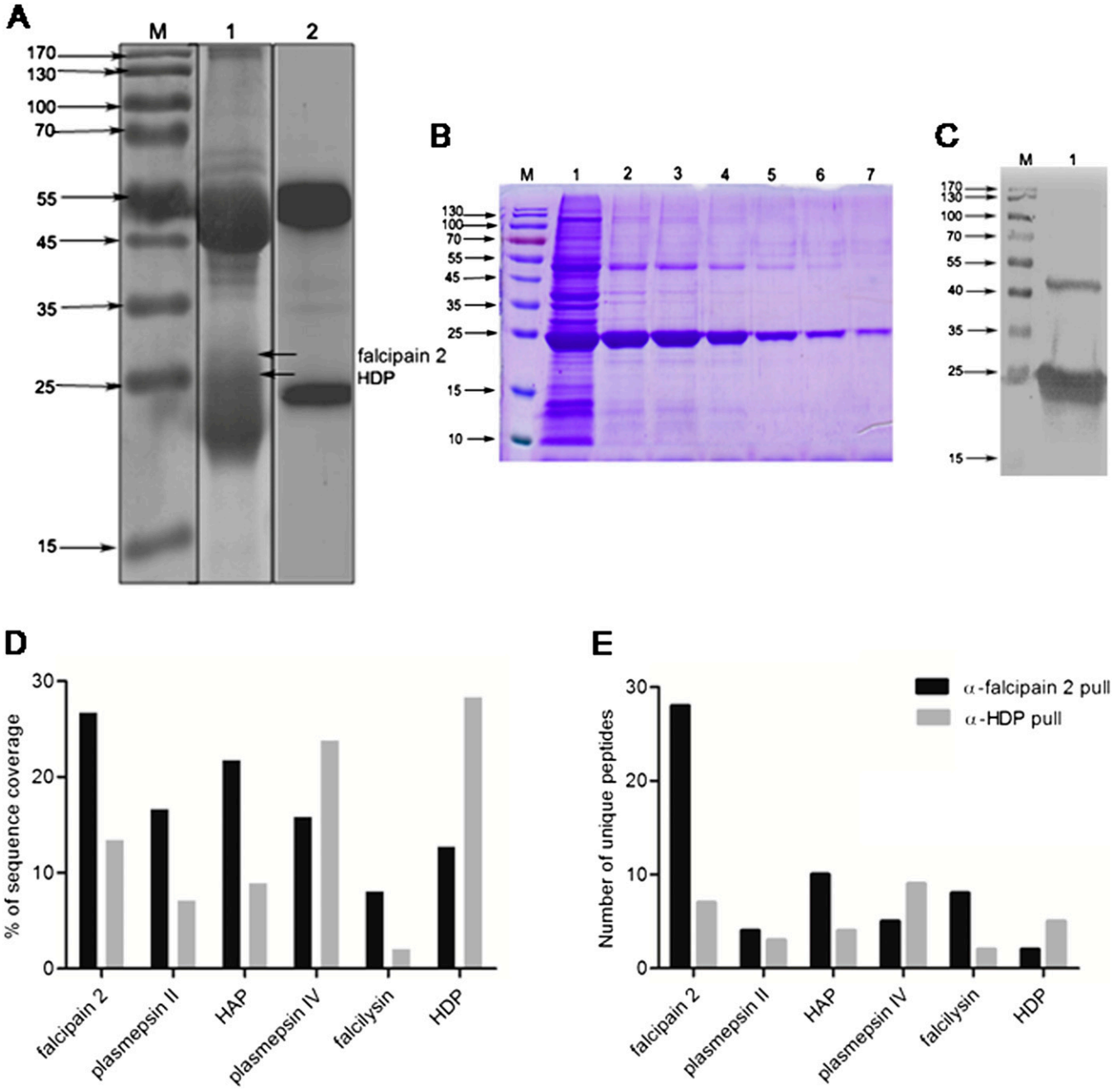

Fig. S1. Expression of HDP and association with hemoglobinases. (*A*) Silver-stained SDS/PAGE of proteins immunoprecipitated from purified food vacuole lysate using anti-falcipain 2 antibody (lane 1) or preimmune sera control (lane 2). Lane M, molecular mass markers. Arrows indicate the positions of falcipain 2 and HDP. (*B*) Coomassie Blue stained reducing 12% SDS/PAGE of HDP expressed in *E. coli* and purified on a Ni-NTA+ column. Lane 1, flow through; lanes 2–4, 50-mM imidazole eluates; lanes 5–7, 100-mM imidazole eluates; and lane M, molecular mass markers. (*C*) Western blot analysis using anti-HDP antibody. (*D* and *E*) Percentage of sequence coverage and number of unique peptides for each protein identified by LC-MS/MS from lanes 2 and 3 of the Sypro Ruby–stained gel.

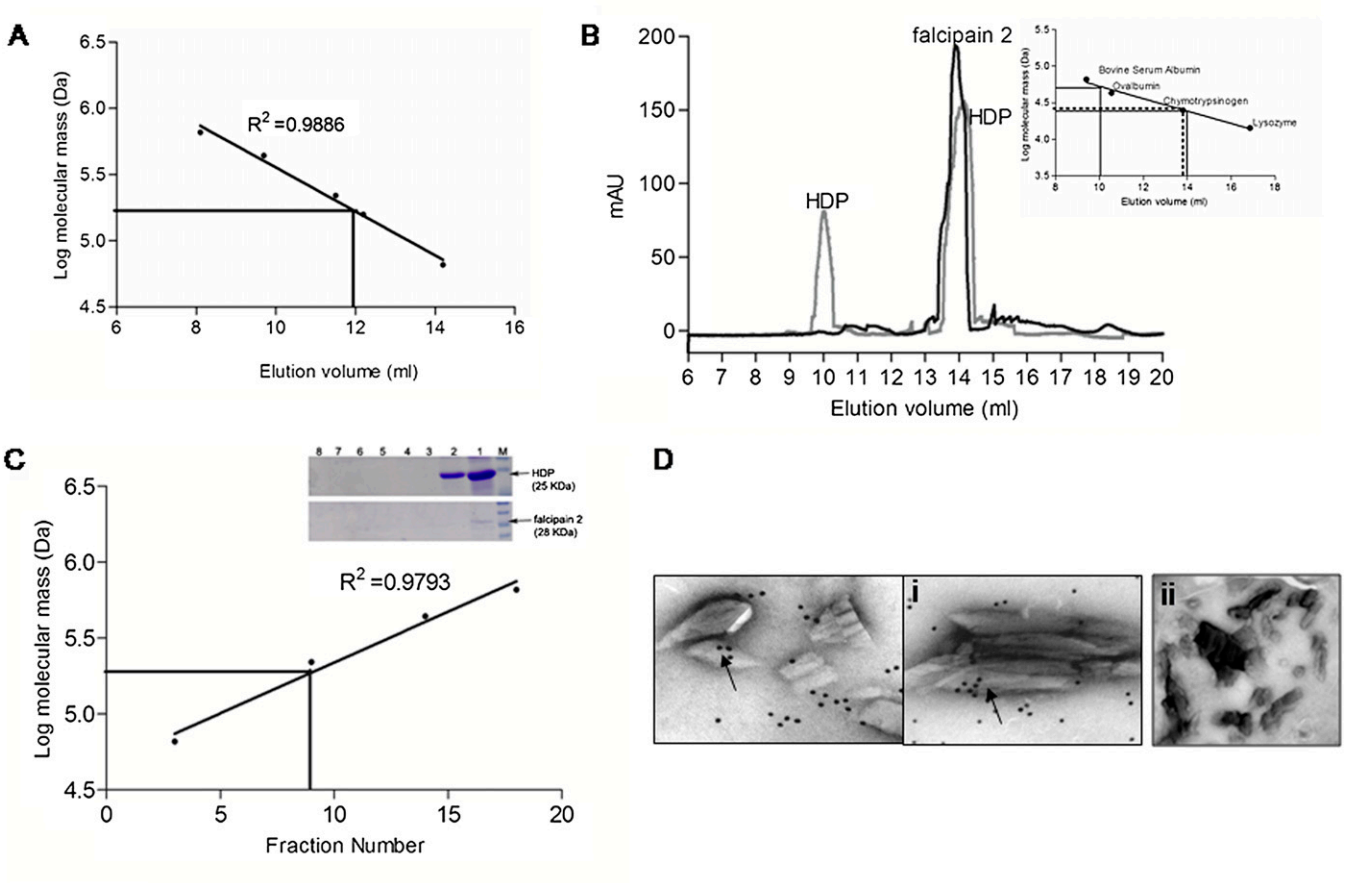

Fig. S2. Native complex of falcipain 2 and its localization with Hz. (*A*) Molecular mass calibration curve for the size determination of native falcipain 2 complex by gel permeation chromatography of food vacuole extract using a Superose-6 column. The molecular mass standards used to calibrate the column were thyroglobulin (660 kDa), ferritin (440 kDa), catalase (220 kDa), aldolase (158 kDa), and BSA (66 kDa). The peaks of falcipain 2, plasmepsin II, plasmepsin IV, and HDP from the food vacuole lysate in fraction 16 (elution volume 12 mL) are marked on the curve and correspond to the antilog of 5.25 and molecular mass of 191 kDa. (*B*) The recombinant proteins falcipain 2 and HDP were run on the Superdex-75 column. Falcipain 2 eluted at a size corresponding to 28 kDa as a monomer. HDP eluted at a size corresponding to 25 kDa (monomer) and 50 kDa (dimer). The inset shows the molecular mass calibration curve for the Superdex-75 column and the molecular mass standards used to calibrate the column: lysozyme (14 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), and BSA (66 kDa). The dotted line presents the elution of falcipain 2 and the black line presents the elution of HDP as monomer and dimer on the column. (*C*) The sedimentation curve of the molecular mass standards, BSA (66 kDa), catalase (220 kDa), ferritin (440 kDa), and thyroglobulin (660 kDa), run on a glycerol gradient. The sedimentation peaks of falcipain 2, plasmepsin II, plasmepsin IV, and HDP from the food vacuole lysate in fraction 9 are marked on the curve and correspond to the antilog of 5.29 and molecular mass of 198 kDa. The inset shows the sedimentation of recombinant proteins falcipain 2 in fraction 1 and HDP in fraction 1 and 2 of glycerol gradient. (*D*) Immuno-electron microscopy of trophozoite stage parasites with (*i*) anti-falcipain 2 antibody and (*ii*) preimmune sera. The arrows indicate the Hz crystals.

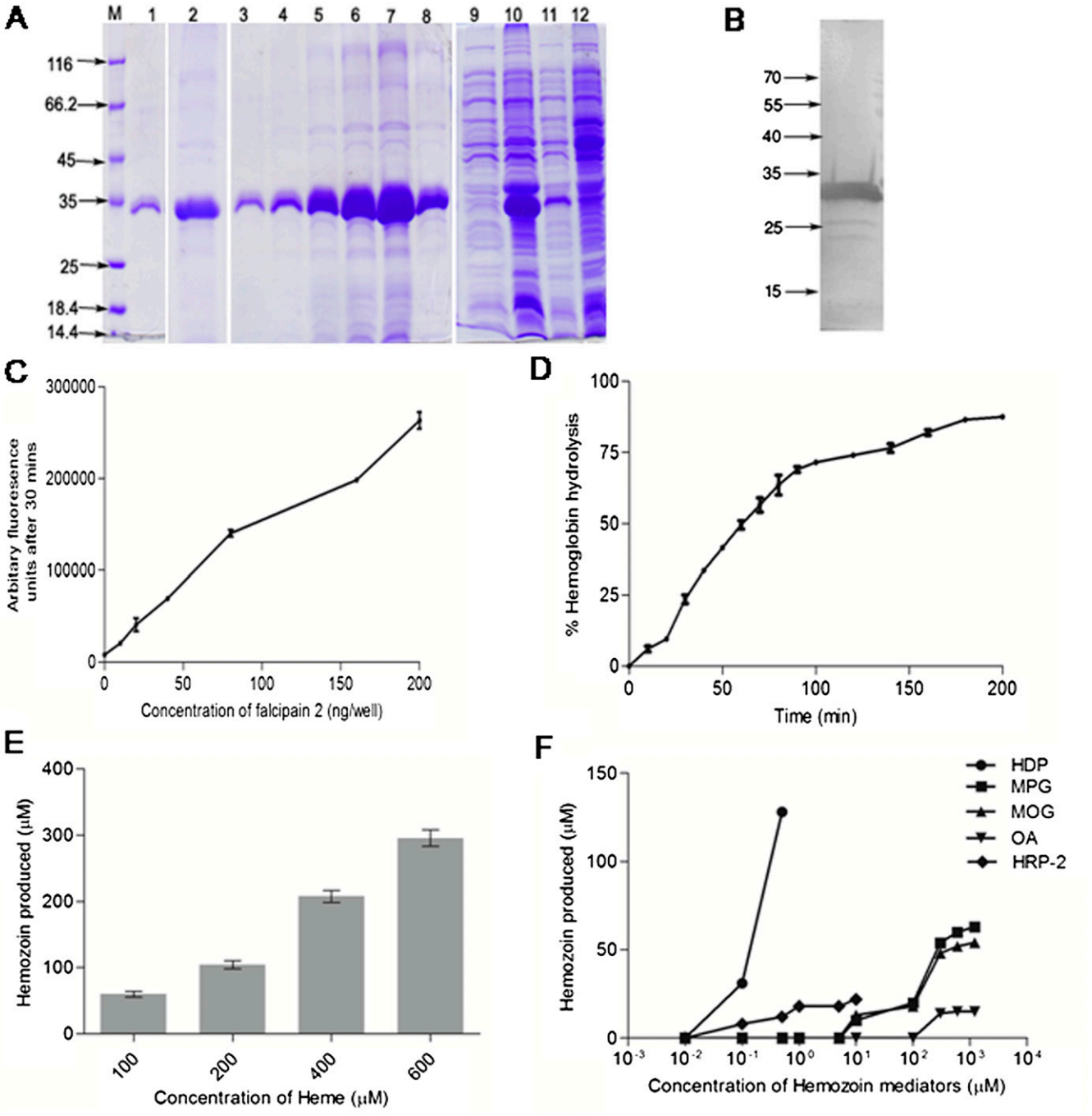

Fig. S3. Expression and activity analysis of recombinant falcipain 2 and HDP. (*A*) Falcipain 2 was expressed in *E. coli* and examined on reducing 12% SDS-polyacrylamide gel stained with Coomassie Blue. Lane 1, refolded and activated falcipain 2; lanes 2–8, Ni-NTA+ eluates; lane 9, soluble fraction; lane 10, insoluble fraction; lane 11, *E. coli* induced with IPTG; lane 12, uninduced *E. coli*; and lane M, molecular mass markers. (*B*) Western blot analysis using anti-falcipain 2 antibody. (*C*) Proteolytic activity of falcipain 2 with a 7-amino-4-methylcoumarin substrate in a concentration-dependent manner. (*D*) Spectrophotometric assessment of Hb hydrolysis. Native Hb (30 μg) was incubated with falcipain 2 (50 nM), and absorbance taken at 410 nm, indicative of disruption of Hb, was monitored over time. (*E*) Concentration-dependent Hz production from heme by 0.5 μM recombinant HDP. (*F*) Comparative Hz production from heme by different concentrations of Hz mediators: MOG, MPG, O, HRP-2, and HDP. Error bars, SD of triplicate measurements. HDP, heme detoxification protein; HRP-2, histidine-rich protein 2; MOG, mono oleoyl glycerol; MPG, mono palmitoyl glycerol; and OA, oleic acid.

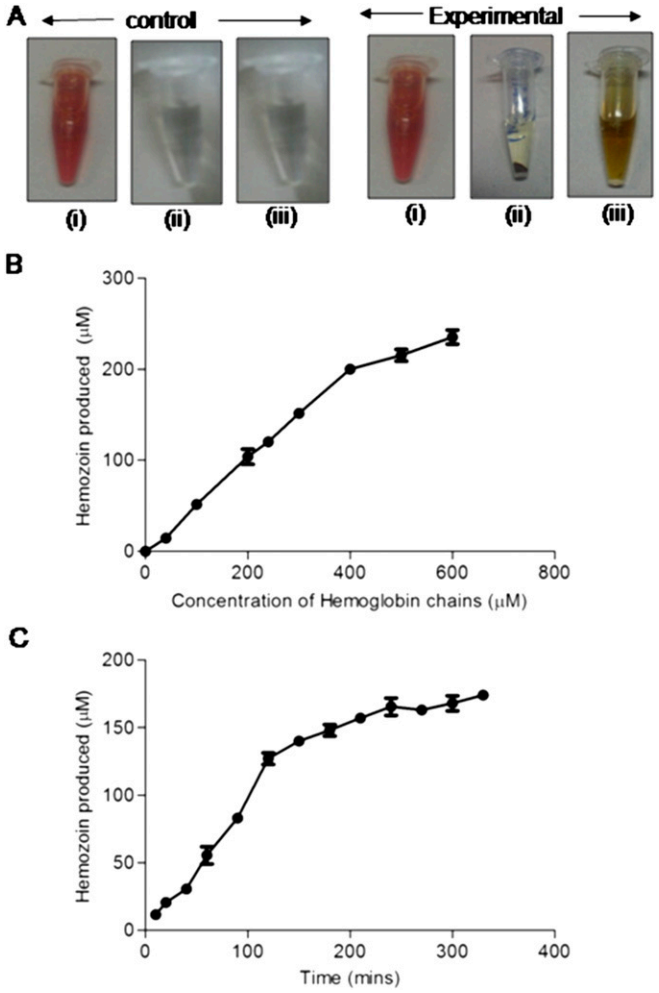

Fig. S4. Hz formation from Hb mediated by falcipain 2 and HDP recombinant proteins. (*A*) In the control reaction, either falcipain 2 or HDP was added to Hb (*i*). Hz formation was not observed with either of the proteins alone (*ii* and *iii*). In the experimental reaction, (*i*) falcipain 2 and HDP (0.5 μM each) were incubated together with purified Hb chains (300 μM) in the reaction at 0 h. (*ii*) Hz formation was observed after incubation of the reaction for 3 h. (*iii*) Solubilized Hz in 0.1 N NaOH for the estimation of the amount of Hz formed. (*B* and *C*) In vitro Hz formation assay showing concentration-dependent and time-dependent Hz formation from Hb chains mediated by falcipain 2 (0.5 μM) and HDP (0.5 μM). Error bars, SD of triplicate measurements.

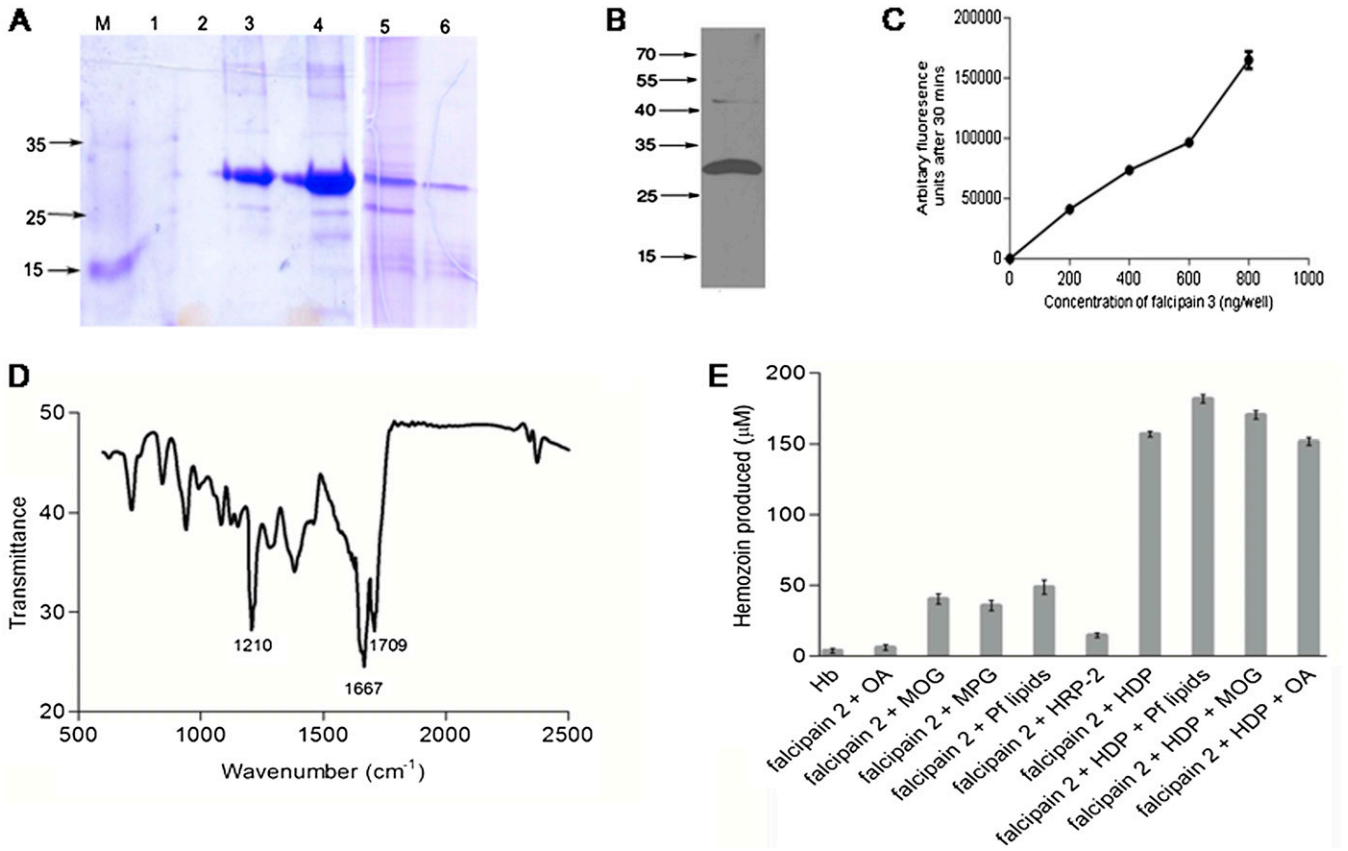

Fig. S5. Recombinant active falcipain 3 expression and Hz characterization. (*A*) Falcipain 3 was expressed in *E. coli* and examined on reducing 12% SDS/PAGE stained with Coomassie Blue. Lane 1, flow through; lane 2, 50-mM imidazole eluate from Ni-NTA+; lane 3, 250-mM imidazole elute from Ni-NTA+; lane 4, refolded falcipain 3; lane 5, refolded falcipain 3 after dialysis; lane 6, activated falcipain 3 using sodium acetate (pH 5.2); and lane M, molecular mass markers. (*B*) Western blot analysis using anti-falcipain 3 antibody. (*C*) Proteolytic activity of falcipain 3 with 7-amino-4-methylcoumarin substrate in a concentration-dependent manner. (*D*) Infrared spectra of the Hz produced in vitro from Hb. (*E*) Comparison of Hz mediators MOG, MPG, OA, HRP-2, and HDP in combination with falcipain 2 for producing Hz from Hb. Parasite-extracted lipids (Pf lipids) from the midtrophozoite stage of *P. falciparum* were used in the assay with falcipain 2. These Pf lipids, along with HDP and falcipain 2, showed a partial additive effect on Hz production from the heme released by falcipain 2. Error bars, SD of triplicate measurements. HDP, heme detoxification protein; HRP-2, histidine-rich protein 2; MOG, mono oleoyl glycerol; MPG, mono palmitoyl glycerol; and OA, oleic acid.

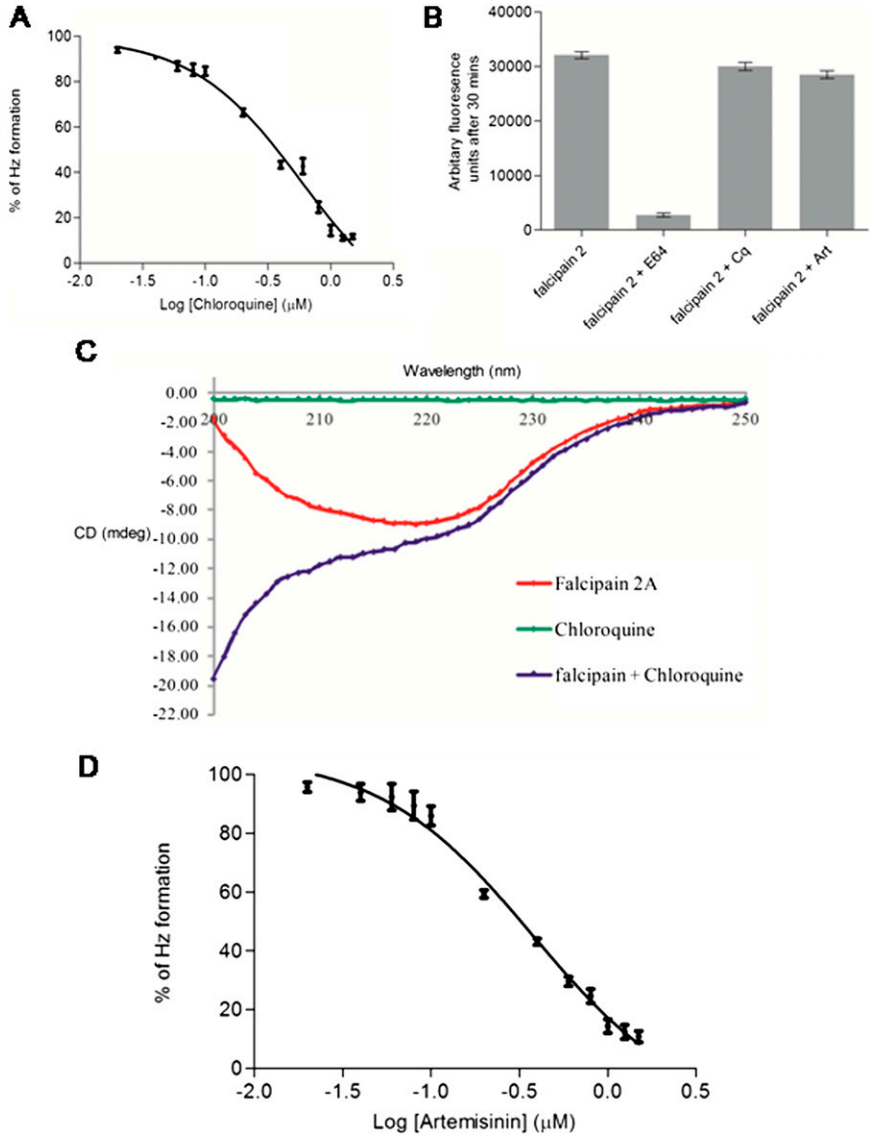

Fig. S6. Effect of chloroquine and artemisinin. (*A*) Concentration-dependent effect of chloroquine on falcipain 2 during the in vitro Hz formation from Hb. The black line corresponds to the approximate IC_{50} value of 590 nM. (*B*) Proteolytic activity of recombinant falcipain 2 on 7-amino-4-methylcoumarin substrate in the presence of E-64 (10 μM), chloroquine (10 μM), and artemisinin (10 μM). (*C*) CD analysis of falcipain 2 alone and in the presence of chloroquine (1 μM). (*D*) Concentration-dependent effect of artemisinin in the in vitro assay of Hz formation from Hb with the approximate IC_{50} value of 380 nM.

Table S1. Identification of malarial proteins immunoprecipitated using anti-falcipain 2 antibody by MALDI-TOF/TOF

Sample no.	Protein	Plasmo DB accession no.	Molecular mass, kDa		Sequence coverage, %	Masses matched/no. of peptides sequenced by MS/MS*	MOWSE (Molecular Weight Search) score
			Proenzyme	Active enzyme			
1	Falcipain 2/2′	PF11_0165/ PF11_161	56.4	28	21	8*	812
2	Plasmepsin IV	PF14_0075	51.2	37	35	7*	405
3	Plasmepsin II	PF14_0077	41.9	37	27	7*	592
4	Heme detoxification protein	PF14_0446	24.5	25	60	15/23	209

Table S2. Sequences of identified peptides corresponding to anti-falcipain 2 antibody immunoprecipitation

Sample no.	Protein name	Peptide matches/peptides sequenced by MS/MS*	Position of the peptide sequence (amino acid)
1	Falcipain 2/2′	K.YKGNENFDHAAYDWR.L*	254–268
		K.GNENFDHAAYDWR.L*	256–268
		K.LITLSEQELVDCSFK.N*	305–319
		K.HYYYIIK.N*	440–446
		K.NSWGQQWGER.G*	447–456
		R.GFINIETDESGLMR.K*	457–470
		R.GFINIETDESGLMR.K* Oxidation (M)	457–470
		R.KCGLGTDAFIPLIE.-*	471–484
2	Plasmepsin IV	K.HLYDASASK.S*	177–185
		K.VEISYGSGTVR.G*	194–204
		K.DVISLGDLSLPYK.F*	210–222
		K.DLSIGSIDPVVVELK.K*	251–265
		K.IDNALFTFYLPVHDK.H*	270–284
		K.ANAVVDSGTSTITAPTSFLNK.F*	330–350
		R.KYFTVFDYEK.E*	429–438
		K.YFTVFDYEK.E*	430–438
3	Plasmepsin II	K.CTTAGCLTK.H *	93–101
		K.DLVTVGNLSLPYK.F *	130–142
		K.DLSIGSVDPIVVELK.N *	171–185
		K.IENALFTFYLPVHDK.H *	190–204
		K.HTGFLTIGGIEER.F *	205–217
		R.FYEGPLTYEK.L *	218–227
		K.VPFLPFYVTLCNNSK.L *	280–294
		K.LPTFEFTSENGK.Y *	295–306
4	Heme detoxification protein	-.MKNRFYYNLIIK.R Oxidation (M)	1–12
		R.FYYNLIIKR.L	5–13
		R.SGGLRKPQKVTNDPESINR.K	18–36
		R.KPQKVTNDPESINR.K	23–36
		R.KPQKVTNDPESINRK.V	23–37
		K.VYWCFEHKPVK.R	38–48
		K.VYWCFEHKPVKR.T	38–49
		K.RTIINLIYSHNELK.I	49–62
		R.TIINLIYSHNELK.I	50–62
		K.EYWLYRDLYGSSYQPWLMYNEKR.E	126–148
		R.DLYGSSYQPWLMYNEK.R	132–147
		R.NLLNNDLIVK.I	156–165
		K.HCNHSIYLNGSK.I	172–183
		K.HCNHSIYLNGSKIIRPNMK.C	172–190
		K.CHNGVVHIVDKPIIF	191–205

Table S3. Sequences of identified peptides corresponding to anti-falcipain 2 and anti-heme detoxification protein antibody immunoprecipitation using liquid chromatography mass spectrometry (LC-MS/MS)

		Peptide sequenced	
Sample no.	Protein name	Anti-falcipain 2	Anti-heme detoxification protein
1	Falcipain 2/2′	GFINIETDESGLM[147]R	
		LITLSEQELVDCSFK	
		LITLSEQELVDC[160]SFK	
		NSWGQQWGER	GFINIETDESGLM[147]R
		YLLDQMNYEEVIK	LITLSEQELVDC[160]SFK
		YLLDQM[147]NYEEVIKK	HYYYIIK
		GFINIETDESGLM[147]R	NYLSVPDNK
		FADLTYHEFK	EIVNPLTK
		NYLSVPDNK	NYLSVPDNKLK
		HYYYIIK	QMNYEEVIKK
		EIVNPLTK	
		YLLDQM[147]NYEEVIK	
		FADLTYHEFK	
		FLGPISISVAVSDDFAFYK	
2	Plasmepsin II		ANC[160]IVDSGTSAITVPTDFLNK
		LPTFEFTSENGK	DLVTVGNLSLPYK
		VEM[147]NYVSGTVSGFFSK	HTGFLTIGGIEER
		DLVTVGNLSLPYK	LPTFEFTSENGK
		M[147]LQNLDVIK	FYEGPLTYEK
		DLSIGSVDPIVVELK	DLSIGSVDPIVVELK
		FYEGPLTYEK	VPFLPFYVTLC[160]NNSK
			CTTAGCLTK
3	Plasmepsin III histo aspartic protease		DLSIGSIDPYIVELK
			GYLTIGGIEER
		DLANVLSFGEAK	NTFVLGDPFM[147]R
		DLSIGSIDPYIVELK	SEFDNVELKDLANVLSFGEAK
		GYLTIGGIEER	KANVILDSATSVITVPTEFFNQFVESASVFK
		IEQAVYSIYLPPENK	LGSEFDNVELKDLANVLSFGEAK
		LPTLEYR	TQNKIEQAVYSIYLPPENK
		NTFVLGDPFM[147]R	FFDGPLNYEK
		NTFVLGDPFM[147]RK	NTFVLGDPFM[147]RK
		AGTISGIFSK	AGTISGIFSK
		DLVTIGK	LPTLEYR
		SEFDNVELK	DLVTIGK
			VPFLSLYVTTC[160]GNTK
			ITVPTEFFNQFVESASVFK
4	Plasmepsin I		ANAVVDSGTSTITAPTSFLNK
			DLSIGSIDPVVVELKK
		ANAVVDSGTSTITAPTSFLNK	DVISLGDLSLPYK
		DVISLGDLSLPYK	IDNALFTFYLPVHDK
		VEISYGSGTVR	IDNALFTFYLPVHDK
		ANAVVDSGTSTITAPTSFLNK	VEISYGSGTVR
		DGTKVEISYGSGTVR	NTFILGDPFM[147]R
		NTFILGDPFM[147]R	FFRDM[147]NVIK
			FYEGPLTYEK
			NTFILGDPFM[147]RK
			NTFILGDPFMR
5	Falcilysin	NIGSM[147]SANVALYSK	
		SIDFVFEM[147]TSK	
		EITNLTYEEFK	RIENFNEQEKEQVIK
		HLFVNSNESLK	LQEQLELAENDFKTLENILVR
		NLM[147]VSVTSDYGALK	
		VSFNGIVYNEM[147]K	
		RIENFNEQEKEQVIK	
		FADLLESK	
6	Heme detoxification protein	DLYGSSYQPWLM[147]YNEK	DLYGSSYQPWLM[147]YNEK
		LSEFVLNHVTK	LSEFVLNHVTK
		TIINLIYSHNELK	TIINLIYSHNELK
		NLLNNDLIVK	NLLNNDLIVK
		FYYNLIIK	FYYNLIIK