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A novel antibacterial peptide derived from *Crocodylus siamensis* hemoglobin hydrolysate induces membrane permeabilisation causing iron dysregulation, oxidative stress and bacterial death

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Abstract

Aims: A novel antibacterial peptide from *Crocodylus siamensis* hemoglobin hydrolysate (CHHs) was characterised for antimicrobial activity.

Methods and Results: CHHs was hydrolysed for 2 h (2h-CHH), 4 h (4h-CHH), 6 h (6h-CHH) and 8 h (8h-CHH). 8h-CHH showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* at concentrations of 20, 20, 20 and 10 mg ml^{-1} (w/v), respectively. Fluorescent microscopy revealed that 8h-CHH had bactericidal activity against *E. coli* and *P. aeruginosa*. β-galactosidase assay supported by RT-qPCR demonstrated that 8h-CHH resulted in differential expression of genes involved in iron homeostasis (*ftnA* and *bfd*) and oxidative stress (*sodA*, *soxR* and *oxyR*). Siderophore assay indicated that 8h-CHH also impaired siderophore production with diminished expression of *pvdF*. This pattern of gene expression suggests that 8h-CHH triggers the release of free ferric ions in the cytoplasm. However, decreased expression of genes associated with the SOS response (*recA* and *lexA*) in combination with neutral comet revealed that no DNA damage was caused by 8h-CHH. Membrane permeabilisation assay indicated that 8h-CHH caused membrane leakage thought to mediate the antibacterial and iron-stress responses observed, due to loss of regulated iron transport. The novel active peptide from 8h-CHH was determined as QAIIHNEKVQAHGKKVL (QL17), with 41 % hydrophobicity and +2 net charge.

Conclusions: The QAIIHNEKVQAHGKKVL fragment of *Crocodylus siamensis* hemoglobin is antibacterial via a mechanism that likely relies on iron dysregulation and oxidative stress which results in bacterial death.

Significance and Impact of the Study: We have described for the first time, a novel peptide derived from *Crocodylus siamensis* hemoglobin hydrolysate, that has the potential to be developed as a novel antimicrobial peptide.

Keywords

Crocodylus siamensis, hemoglobin hydrolysate, antibacterial, peptide, oxidative stress genes, iron homeostasis genes.

Running headline

Antibacterial peptide from *C. siamensis* hemoglobin

Introduction

Bacterial infections account for a significant proportion of the global infectious disease burden, and morbidity and mortality rates caused by infectious microbial agents pose serious public health concerns. This is exacerbated by increasing resistance to antibiotics which is significant in an era where the development of new, synthetic antibacterial drugs lags the emergence of antimicrobial resistance (Mbah *et al*. 2012). It is therefore paramount to broaden the search for new antimicrobial substances, including the exploitation of novel sources where possible (Song *et al*. 2012). A growing area of research has begun to focus on protein hydrolysates of animal origin, such as goat whey protein hydrolyzed by treatment with alcalase, and which shows broad spectrum antibacterial activity (Osman *et al*. 2016). Similarly, an antibacterial peptide derived from acid extract of chicken (*Gallus gallus*) blood has efficacy against *E. coli* by a mechanism that results in toroidal pore formation and subsequent bacterial lysis (Vasilchenko *et al*. 2016). Furthermore, a peptic hemoglobin hydrolysate from bovine hemoglobin has also been shown to possess antibacterial activity (Froidevaux *et al*. 2001; Daoud *et al*. 2005; Arroume *et al*. 2006; Arroume *et al*. 2008; Adje *et al*. 2011). Whilst these areas of research are largely in their infancy, they provide a diverse, yet novel, means of informing the development of new peptide-based antimicrobial treatments.

Crocodylus siamensis is a small, freshwater crocodilian. In the wild these crocodiles experience many traumatic wounds that might be expected to be rife with infection from endogenous environmental bacteria, but this is not seen to be the case. *C. siamensis* (Siamese crocodile) hemoglobin constitutes the most abundant component in crocodile blood and has been long associated with a broad spectrum of biological activity, including antimicrobial (Srihongthong *et al*. 2012; Pakdeesuwan *et al*. 2017), antioxidant (Jandaruang *et al*. 2012; Srihongthong *et al*. 2012; Phosri *et al*. 2014; Maijaroen *et al*. 2016; Pakdeesuwan *et al*. 2017; Phosri *et al*. 2017) and anti-inflammatory activity (Phosri *et al*. 2014; Jangpromma *et al*. 2017; Phosri *et al*. 2017). Antibacterial activity has been attributed to peptides derived from *C. siamensis* hemoglobin and they are currently thought to be targeted to the bacterial surface (Srihongthong *et al*. 2012; Pakdeesuwan *et al*. 2017). However detailed studies of antimicrobial activity, mechanism of action and critical peptide sequences that mediate the observed activity are currently lacking.

This study aimed to investigate the antibacterial activity of hemoglobin hydrolysate from *C. siamensis* blood (CHH), hydrolysed for 2, 4, 6 and 8 h with pepsin, and to identify specific antimicrobial peptide fragments. Minimum Inhibitory Concentration (MIC), Time-Killing kinetics and viability staining revealed both bactericidal and bacteriostatic activity or the crude, hydrolysed peptide cocktail. To establish a mechanism of activity, the effect of CHHs on bacterial iron homeostasis was investigated using *ftnA* and *bfd* linked reporter strains, and analysis of siderophore production. Due to the close association of iron homeostasis and oxidative stress, the expression levels of oxidative stress response genes (*oxyR*, *sodA* and *soxR*) were also investigated but ruled out the inclusion of the SOS response genes or associated DNA damage as a mechanism of action. However, membrane permeability assay indicated the hemoglobin fragments disrupted the cell envelope; the active peptide was found to be positively charged and therefore likely functioned in a similar manner to cationic antimicrobial peptides.

Materials and methods

Bacterial strains

Escherichia coli (NCTC 10418), *Pseudomonas aeruginosa* (PAO1), *Staphylococcus aureus* (NCTC 13141), *Klebsiella pneumonia* (ATCC 13883), *Bacillus subtilis* were maintained on nutrient agar (NA) or nutrient broth (NB), aerobically at 37ºC throughout the study, unless otherwise stated. *E. coli* MC4100, *E. coli* MC4100 *ftnA-lacZ*, *E. coli* MC4100 *bfd-lacZ, E. coli* H1914 Δ*fur-ftnA-lacZ*, *E. coli* H1914 Δ*fur-bfd-lacZ* carried stable chromosomal mutations that allowed them to be maintained on NB or NA as described above; these strains were provided by Prof. Simon Andrews (University of Reading).

Hemoglobin extraction

The extraction of hemoglobin from red blood cells (RBCs) was performed following the method of Srihongthong *et al*. (2012). The RBCs were washed three times with phosphate buffered saline (PBS) pH 7·0 and centrifuged at 3000 × ***g*** for 5 min at 4°C. Ice-cold distilled water with five-fold volume was added to the RBCs pellet, vigorously mixed and allowed to settle for 10 min. After centrifugation at 10000 × ***g*** for 20 min at 4°C the supernatant was collected, lyophilized and stored at –70°C.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed according to the method of Yu *et al*. (2006). Briefly, the hemoglobin solution was digested with pepsin (at pH 2·0) using a ratio of enzyme to substrate (1:100 w/w) at 37°C for 2, 4, 6 and 8 h and boiled at 95°C for 10 min to inactivate the enzyme. The insoluble material was removed by centrifugation at 7168 × ***g*** for 20 min. The supernatant was collected and adjusted to pH 7·0 by addition of 1 M HCl or 1 M NaOH. Finally the supernatant (hydrolysate) was lyophilized and stored at –20°C.

Minimum inhibitory concentration (MIC) and time –killing assay (TKA)

The minimum inhibitory concentration (MIC) of CHHs was determined by microbroth dilution in a microtiter plate assay system using a total volume of 110 *µ*l per well. Each well contained 10 *µ*l bacterial suspension (*E. coli* NCTC 10418, *S. aureus*, *K. pneumoniae* and *P. aeruginosa*; adjusted to OD 0·1 A_{650}) and 100 *µ*l of CHHs. Plates were incubated for 24 h (MIC assay) or 9 h (TKA) at 37°C. An end point reading was taken for MIC, and hourly readings were taken for TKA (A_{650}) (Spectrostar^{Nano}, BMG Labtech).

Fluorescent microscopy using BacLight™ to assess bacterial viability

Test microorganisms (*E. coli* NCTC 10418, *S. aureus*, *K. pneumoniae*, *B. subtilis* and *P. aeruginosa*) were incubated for 16 h with 10, or 20 mg ml^{-1} CHHs (determined from MIC). Cultures were centrifuged at 9000 × ***g*** for 5 min and the supernatant was discarded. The cell pellets were re-suspended in 100 *µ*l dH_2O containing SYTO9 and PI at a ratio of 2:1. Cells were incubated in the dark at room temperature for 45 min and 10 *µ*l transferred to a glass slide. Cells were visualized by fluorescent microscopy (Nikon eclipse 80i) using oil immersion and ×100 lens. SYTO9 detection (viable cells) was used a 488 nm excitation and 520 nm emission filter. Propidium iodine (PI) detection (non-viable cells) was used 543 nm excitation and 572 nm emission filter.

β-galactosidase assay

Test microorganisms (*E. coli* MC4100, *E. coli* MC4100 *ftnA-lacZ*, *E. coli* MC4100 *bfd-lacZ*, *E. coli* H1914 Δ*fur-ftnA-lacZ*, *E. coli* H1914Δ*fur-bfd-lacZ*) were cultured in 100 *µ*l NB in a 96-well microtiter plate until mid-log was reached. Then 100 *µ*l of 2, 4, 6 and 8h-CHH (20 mg ml^{-1}) was added to each well followed by an additional incubation for 1.5 h at 37°C. Bacteria were permeabilised with buffer containing 60 mM $Na_2HPO_4 \cdot 7\, H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol, 0·1 % SDS and 4 *µ*l of chloroform for 5 min. Bacterial lysate was transferred to a fresh MTP and 20 *µ*l of ONPG was added to each sample. The plate was incubated in the dark at 37°C for 10 min, and absorbance values were read at 420 nm and 550 nm (Spectrostar^{Nano}, BMG Labtech). Miller units were calculated based on the following formula (Miller 1972):

$$\text{Miller Units} = 1{,}000 \times [OD_{420} - (1{\cdot}75 \times OD_{550})] / [T \times V \times OD_{600}]$$

where T determines the reaction time in minute and V determines the volume of cultured assayed in ml.

CAS agarose diffusion (CASD) assay

CAS agarose diffusion assay followed the method of Schwyn and Neilands (1987). Pre-cultures of (*E. coli* MC4100, *E. coli* NCTC 10418, *S. aureus*, *K. pneumoniae*, *B. subtilis* and *P. aeruginosa*) were equilibrated to OD 0·1 (A_{650}) and supplemented with 20 mg ml^{-1} of 2, 4, 6 and 8h-CHH and 1 mM DTPA. Treated cultures were incubated for a further 24 h at 37°C. After centrifuging at 9000 × ***g*** for 3 min (Rotina 380R centrifuge; Hettich, Germany) 50 μl aliquots of the sample supernatant were added to wells bored into the center of Petri dishes filled with 25 ml CAS agarose agar. After incubating in the dark at room temperature for 2 h, digital calipers were used to measure the diameter (mm) of the yellow diffusion zone diameters which is an indicator of siderophore production and the reduction of ferric iron.

RNA extraction and cDNA synthesis

Total RNA was extracted from bacterial cells per the manufacturer's instructions using the SV Total RNA extraction kit (Promega, USA). Total RNA concentration and purity were determined using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA). RNA samples with an absorbance ratio at OD 260/280 between 1·8-2·2 and OD 260/230 < 2·0 were used for further analysis. For each sample, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystem Inc).

Reverse transcription - real time PCR (RT-qPCR) assay

Primers used in this study are shown in Table 1. All PCR reactions were performed using 7500 Fast Real-Time PCR System machine under following conditions: 15 min at 95ºC and 40 cycles of 3 s at 95ºC, 30 s at 68ºC in 96-well clear optical reaction plates (Applied Biosystem, USA). The procedure ended by melt-curve ramping from 60 to 95ºC for 20 min to check the PCR specificity. All RT-qPCR reactions were carried out in biological and technical triplicate. A non-template control was also included in each run for each gene.

Neutral comet assay

Neutral comet assay was determined following the method of Solanky and Haydel (2012) with some modifications. Frosted microscope glass slides with a clear window were pre-coated by dipping in 1% agarose solution prepared with sterile water and were dried in an incubator at 40°C for 30 min. *E. coli* MC4100 cultures were incubated with rotary agitation at 37°C until logarithmic phase of growth (OD ~0·1; A_{600}), and then diluted with NB broth to achieve a concentration of ~10^7 CFU ml^{-1}. The cells were collected by centrifugation (9000 × ***g***, 15 min), washed with 0.1× phosphate-buffered saline (PBS), and re-suspended in one of the following solutions: 1% TritonX-100; 5 mM H_2O_2; deionized H_2O and 8h-CHH 20 mg ml^{-1}. Exposed cells were then incubated for 30 min at 37°C.

After incubating the slide at 4°C for 10 min to allow the initial layer of agarose to cool, the coverslip was removed and a subsequent agarose layer was added. The first layer consisted of 200 μl of 0.5% agarose prepared in 0.1× PBS and maintained at 55 –60°C for 30 min. For the second layer, 2 μl of exposed cells was mixed thoroughly with 200 μl of 0.5% agarose solution and 100 μl of this mixture was transferred to the slide. A third layer was consisted of 5 μg ml^{-1} RNaseA and 1 mg ml^{-1} lysozyme in 0.5% agarose solution. Slides were refrigerated for 10 min at 4°C and incubated for 30 min at 37°C. Embedded cells were then lysed by immersing slides in a solution containing 2·5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10 and 1% TritonX-100 for 1 h at room temperature. Following lysis, slides were immersed in an enzyme digestion solution prepared with 2·5 M NaCl, 10 mM EDTA, 10 mM Tris pH 7·4, and 0·5 mg ml^{-1} of proteinase K for 2 h at 37°C. Lysis and enzyme digestion steps were carried out in the dark to prevent light exposure. Slides were immersed in buffer containing 300 mM sodium acetate and 100 mM Tris, pH 9 for 20 min. Slides were electrophoresed at 12 V for 50 min. Following electrophoresis, slides were sequentially immersed in 1 M ammonium acetate prepared in ethanol for 20 min and 75% ethanol for another 30 min. Slides were then allowed to dry. Prior to staining, slides were pretreated with a freshly prepared solution of 5% DMSO and 10 mM NaH_2PO_4. While the slides were still wet, DNA was stained with 50 μl of 1 μM YOYO-1 in 5% DMSO and visualized using a Nikon eclipse 80i fluorescent microscope at 100× magnification with the appropriate filter set for YOYO-1 (excitation 491 nm and emission 509 nm). Comets were imaged and comet lengths were measured using Volocity software version 5·5.

Cytoplasmic membrane permeability assay

Cytoplasmic membrane permeability assay was determined following the method of Chitemerere and Mukanganyama (2014) with modifications. Briefly, bacteria (*E. coli* MC4100, *P. aeruginosa*, *S. aureus*) were grown to mid-exponential phase (OD 0·2-0·4; A_{650}); 2 ml were mixed with an equivalent volume of 1 μM diSC3-5 dyes and incubated 1 h, for maximal uptake of dye, then were collected by centrifugation (3000 × ***g*** for 5 min). Cells were wash and re-suspended in 2 ml of buffer (5 mM HEPES, pH 7·2, 5 mM glucose) and the absorbance was measured at an excitation wavelength of 622 nm and emission wavelength of 670 nm. Afterward, 2 ml of 100 mM KCl was added to equilibrate the cytoplasmic and external K^+ ion concentrations. Cells were mixed with and equal volume of sample then the fluorescence was monitored at an excitation wavelength of 622 nm and emission wavelength of 670 nm at 1 h intervals. Dye released with de-ionised water was used as a negative control.

Amino acid sequence analysis

The active fraction of 8h-CHH was selected and the contained peptides identified using LTQ Orbitrap XL Mass spectrometry employing the following search parameters: non-specified enzymatic cleavage with three possible missed cleavages, +/−0·8 Da mass tolerances for MS and MS/MS, a peptide mass tolerance of +/−5 ppm, methionine oxidation and Gln->pyro-Glu (N-term Q) variable modification and monoisotopic mass. Data were additionally processed at the Mascot Server (http://www.matrixscience.com/) using MS/MS ion searches against SwissProt (current release).

Statistical analysis

Statistical analysis was performed using ANOVA and followed by Dunnett's test. Data are presented as mean ± SEM. A value of $P < 0{\cdot}05$ was accepted to be significant (*$P < 0{\cdot}05$, ** $P < 0{\cdot}01$, ***$P < 0{\cdot}001$).

Results

Bacteriostatic and bactericidal effects of hemoglobin hydrolysate

Bacteriostatic and bactericidal effects of hemoglobin hydrolysate were investigated by minimum inhibitory concentration (MIC), time–killing assay (TKA) and fluorescent microscopy using BacLight^{TM}. The MIC was determined using 2h-CHH, 4h-CHH, 6h-CHH and 8h-CHH, and results are presented in Table 2. Gram-negative microorganisms generally required higher concentrations of hydrolysed CHHs to inhibit growth. The results showed that all hydrolysed CHHs were antibacterial at concentrations of 20 mg ml^{-1} (w/v) against *E. coli* and *S. aureus*. Furthermore, there was antibacterial activity for all CHHs at concentrations of 10 mg ml^{-1} (w/v) against *K.*

pneumoniae and *P. aeruginosa*. Significantly, the dose required to inhibit growth decreased as the length of time that the hemoglobin was hydrolysed increased, suggesting that higher concentrations of shorter peptide fragments mediated the inhibitory activity. This was verified by TKA which indicated that 8h-CHH resulted in the highest percentage of bacterial death (Table 3). Samples analysed by BacLight ™ viability staining and fluorescent microscopy confirmed that 8h-CHH at a dose of 20 and 10 mg ml^{-1} caused bacterial death (*E. coli* NCTC 10418 and *P. aeruginosa*; Fig. 1a and 1b, respectively). At lower doses and shorter hydrolyze times, CHH treatments tended to be bacteriostatic rather than bactericidal. Both mechanisms were investigated as described below.

Hemoglobin hydrolysate alters bacterial iron homeostasis and causes oxidative stress without inducing irreversible DNA-damage or the SOS response

The β-galactosidase assay demonstrated that 2h-CHH, 4h-CHH, 6h-CHH and 8h-CHH at 20 mg ml^{-1} decreased the expression of *ftnA* while increasing the expression of *bfd*, under iron rich conditions (Fig. 2). Decreased expression of *ftnA* is indicative of iron restriction whereas increased expression of *bfd* is affiliated with iron repletion and is known to have a role in haem-iron handling. The expression profile was not altered in a Δ*fur* background suggesting that the dysregulated expression of *ftnA* and *bfd* was not the result of Fur-dependent iron-mediated regulation (Fig. 2). However, CASD assay indicated that CHHs decreased the production of siderophores (Fig. 3) and following treatment with 8h-CHH, the expression of *pvdF* (associated with siderophore synthesis) also significantly decreased ($P < 0.05$) (Fig. 4b) suggesting some degree of altered iron homeostasis.

Bacterial iron metabolism and oxidative stress are inextricably linked; RT-qPCR demonstrated that following treatment with 8h-CHH, the expression of oxidative stress response genes *sodA* and *soxR* significantly increased while the expression of *oxyR* decreased (Fig. 4a). Oxidative stress is correlated with DNA damage and subsequent induction of the SOS system of repair. However, the expression of *recA* was diminished and *lexA* unchanged following treatment with 8h-CHH suggesting that the SOS response was not initiated (Fig. 4c). Therefore, the dysregulated iron homeostasis observed might be mediated by transcriptional responses associated with oxidative stress, rather than in response to environmental iron availability.

Hemoglobin hydrolysate does not cause DNA damage

Neutral Comet Assay verified that CHH did not cause DNA damage. Treatment with 1% TritonX-100 and 5 mM H_2O_2 (positive controls) produced an increase in comet length, and therefore DNA degradation, relative to the negative control (DI sterilized water). Exposure of *E. coli* MC4100 to 1% TritonX-100 and 5 mM H_2O_2 for 30 min increased the comet length values of 49·79 μm and 21·12 μm, respectively. Meanwhile the exposure of 8h-CHH resulted in comet length values of 11·24 μm and the negative control, 10·78 μm as shown in Table 4. The 1% TritonX-100 also yielded higher a comet length value, demonstrating higher DNA double strand break (DSB) levels while DNA double strand break wasn't caused by 8h-CHH upon exposure in bacterial cells.

Hemoglobin hydrolysate causes bacterial membrane leakage

Many short peptides aggregate in bacterial membranes resulting in a loss of integrity, such loss of integrity allows influx and efflux of various solutes and ions, including metal ions such as iron. It was hypothesized that membrane disruption might underpin the iron homeostatic dysregulation and concurrent oxidative stress response described above. Indeed, results from cytoplasmic membrane permeability assay, showed a gradual increase of diSC3-5 dye release over time in the presence of 8h-CHH as well as in the presence of the 1% TritonX-100 positive control (Fig. 5a, 5b and 5c). Similarly, diSC3-5 was released from all test microorganisms following exposure to 8h-CHH for 60 min (Fig. 5). The effect was most marked for the Gram-negative test microorganisms (*E. coli* and *P. aeruginosa*) indicating that 8h-CHH can permeabilise the cell envelope more effectively than that of Gram-positive microorganisms (*S. aureus*).

Active peptide was identified by LTQ Orbitrap XL mass spectrometry

To establish the identity of the active CHH peptide, the primary sequence was determined using LTQ Orbitrap XL mass spectrometry. As the most active CHH, the amino acid sequences of 8h-CHH was determined as QAIIHNEKVQAHGKKVL (QL17) corresponding to a molecular mass of 1895·07 Da. The obtained sequence was uploaded to the antimicrobial database (http://aps.unmc.edu/AP/main.php) and the protein databank (https://www.ncbi.nlm.nih.gov) for further characterization. As shown in Table 5, antibacterial peptide QL 17 had hydrophobicity values of approximately 41%, with a net charge of +2. Alignment of the amino acid sequences of the peptide fragments with *C. siamensis* hemoglobin indicated the antibacterial peptide originated from the β-subunit of

C. siamensis hemoglobin. The short length and positive charge of the peptide indicates that it might aggregate in the bacterial cell envelope in much the same way as cationic antimicrobial peptides.

Discussion

Protein hydrolysates are gaining popularity for their potential therapeutic effects due to demonstrable efficacy and low toxicity. This study used MIC, time-killing kinetics and viability staining to ascribe antimicrobial activity to hemoglobin hydrolysate from *C. siamensis.* CHHs were either bacteriostatic or bactericidal depending on the species of bacteria, dose and length of hydrolyzed time. Longer hydrolysis times correlated with higher inhibition of growth, with 8h-CHH proving to be the most efficacious. The antibacterial mechanism was investigated with focus on iron homeostasis and oxidative stress which in numerous pathogens is associated with host haem-iron availability.

The expression of *ftnA* was increased and that of both *bfd* and *pvdF* decreased following exposure to 8h-CHH. Under iron repleted conditions expression of *ftnA* and *bfd* is ordinarily repressed via a process relying on the ferric uptake regulator (Fur). The observed expression profile was no concordant with typical iron-Fur regulation which was verified using a Δ*fur* background, which showed the same expression profile as the wild-type background. However, differential expression of *ftnA*, *bfd* and *pvdF* also occurs in response to oxidative stress, which is closely allied to iron homeostasis (Zheng *et al*. 1999). Quantitative analysis of the expression of *soxR* and *oxyR* indicated differential expression in response to CHH-treatment akin to that observed during oxidative stress, indicating that CHH induced a state of oxidative stress in the microorganisms tested.

Uncontrolled oxidative stress results in widespread lipid, protein and DNA damage (Gault *et al*. 2016). If DNA damage becomes too great, the bacterial SOS system is activated. *LexA* is the master regulator of the SOS response; under normal conditions it represses the expression of genes encoding several DNA repair proteins, including *recA*. *LexA* has a negative auto-regulatory function and when the SOS response is triggered, increased levels of *lexA* are also produced which ultimately serves as a negative feedback mechanism to switch of the SOS response once rescue is achieved (Michel 2005). In this study, the expression of *lexA* was unchanged and *recA* decreased following treatment with CHH indicating that while oxidative stress occurred it was below the threshold to necessary to induce the SOS system. This was verified by the absence of DNA damage (a key signal for SOS induction) observed by neutral comet assay.

The bacterial response to outer membrane stress is distinct to the oxidative stress and SOS response, but triggers several stress-associated, damage repair pathways to maintain membrane integrity and prevent influx/efflux between the cytoplasm and external environment. Cationic antimicrobial peptides are well known to disrupt the bacterial cell envelope by aggregating to form pores within the membrane. Ordinarily this causes catastrophic damage resulting in bacterial death by lysis. Analysis of membrane integrity following treatment with CHH indicated that 8h-CHH could permeabilise *E. coli*, *P. aeruginosa* and *S. aureus* (Chitemerere and Mukanganyama 2014). The active peptide from 8h-CHH was identified using LTQ-Orbitrap XL mass spectrometry and the sequence determined as QAIIHNEKVQAHGKKVL (QL17) corresponding to a molecular mass of 1895·07 Da. The peptide QL17 had hydrophobicity values of about 41%, together with a net charge of +2. Given its positive charge, this fragment is hypothesized to aggregates within the bacterial membrane, in the same way as cationic antimicrobial peptides (Sato and Feix 2006). It is known that antimicrobial peptides positive charge combined with hydrophobicity is critical for partitioning of the peptide into the bacterial cell membrane (Pata *et al*. 2011).

Alignment the amino acid sequences of the peptide fragment with *C. siamensis* hemoglobin indicated the antibacterial peptide originated from the β-subunit of *C. siamensis* hemoglobin. This result agrees with Arroume *et al*. (2008), who reported that antimicrobial products derived from hemoglobin hydrolysis are cleaved from the α- and β-subunits. These products mostly consist of ~15–30 amino acid residues and have a molecular weight < 10 kDa. Of note, was the correlation between length of hydrolysis time and antimicrobial activity. As expected, hemoglobin hydrolysed for longer periods of time comprised a higher concentration of small peptide fragments, which in turn exhibited better antimicrobial efficacy, highlighting the importance of peptide fragment size for activity.

Taken collectively, the data derived from this study indicates that the hemoglobin of *C. siamensis* can be hydrolyzed to produce a novel antimicrobial peptide that at high concentrations mediates bacterial death by aggregating in the cell envelope and damaging membrane integrity. The consequent influx of exogenous material combined with an efflux of cytoplasmic content likely underpins the dysregulation of iron homeostasis and concurrent oxidative stress. Whilst the potential to utilise antimicrobial peptides derived from hemoglobin warrants further study, blood derived from *C. siamensis* is currently a waste-product of farming. Therefore, with larger studies drawn from this exploratory research, it could instead be developed as a natural or synthetic antimicrobial peptide, engineered to ensure maximum efficacy and minimal toxicity.

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Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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Table 1 Primer pairs for RT-qPCR

Species	Gene names	GeneBank accession no.	Region	Product size (bp)	Forward primer (5́→3́)	Reverse primer (5́→3́)
E. coli	*oxyR*	HG738867	2620691..2621608	197	CCCCGGCTTCAAAACAGAAA	GCTGGTGAAAGAGAGCGAAG
	sodA		2678668..2679288	187	CGAAGTCACGTTCGATAGCC	CTGCCAGAATTTGCCAACCT
	soxR		4163425..4163889	172	CAGCGGCGATATAAACGTGA	CCAACTCTTCTCGCCATTGG
	rec A		3960012..3961073	173	GAAGAACAAAATCGCTGCGC	CATTCGCTTTACCCTGACCG
	lex A		4143071..4143679	158	GCAGGAAGAGGAAGAAGGGT	CTTTCATCGACATCCCGCTG
	rpoD	BA000007	3952578..3954419	220	TTCGTACGCAAGAACGTCTG	AGGCCGGTTTCTTCTTCAAT
P. aeruginosa	*pvdF*	NC_002516	2652230..2653057	181	CGTACCAGCTCATCGAGGAT	AGACCCTGAACGACCTCTTG
	rpoD		634371..636224	186	GGGTCACATCGAACTGCTTG	TCATCGAGGACTCCACCATG

Table 2 The MIC values of sample CHHs

Sample	MIC values (mg ml^{-1})				% Growth inhibition			
	E.coli	*K.pneumoniae*	*P.aeruginosa*	*S.aureus*	*E.coli*	*K.pneumoniae*	*P.aeruginosa*	*S.aureus*
2h-CHH	20	20	10	20	33·33***±0·003	57·14***±0·015	34·11***±0·004	38·87***±0·005
4h-CHH	20	10	10	10	21·74***±0·025	34·29***±0·002	25·10***±0·002	21·11***±0·031
6h-CHH	20	10	10	20	37·86***±0·007	45·14***±0·008	35·03***±0·008	25·53***±0·020
8h-CHH	20	20	10	20	24·64***±0·003	41·10***±0·113	44·61***±0·020	31·20***±0·195

*** denotes $P < 0·001$. Data expressed as a mean ± SEM of 3 independent experiments. Significance was measured using ANOVA followed by Dunnett’s test.

Table 3 Time-killing assay (TKA) of sample CHHs

Sample	%Growth inhibition at 9 h intervals			
	E.coli	*K.pneumoniae*	*P.aeruginosa*	*S.aureus*
2h-CHH	48·07***±0·001	3·21***±0·011	6·07***±0·004	31·23***±0·006
4h-CHH	55·64***± 0·007	4·06***±0·002	4·98***±0·007	56·31***±0·007
6h-CHH	73·37***±0·001	0·06±0·002	10·65***±0·011	33·23***±0·013
8h-CHH	48·54***±0·020	12·00***±0·015	21·73***±0·004	17·88***±0·115

*** denotes *P* < 0·001. Data expressed as a mean ± SEM of 3 independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

Table 4 Neutral bacterial comet assay following *E.coli* exposure to 1 % TritonX-100, 5 mM H_2O_2, 8h-CHH and DI water.

	E.coli treated with			
	1% (v/v) TritonX-100	5 mM H_2O_2	8h-CHH	DI water
Comet length values (μm)	49·79***±0·002	21·12***±0·010	11·24±0·014	10·78±0·009

*** denotes *P* < 0·001. Data expressed as a mean ± SEM of 3 independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

Table 5 Structural characteristics of the antibacterial peptide from 8h-CHH

Property	Peptide sequence	Hydrolysate	%Hydrophobicity	Net charge	Sequence alignment
Antibacterial	QAIIHNEKVQAHGKKVL (QL17)	8h-CHH	41%	+2	*C. siamensis* Hb β-subunit (position 53-69)

Figure captions

Figure 1 Representative images of Live and Dead staining assay (BacLight^{TM} Fluoresent microscopy), red coloured cells are dead cells and green coloured cells are live cells, showed the bactericidal effect of 8h-CHH at a dose of 20 and 10 mg ml^{-1} against (a) *E.coli* and (b) *P.aeruginosa* PAO1, respectively.

Figure 2 β- galactosidase assay. Expression of *ftnA* and *bfd* in response to treatment with CHHs does not exhibit iron-dependent expression in a wild-type or Δ*fur* background. Each bar represents the mean ± SEM (n=3). *** significant at $P < 0{\cdot}001$.

Figure 3 The effect of CHHs on siderophore production by CASD assay. Sample CHHs decreased the production of siderophore from all of bacterial strains over a time frame of 2 h. Each bar represents the mean ± SEM (n=3). *** significant at $P < 0{\cdot}001$.

Figure 4 Effect of 8h-CHH on gene expression levels in *E.coli* and *P.aeruginosa* were analyzed by real time PCR (RT-qPCR). (a) A fold change gene expression of *oxyR*, *sodA* and *soxR* were validated for oxidative stress response. (b) A fold change gene expression of *pvdF* was validated for siderophore production and (c) a fold change gene expression of *recA* and *lexA* were validated for DNA damage (SOS response). Each bar represents the mean ± SEM (n=3). *significant at $P < 0{\cdot}05$, **significant at $P < 0{\cdot}01$ and *** significant at $P < 0{\cdot}001$.

Figure 5 Measurement of 3'3 dipropylthiadicarbocyanine (diSC3-5) dye release overtime from (a) *E.coli*, (b) *P.aeruginosa* PAO1 and (c) *S.aureus* membranes in the presence of 8h-CHH, permeabilizing agent 1% TritonX-100 and negative control Deionize water (DI).