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# Neutralization of toxic haem by *Porphyromonas gingivalis* haemoglobin receptor

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**Free haem is known to be toxic to organs, tissues and cells. It enhances permeability by binding to a cell membrane, which leads to cell death, and damages lipids, proteins and DNA through the generation of reactive oxygen species. Lysine- and arginine-specific gingipains (Kgp and RgpA/B) are major proteinases that play an important role in the pathogenicity of a black-pigmented periodontopathogen named *Porphyromonas gingivalis*. One of the adhesin domains of gingipain, HbR could bind haem as an iron nutrient source for *P. gingivalis*. Using erythrocyte and its membrane as a model, results from the present study demonstrate that recombinant HbR expressed in *Escherichia coli* could inhibit haem-induced haemolysis, probably through removing haem from the haem–membrane complex and lowering free haem toxicity by mediating dimerization of haem molecules. The ability to protect a cell membrane from haem toxicity is a new function for HbR.**

*Keywords*: gingipain/HbR/haem/membrane damage/*Porphyromonas*.

*Abbreviations*: BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; HA, haemaglutinin/adhesin domain; Hb, haemoglobin; HbR, haemoglobin receptor; Hp, haptoglobin; rHbR, recombinant HbR.

Haem (ferroprotoporphyrin IX) is a prosthetic group for indispensable cellular functions of various proteins including haemoglobin (Hb), myoglobin, cytochromes and nitric oxide synthase *(1)*. However, free haem causes undesirable damage to organs, tissues and cells by binding to cell membrane and enhancing permeability that lead cells to death. It also damages lipids, proteins and DNA through the generation of reactive oxygen species. Haem is a potent haemolytic agent that impairs the erythrocyte's ability to maintain cation gradients, affects colloid-osmotic mechanism (*2*, *3*), stimulates potassium loss and swelling (*4*), alters the conformation of cytoskeletal proteins (*5*) and prohibits some enzymes of erythrocytes, eventually leading to haemolysis (*6*, *7*). Haem induces lipid oxidation of a human erythrocyte ghost (*8*), aggregates in the membrane and promotes oxidation leading to the enhancement of permeability and membrane disorder, subsequently causing cell lysis and death (*9*, *10*). It also catalyses the oxidation of protein, triggering covalent cross-linking and aggregation, and degradation to small peptides (*10*, *11*). Some compounds such as Vitamin E, glutathione, iron chelator desferrioxamine and histidine-rich proteins can protect against or inhibit haem-induced haemolysis by stabilizing membranes and degrading haem, or by binding to haem (*12–16*).

*Porphyromonas gingivalis* is a black-pigmented Gram-negative anaerobic bacterium associated with the initiation and progression of adult periodontal disease (*17*). *P. gingivalis* produces a variety of enzymes, particularly trypsin-like proteases, the activity of which has been found to be critical for this pathogen. Among these enzymes, lysine- and arginine-specific gingipains (Kgp and RgpA/B) are the major proteinases (*18*) and play an important role in pathogenicity, either by degrading or inactivating proteins essential for host immunity and connective tissue integrity (*19*). Gingipains are implicated in haem acquisition by proteolytic degradation of haemoglobin (*20*, *21*), and they are quite essential for the growth and survival of the bacterium by providing a source of iron nutrients under iron-depleted conditions (*22*) and taking part in detoxification in an iron-rich environment through intracellular iron storage (*23*, *24*). Besides playing a crucial role in growth, iron has also been important in the virulence of *P. gingivalis* (*25*). RgpA- and Kgp-encoding genes were found to encode the polyprotein consisting of three parts: propeptide, catalytic domain and C-terminal adhesion domains (HGP44, HbR, HGP17 and HGP27) (*26–28*). HbR (also called HA2 or HGP15) is able to bind Hb (*29*).

Based on the function of HbR as a receptor for both haem and Hb (*29*, *30*), as well as on its role in converting monomeric haem into an μ-oxo dimeric complex (*31*) that is accumulated on the bacterium cell surface as a black pigment (*32*), we examined the neutralization effect of recombinant HbR (rHbR) on the toxicity of haem in biological cell membrane damage. In the present study, an erythrocyte, or its membrane, was used as a model for cells or cell membranes. Haptoglobin (Hp) and bovine serum albumin (BSA) were used as control proteins. Hp is a plasma $\alpha_2$-glycoprotein known as an Hb-binding protein (*33*), and BSA reportedly inhibits haem-induced haemolysis (*34*) and binds to the haem molecule (*35*).

## Materials and Methods

### Materials

Haemin chloride, Hp (from human plasma, phenotype 1-1, salt free), porcine haematin and hen egg white lysozyme were purchased from Sigma (St Louis, MO, USA). BSA was from Nacalai Tesque (Japan). All chemicals were high analytic grade. Hp was dissolved and dialysed against PBS pH 7.4 before use, while lysozyme and BSA were used directly after dissolving.

### Haem preparation

Haemin chloride (16.3 mg) was dissolved in 1 ml of dimethyl sulphoxide (DMSO), and then the insoluble haem was removed by centrifugation for 10 min at 7,000*g*. The concentration of haem in the solution was estimated from the absorbance at 400 nm after dilution with 100 mM NaOH–2.5% SDS solution. The molar extinction coefficient for haem is $10^5$ at 400 nm, as described previously (*36*). This monomeric haem solution was stored at 4°C in the dark until used, and diluted with phosphate buffer saline (PBS), Tris-buffer saline (TBS) or HEPES buffer to the desired concentration immediately prior to use.

For analysis of the interaction between haem and rHbR, monomeric haem stock was prepared by dissolving haemin chloride in Tris saline buffer (0.1 M Tris–HCl, pH 9.8, containing 0.14 M NaCl), followed by adjustment to pH 7.5 by slow drop-wise addition of diluted HCl. This was further diluted with phosphate buffer (0.2 M phosphate buffer, pH 6.5, containing 0.14 M NaCl) to yield a solution composed of predominantly monomeric haem, as described previously (*37*). The UV-visible absorption spectrum of this solution was then recorded to confirm the presence of the monomeric form, which can be seen by the presence of prominent Soret bands at 365 nm (*38*). This monomeric solution was used within 1 h of preparation.

Sodium salt of μ-oxo dimeric haem was prepared chemically from haematin as shown in the previous study (*38*). The μ-oxo dimer form was confirmed by attenuated total reflectance Fourier transformed infrared (ATR FTIR) spectroscopy that revealed an absorbance band located at about 896 $cm^{-1}$, as described previously (*31*, *39*).

### Purification of rHbR

Expression vector pHbR was constructed as shown in a previous study (*29*). rHbR was expressed in *Escherichia coli* BL21(DE3) and purified as described previously (*29*). Then, rHbR was dialysed against PBS (10 mM $Na_2HPO_4$–2 mM $KH_2PO_4$, pH 7.4, containing 137 mM NaCl and 2.7 mM KCl).

### Erythrocyte preparation

Fresh blood from healthy donors was heparinized (1 mg heparin/ml) to suppress clotting. The erythrocytes were separated from plasma by centrifugation at 1,500*g* for 3 min. Then they were washed six times with PBS and were either used to prepare membrane ghosts or resuspended in PBS for haemolysis assay.

### Erythrocyte white ghost membrane preparation

Human erythrocyte white ghost membrane was prepared on ice with slight modifications as described previously (*40*, *41*). Washed erythrocytes were haemolysed in 25 ml of 5 mM phosphate buffer, pH 8. After shaking vigourously, the haemolysate was centrifuged at 10,000*g* for 20 min to sediment membranes. This step was repeated, and then the membrane was washed repeatedly with 50 mM Tris–HCl, pH 7.4, until the pellet turned white. The brown pellet that had accumulated at the bottom of the membrane fraction was carefully removed to avoid contamination with proteinases. Finally, the membrane was resuspended in TBS (50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl) or HEPES buffer (200 mM HEPES buffer, pH 7.4). The erythrocyte white ghost membrane thus obtained was stored on ice and used within 24 h. The amount of membrane was determined from the membrane protein concentration using a protein assay kit (Bio-Rad, USA), with BSA as the standard.

### Haemolysis assay

The haemolytic assay was based on our previous method (*42*), with a slight modification. Briefly, 60 μl of erythrocyte suspension in PBS (final suspensions of cells ranged from 0% to 2% in serial 1:2 dilutions) was dispensed in a 96-well plate, and then 200 μl of distilled water or PBS was added. After shaking for 15 min, the plate was scanned at 750 nm using an MTP-120 microplate reader (Corona Electric Co., Ibaragi, Japan) to measure the turbidity of cells. Adding distilled water into the wells caused 100% haemolysis of the erythrocytes, as shown by the turbidity at the baseline, while intact erythrocytes exhibited a linear turbidity in the range of 0–2% suspension (Fig. 1A). Thus, we used a 0.5% suspension of erythrocytes in all haemolytic assays.

The erythrocytes were haemolysed by adding distilled water to get a 0.5% suspension. A series of mixtures containing the haemolysed erythrocytes and intact erythrocytes at various ratios were prepared in 96-well plates. The total final combined suspension of cells in the mixtures was constant at 0.5%. The absorbance of mixtures at 750 nm was measured by an MTP-120 microplate reader. As shown in Fig. 1B, a good positive correlation between the percentage of haemolysed erythrocytes and reduction of turbidity over a wide range was observed, indicating that this method can be used for the quantitation of the haemolytic activity of protein.

We conducted our haemolysis assay as follows. Fifty microlitres of haem (final concentrations; 10 or 15 μM) was incubated with various concentrations of rHbR or Hp (final concentration ranged from 0 to 5 μM) in 50 μl of PBS on a 96-well plate for 10 min. One hundred microlitres of erythrocyte suspension (0.5%) in PBS was then added to the wells. After shaking for 2 h at 37°C, the plate was scanned at 750 nm. The control well, which contained only erythrocytes, was assumed to be of 0% haemolysis, and the percentage of haemolysis was expressed as the percentage of absorbance at 750 nm of sample when compared with that of the control.

The effect of monomeric or synthesized μ-oxo dimeric haem (final concentration ranged from 0 to 40 μM) on haemolysis was also studied as described above. Concentration of μ-oxo dimeric haem was calculated by equivalent monomeric haem in SDS solution as describe above. After shaking for 2 h at 37°C, the haemolysis percentage was assumed.

### Effects of rHbR and Hp on haem-erythrocyte white ghost membrane binding

Haem (10 μM) was pre-incubated either with or without 2.5 μM of rHbR or Hp in 200 mM HEPES, pH 7.4, for 30 min at 37°C. Suspensions of white ghost membranes containing 20 μg membrane protein were added and mixed with 0.6 ml of 0.2 M HEPES, pH 7.4, and then incubated for 7 min at room temperature, as suggested in our previous work (*16*). Then the membranes were collected as pellets by centrifugation at 7,000*g* for 10 min, and washed twice with the same buffer. After solubilization of the membrane pellet in 2.5% SDS buffered with 50 mM Tris–HCl, pH 7.4, the absorbance of haem at 402 nm was measured with a spectrophotometer (Hitachi U-3300, Tokyo, Japan). The amounts of haem bound to erythrocyte membranes were calculated as previously described (*16*), and expressed as nanomoles of haem per milligram membrane protein.

### Effects of rHbR and Hp on removal of haem from haem–membrane complexes

In our previous study, the amounts of haem bound to membranes were determined after incubating a mixture of white ghost membranes and haem for various periods (*16*). The results demonstrated that incubation for 7 min was enough for haem to bind with

membranes, after which no further haem bound to the membranes. Thus, we determined that the optimal condition to evaluate the effects of rHbR, Hp, BSA and hen egg white lysozyme on haem removal from haem–membrane complexes is as follows. Suspensions of white ghost membranes containing 250 μg membrane proteins were pre-incubated with 50 μM of haem in 1 ml of 50 mM Tris–HCl buffer, pH 7.4, for 7 min at room temperature. After incubation, the membranes were washed three times with the same buffer by centrifugation to remove unbound haem. The haem-bound membrane was re-suspended in 1.25 ml of the same buffer, divided into five tubes (each containing 250 μl of the sample), and incubated with 2.5 μM of rHbR, Hp, BSA, lysozyme or buffer as a control. After incubation at room temperature for 30 min, the mixtures containing the membranes were separated into pellets and supernatants by centrifugation at 7,000*g* for 10 min. Then, the pellets were dissolved in 250 μl of 50 mM Tris–HCl, pH 7.4, containing 2.5% SDS. The absorption spectrum of haem in the solution was recorded with a Hitachi U-3300 double beam spectrophotometer to analyse the haem state in the membrane. The absorption spectrum of haem released into the supernatants from the membranes was also analysed.

#### *Effect of rHbR and Hp on membrane lipid peroxidation*

The extent of lipid peroxidation by haem was determined by measuring the malonyldialdehyde (MDA) formed by the thiobarbituric acid (TBA) reaction as described previously (*9*). Ten micromolar of haem was incubated with 2.5 μM of rHbR or Hp in TBS buffer for 30 min. Thereafter, suspensions of 20 μg erythrocyte white ghost membranes in a total of 0.3 ml of TBS were added and incubated at 37°C for 2 h. The samples were mixed with 0.3 ml of 0.375% TBA (v/v) solution containing 0.25 M HCl and 1% Triton X-100 (v/v). The mixtures were heated for 30 min in a boiling bath, immediately cooled on ice and centrifuged at 7,000*g* for 10 min. The amount of MDA produced in the supernatant was determined by measuring the absorbance at 532 nm, and was calculated using a molar extinction coefficient of $1.56 \times 10^5$. Samples lacking membranes but containing either haem, haem/rHbR or haem/Hp was used as blanks.

#### *Effect of rHbR and Hp on membrane protein oxidation*

Suspensions of 0.2 mg erythrocyte white ghost membranes were incubated at 37°C for 2 h in 0.2 M HEPES, pH 7.4, in the presence of either haem (10 μM) or haem–protein mixture (10 μM haem and 2.5 μM rHbR or Hp). In the control experiment, the membrane was incubated with 0.2 M HEPES, pH 7.4. The membrane was sedimented by centrifugation at 7,000*g* for 10 min, and then lysed with 0.6 ml of 0.3 M phosphate buffer, pH 7.4, containing 0.8 M urea and 2% SDS. The membrane thiol content was determined by measuring the difference in absorption at 412 nm before and 10 min after the addition of 12 mg of dithionitrobenzoic acid in 0.6 ml of 0.3 M phosphate buffer, pH 7.4, containing 0.8 M urea and 2% SDS (*43*). Thiol concentration was determined by using a molar extinction coefficient of 13,600.

#### *Interaction of* haem *and rHbR or Hp*

Either purified rHbR or Hp (2 μM) was incubated at 37°C, with an excess concentration of monomeric haem (20 μM) in 0.2 M phosphate, pH 6.5, containing 0.14 M NaCl, and the spectra were recorded periodically from 0 to 6 h during incubation with a Hitachi U-3300 double beam spectrophotometer.

#### *Confirmation of micro-oxo dimeric haem production*

Monomeric haem (900 μM) was incubated with or without rHbR (9 μM) at 37°C in 5 mM Tris–HCl buffer, pH 6.5, for 24 h. Samples were freeze-dried and subjected to ATR FTIR on a Thermo Nicolet instrument using a Smart Orbit diamond ATR, and 128 spectra were collected at a resolution of 1 $cm^{-1}$. Buffer or rHbR was used as control sample.

#### *Statistical analysis*

Data represent the mean ± SD of three independent experiments in triplicate. A Student's *t*-test was used to compare mean values, and a $P < 0.05$ was considered to be significant.

## Results

### *Recombinant HbR inhibited haem-induced haemolysis*

To explore the effects of rHbR on haem-induced haemolysis, a haemolytic experiment was also performed as described above. The results showed that rHbR significantly inhibited haem-induced haemolysis (Fig. 1C), and the inhibition of haemolysis depended on the concentration of both rHbR and Hp. After 2 h incubation, 10 μM of haem caused ~24% haemolysis. The haem-induced haemolysis was suppressed even by 1.25 μM of rHbR, and 50% of haemolysis was inhibited by 5 μM of rHbR. In addition, commercial Hp also inhibited haem-induced haemolysis but was less effective than rHbR, probably because of its lower affinity to haem than that of rHbR. Both rHbR and Hp are known as Hb-binding proteins (*29*, *33*) and they alone have no haemolytic activity (data not shown).

### *Effect of rHbR and Hp on haem–membrane binding*

The binding of haem to erythrocyte white ghost membranes in both the absence and presence of either rHbR or Hp were compared. The results, as shown in Table I, revealed that about 23% of the haembinding to erythrocyte membranes was inhibited by rHbR and Hp ($P < 0.05$). No significant difference was observed between the effect of rHbR and Hp on haem–membrane binding ability ($P > 0.05$).

### *Effect of rHbR and Hp on haem removal from a haem–membrane complex*

The effects of rHbR and Hp on the haem–membrane complex were further investigated. First, a haem-bound membrane was prepared. After the incubation of rHbR with a haem–membrane complex, the membrane and supernatant were separated by centrifugation, and then the haem contents were determined by means of absorption spectra. In the case of the membrane fraction, the absorption spectra were recorded after dissolving the membranes by SDS.

After the incubation of haem-bound membranes without proteins, the haem remaining in the membranes, which was pelleted by centrifugation and then dissolved using 50 mM Tris–HCl, pH 7.4, containing 2.5% SDS, gave an absorption spectrum, with a relatively sharp Soret-band, the Soret- and Q-band absorption being maximal at 400, 520 and 610 nm, respectively (spectrum 1 in Fig. 2B). The increase in absorption coefficient for the Soret band (400 nm) also supported the contention that the aggregated form of haem can be converted to the monomeric form in the presence of surfactant SDS and lipids, which is consistent with a previous study (*16*). The spectrum of the supernatant obtained after incubating haem-bound membranes without proteins (spectrum 1 in Fig.2A) slightly increased, probably due to the basal release of haem from the membrane. In contrast, after incubation with either rHbR or Hp, the absorbed haem in the membranes significantly decreased (spectra 2 and 3 of Fig. 2B), and the haem released into the supernatant significantly increased (spectra 2 and 3 in Fig. 2A). Moreover, the amount

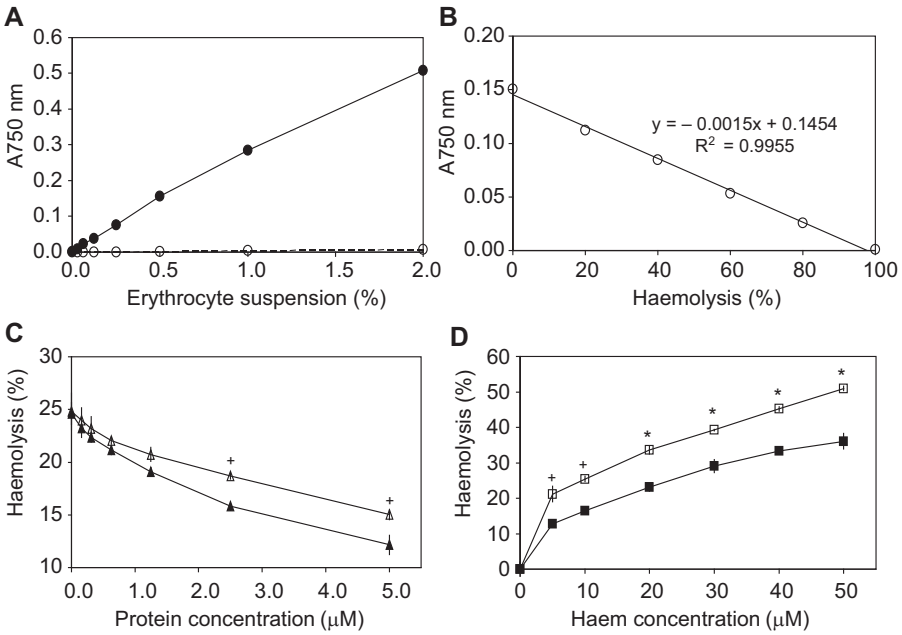


**Figure 1 Effect of rHbR and Hp on haem-induced haemolysis.** Haemolysis of erythrocytes was monitored by measuring turbidity (absorbance at 750 nm) using a 96-well plate. (A) Turbidity of haemolysed erythrocytes (open circles) was on the baseline, while that of intact cells (closed circles) was linear in the range of 0–2% suspension. (B) Turbidity of 0.5% erythrocyte suspension is linear over the entire range of haemolysis. (C) Effect of rHbR and Hp on haem-induced haemolysis. Suspension of erythrocytes was incubated in the presence of haem (10 μM) with various concentrations (final concentration ranged from 0 to 5 μM) of rHbR (closed triangle) or Hp (open triangle). (D) Haemolysis by monomeric (open square) and synthesized μ-oxo dimeric haem (closed square). Concentration of haem was expressed by equivalent monomeric haem. Significant different between two effects on haemolysis of rHbR and Hp or monomeric and μ-oxo dimeric haem at each point was indicated by $^{+}P<0.05$ and $^{*}P<0.01$.

**Table I. Effect of rHbR and Hp on haem binding to a white ghost membrane.**

| Additives | Membrane-bound haem (nmol/mg membrane protein) | *P*-values[a] |
|---|---|---|
| None | 1.18 ± 0.34 | *0.001* |
| Haem (10 μM) | 99.28 ± 8.85 | 1 |
| Haem–Hp (10–2.5 μM) | 72.95 ± 5.06 | *0.004* |
| Haem–rHbR (10–2.5 μM) | 76.21 ± 3.69 | *0.005* |

Results are expressed as mean ± SD from three experiments in triplicate.
[a]*P*-values for the comparison with haem group, *NS: P > 0.05*

of haem released into the supernatant in the presence of rHbR was similar to that with Hp, suggesting that the two proteins have an identical ability to remove haem from a haem–membrane complex. In addition, upon adding BSA into the haem–membrane complex, the spectrum of the supernatant showed a maximum at 403 nm and the height was higher than that of the spectra in the presence of both Hp and rHbR (spectrum 4 in Fig. 2A), indicating that the presence of the haeme–BSA complex was formed as reported previously (*35*), and the affinity of BSA for haem may be higher than that of Hp and rHbR. Lysozyme was used as negative control of non-specific binding of haem to a protein (*44*). The spectra of both supernatant and pellet after incubating haem-bound membranes with lysozyme (spectrum 5 in Fig. 2) were almost the same with the spectra of those without proteins (spectrum 1 in Fig. 2), indicating that the haem removal activity by rHbR and Hp is specific.

### *Interaction between haem and rHbR or Hp*

To further explore the differential effects of rHbR and Hp on haem-induced membrane damage, the interaction between monomeric haem and rHbR and Hp was also studied by recording the change of prominent Soret bands at 365 nm after incubation of the mixture of monomeric haem and either rHbR or Hp in phosphate buffer, pH 6.5. The result showed that during short-term incubation of monomeric haem with rHbR, a progressive drop in $A_{365}$ was observed, accompanied by a broadening and lowering of the Soret band and a red-shift of the Soret band from 365 to 385 nm (Fig. 3A), which was in good agreement with previous work (*31*, *37*). These changes in the spectra indicated a depletion of monomer haem and an increase in the haem dimer and aggregated forms (*31*, *37*). In both the control sample with only haem (Fig. 3C) and the monomeric haem with Hp (Fig. 3B), there were significant reductions in time in the Soret band intensity but no shift of the Soret band, suggesting the aggregation of haem. As another control, monomeric haem was incubated with BSA, which resulted in a Soret band at 403 nm (data not shown), indicating the formation of a haem monomer–albumin complex, rather than a dimeric haem, as described previously (*35*).

Moreover, the μ-oxo dimeric haem formed after incubating monomeric haem and rHbR was further confirmed by ATR-FTIR spectroscopy. To improve the IR spectroscopic detection of μ-oxo dimeric

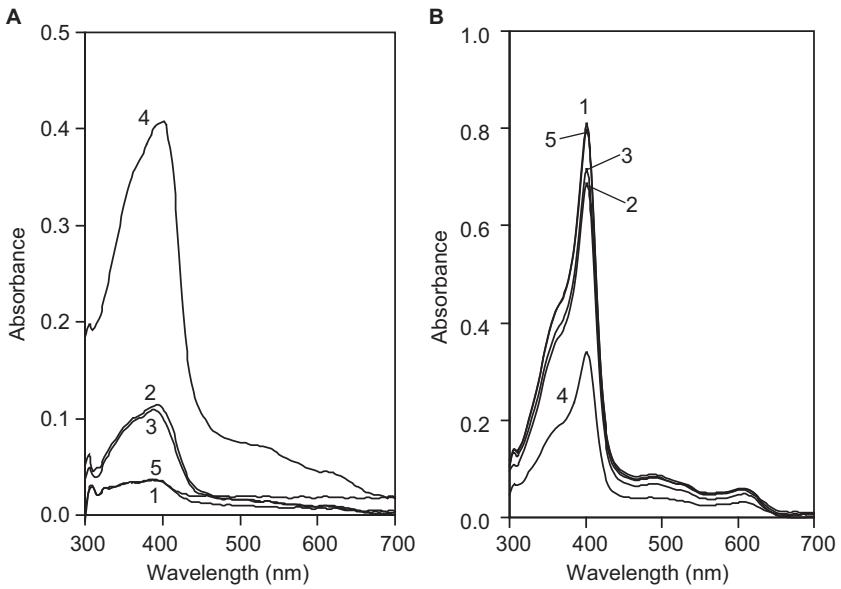


**Figure 2 Removing haem from haem-bound membrane by rHbR or Hp.** Haem-bound membranes were incubated in the presence or absence of rHbR, Hp or BSA, dissolved with 50 mM Tris–HCl and 2.5% SDS, pH 7.4, and then the absorption spectra of supernatants (A) and pellets (B) were recorded: 1, without protein; 2, with rHbR; 3, with Hp, 4, with BSA, 5, with lysozyme.

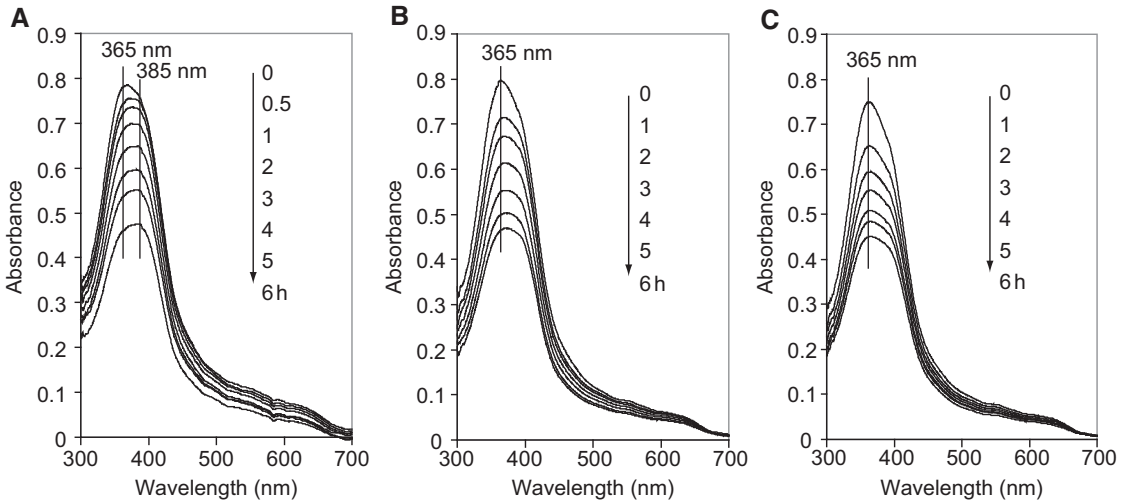


**Figure 3 Interaction between monomeric haem and rHbR or Hp.** UV spectra of monomeric haem incubated with rHbR (A), Hp (B), or buffer (C) was recorded periodically over 6 h in 0.20M phosphate containing 0.14 M NaCl, pH 6.5. The concentrations of monomeric haem and protein were 20 and 2 μM, respectively.

haem, rHbR was incubated for 24 h with high concentration of monomeric haem (900 μM) at a haem: protein molar ratio of 100:1 (Fig. 4A). This revealed an absorbance band at about 896 $cm^{-1}$, which attributed to the asymmetric stretching frequency of the oxo-bridge Fe-O-Fe dimer, consistent with previous studies (*31*, *39*). As negative controls, the sample of monomeric haem without rHbR, rHbR or Tris buffer alone did not reveal the band presenting for μ-oxo dimer formation (Fig. 4B–D).

### *Effect of rHbR and Hp on* haem*-induced membrane protein oxidation and lipid peroxidation*

The oxidative action of haem on membrane protein is thought to be a mechanism of haem-induced membrane damage (*9*). Therefore, we evaluated the effects of rHbR or Hp on haem-induced oxidation of membrane protein by analysing thiol contents. As shown in Table II, the thiol content in erythrocyte white ghost membrane was 144.28 nmol/mg membrane proteins when incubated with only buffer. Oxidation with haem reduced the thiol content by approximately 61.8%, which was consistent with a previous study (*40*). We found that rHbR slightly suppressed the haem-induced protein oxidation by 11.8% ($P<0.05$), while Hp had no effect on the haem-induced protein oxidation. Alone, neither rHbR nor Hp showed any membrane oxidant activity.

We also examined the effect of rHbR and Hp on haem-catalysed peroxidation of erythrocyte membrane by measuring MDA production by the TBA reaction. In the present study, our results showed that haem

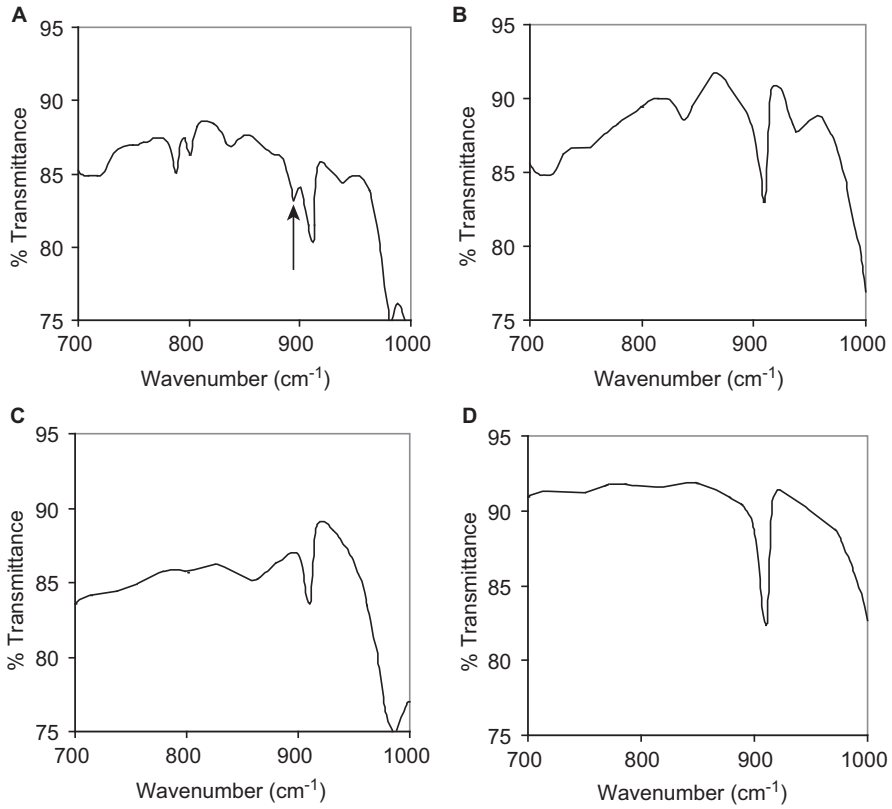


**Figure 4 Confirmation of μ-oxo dimer production**. ATR-FTIR spectra of monomeric haem after incubation with rHbR (A) or without (B), of rHbR (C) or 5mM Tris–HCl pH 6.5 buffer (D). Incubations were carried out for 24 h. The concentrations of monomeric haem and protein were 900 and 9 μM, respectively.

**Table II. Effect of rHbR and Hp on haem-induced oxidation of thiols in membrane protein.**

| Additives | Thiol groups (nmol/mg membrane protein) | *P*-values[a] |
|---|---|---|
| None | 144.28 ± 7.13 | *0.001* |
| Haem (10 μM) | 55.38 ± 3.65 | 1 |
| Haem–Hp (10–2.5 μM) | 54.92 ± 6.64 | *NS* |
| Haem–rHbR (10–2.5 μM) | 65.89 ± 4.97 | *0.002* |
| Hp (2.5 μM) | 139.01 ± 5.7 | *0.001* |
| rHbR (2.5 μM) | 139.03 ± 7.8 | *0.001* |

Results are expressed as mean ± SD from three experiments in triplicate.
[a]*P*-values for the comparison with haem group, *NS: P > 0.05*

**Table III. Effect of rHbR and Hp on haem-catalysed lipid peroxidation.**

| Additives | MDA (nmol/mg membrane protein) | *P*-values[a] |
|---|---|---|
| None | 3.53 ± 1.69 | *0.012* |
| Haem (10 μM) | 24.89 ± 8.52 | 1 |
| Haem–Hp (10–2.5 μM) | 14.71 ± 2.78 | *0.041* |
| Haem–rHbR (10–2.5 μM) | 21.01 ± 5.33 | *NS* |
| $H_2O_2$ (0.25%) | 198.29 ± 14.91 | *0.001* |

Results are expressed as mean ± SD from three experiments in triplicate.
[a]*P*-values for the comparison with haem group, *NS: P > 0.05*

itself could induce erythrocyte membrane lipid peroxidation, as shown in Table III. We also found that after 2-h incubation, rHbR did not suppress haem-induced lipid peroxidation in comparison with haem alone ($P > 0.05$), while Hp slightly reduced lipid peroxidation by haem ($P < 0.05$). In addition, $H_2O_2$, a typical oxidant, caused much higher lipid peroxidation, as determined by MDA formation.

### *Haemolysis by monomeric and synthesized μ-oxo dimeric* haem

To study the effect of monomeric and μ-oxo dimeric haem on membrane destabilization, a haemolytic experiment was performed using fresh erythrocyte as described above. The results showed that both monomeric and μ-oxo dimeric forms of haem could induce haemolysis (Fig. 1D). As higher haem concentrations were used, more haemolysis was induced. After 2 h incubation, haemolysis increased from about 22% to 51% and from 12% to 36%, respectively, when the monomeric and μ-oxo dimeric haem concentrations were increased from 5 to 50 μM. In the comparison of effects of the two forms of haem, μ-oxo dimeric has less effect than the monomeric form in inducing haemolysis ($P < 0.05$).

## Discussion

HbR is one of the adhesion domains of gingipains in *P. gingivalis* that are very essential for the growth, survival and virulence of this bacterium (*22–25*). In the present study, we found an interesting activity

of the Hb-binding domain HbR that has a protective effect against haem-induced membrane damage, using an erythrocyte haemolytic assay as an experimental model (Fig. 1C). Hp, another Hb-binding protein (*33*), was also studied but it showed less inhibitory effect against haem-induced haemolysis than rHbR. The difference in inhibition activity of these two proteins may be explained through their interaction with Hb. During haemolysis, released Hb is a potent oxidant that can catalyse various oxidative and peroxidative reactions (*45*). Hp, an antioxidant protein that plays an essential role in capturing free Hb (*33*) and can block induced oxidative damage (*46*, *47*), interacts with Hb to form a stable Hp–Hb complex, as opposed to haem, so it reduces the toxicity of free Hb after haemolysis more than free haem. On the contrary, at neutral pH (pH 7.4), HbR has less ability to bind Hb (*29*), so the impact of Hp and HbR on haem and Hb is different.

Even though rHbR and Hp showed different effects on haem-induced haemolysis, both can inhibit haem binding to a white ghost membrane (Table I), and both can remove haem from a haem–membrane complex (Fig. 2), indicating the same effects of lowering haem affinity to a membrane. Thus, the inhibition of haem binding to a membrane or removal of haem from a membrane may not cause the differing effects of rHbR and Hp on haem-induced membrane damage.

Free haem reportedly damages lipids and proteins, such as by inducing lipid oxidation of a human erythrocyte ghost (*8*), aggregation in the membrane and promoting oxidation, causing cell lysis and death (*9*, *10*). The effect of rHbR and Hp on haem oxidant activity on proteins and lipids was further studied. Besides inhibiting haem-induced haemolysis, rHbR also reduces haem oxidant activity (Table II), but it has no significant effect on haeme-mediated erythrocyte membrane lipid peroxidation (Table III). Both rHbR and Hp showed the same effect on lipid peroxidation but rHbR had more impact on reducing membrane protein oxidation, which suggests that the suppression of protein oxidation is at least partially responsible for the inhibition mechanism of HbR against haemolysis.

Using UV-visible spectroscopy, the present study also demonstrated the ability of rHbR to convert monomeric haem to the dimeric form (Fig. 3), which was also confirmed by the covalent Fe-O-Fe bridged haem complex using FTIR spectroscopy (Fig 4). The mechanism of rHbR-mediated dimerization is not clear, but it was thought that the HbR domain may serve as a template to transiently bind monomeric haem, that they may react with other haem molecules, either free in solution or bound to protein, to form μ-oxo dimer complex (*31*). Activity of both μ-oxo dimeric and monomeric haem in haemolysis was also studied and it indicated that the μ-oxo dimeric form has less activity in inducing haemolysis than the monomeric form (Fig. 1D). Taken together, since cellular permeability induced by haem also depends on the state of haem, the inhibitive ability of HbR on haem-induced membrane damage may be a function of its mediating dimerization of haem molecules.

Both rHbR and Hp have protective effect against haem-induced membrane damages by lowering the affinity of haem molecule and removing it from the membrane. However, rHbR show more inhibitive effect than Hp. Besides the ability to bind to Hb, which is discussed above, it could be explained that rHbR can convert haem molecules to the μ-oxo dimeric form, which has less activity in inducing haemolysis compared to the monomeric form, thereby lowering the toxicity of the haem molecule. That causes the difference in protective function between rHbR and Hp against haem-induced membrane damages.

HbR proved to be a haem- and Hb-binding protein, associated with the outer membrane of *P. gingivalis* (*29*, *30*). This protein played a role in capturing haem (*48*) as an iron nutrient source for the bacterium. Because of the toxic properties of this nutrient in an iron overload environment, as well as some membrane receptor and iron/haem uptake regulation systems, HbR is also very important for bacterium survival, by converting monomeric haem into the μ-oxo dimeric complex (*31*), which was stored intracellularly as a black pigment (*32*). μ-Oxo dimeric haem can protect *P. gingivalis* against hydrogen peroxide and may function as a protective barrier against assault by reactive oxidants generated by neutrophils (*32*, *49*), which makes it an important virulence factor for this bacterium.

In conclusion, HbR played a role in capturing haem (*48*) as an iron nutrient source for *P. gingivalis*. We suggest that HbR may play an important role in protecting the bacterium in the infection pocket by reducing haem-induced membrane damage. It is suggested that the mechanism of HbR inhibition on haem-induced membrane damage is through removing haem from haem–membrane complex and lowering free haem toxicity by mediating dimerization of haem molecules.

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### Conflict of interest

None declared.

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