Antibacterial and antioxidant activity of plasma from crocodile (*Crocodylus siamensis*) and human

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Abstract

Crocodylus siamensis is a small freshwater crocodilian. In the wild, crocodiles live in environments with high risk of bacterial infection, but they normally suffer no adverse effects. Even though the immune system of crocodiles has not been well characterized, it was believed that the powerful innate immunity of crocodiles was derived from their blood, especially plasma. In the present study, we have examined both antibacterial and antioxidant properties of *C. siamensis* plasma by comparing those activities with human plasma. In terms of antibacterial properties, broth dilution assay revealed that *C. siamensis* plasma had significantly higher potent effect on *Staphylococcus* spp. than human plasma. At a concentration of 1000 μg/ml, the percentage of *S. aureus* ATCC 25923 inhibition of *C. siamensis* plasma was determined as 97%, while 60% inhibition was observed in human plasma. In addition, 1,000 μg/ml *C. siamensis* plasma strongly inhibited growth of bacterial drug-resistant strain (methicillin-resistant *S. aureus* DMST 20652 (MRSA)) with the percentage of inhibition at 99%. However, 1,000 μg/ml human plasma was able to inhibit growth of *S. aureus* (MRSA) by only 60%. To investigate antioxidant activity, an ABTS assay was performed, which revealed the ability of crocodile plasma to possess antioxidant activity higher than human plasma. Our data directly indicates that *C. siamensis* plasma is potentially useful as a pharmaceutical agent against disease.

Introduction

Bacterial infection is a worldwide problem at present and is a cause of death from infectious disease, eventually leading to a decreasing population. *Staphylococcus aureus* is a gram positive bacterium which is a member of normal flora, and also an opportunistic bacteria that can be found on the skin[1]. *S. aureus* is one important strain to have acquired resistance to antibiotics and is a leading cause of death from infection via a wound, especially the methicillin-resistant *S. aureus* (MRSA) strain has been found spreading worldwide up to the present[1,2]. Moreover, the infection could be stimulating oxidant production and having an effect on oxidative stress in an infected cell[3].

Antibiotics are common broad spectrum or specific drugs used for bacterial infection therapies. They are classified by their action or their mechanism to kill bacterial cells via inhibition of biosynthesis molecules inside the cells [4,5]. Most antibiotic drugs are naturally-produced molecules[6], however commercial antibiotics in clinical use have problems which can cause bacterial resistance. In the last decade, antimicrobial peptides (AMPs) have the greatest potential to represent a new class of antibiotics, because of the non-specific mechanisms of antimicrobial action which result in membranolytic activity, in addition to also stimulating the innate immune response. Numerous studies have demonstrated that AMPs have been discovered from a wide range of sources such as plant[7], human[8] and animal, especially from crocodiles[9,10]. Generally, crocodiles always have injuries from battle for territory, food and breeding. However, there have not been reports of crocodile disease or death from infection. This evidence has led to the hypothesis that a crocodile's immune system has high efficacy. Thus, most researchers believe that the innate immunity in crocodile is much stronger than humans, since it can survive for such a long time[10]. Blood circulation is the first line of defense in the immune system[11]. With regard to our previous studies about *C. siamensis* blood, they have found potent broad spectrum antimicrobial activity in blood components, including hemoglobin, white blood cell extract, serum and also plasma[10,12]. Most of these blood components also show antioxidant activity, but are limited to the plasma. In fact, plasma is the main component of blood and consists of protein or nutrients that circulate in the bloodstream, especially substances involved with the immune system[14]. However, human plasma components have been reported to be AMPs against bacterial resistance, including α-defensin, lysozyme or cathelicidin[15]. To confirm the potential biological activites, *C. siamensis* plasma was evaluated and compared with human plasma.

Thereby this report will be comparing the biological ability between *C. siamensis* plasma and human plasma, in terms of antibacterial activity and antioxidant activity by using the bacterial stain *S. aureus* and the ABTS radical, respectively. All of these experiments can be used to explain the concept of biological activities of *C. siamensis* plasma and corroborate antibiotic drug development for clinical and industrial use.

Methodology

Plasma preparation

The whole blood of Siamese crocodile was provided from Sriracha Moda Farm, Chonburi, Thailand. Blood plasma was prepared by the method of Preecharram et al.[10]. Briefly, whole blood of *C. siamensis* was added to an anticoagulant tube, containing 0.05 M EDTA, and human whole blood was collected from a healthy donor, O Rh+ blood group, in a heparin tube. Both blood types were kept at 4 °C until blood layer separation. The blood plasma layer was collected and stored at -20 °C.

Bacterial strain

Staphylococcus aureus ATCC 25923 and Methicillin-resistant *Staphylococcus aureus* DMST 20652 (MRSA) were maintained in nutrient agar slant at 4°C.

Antibacterial assay

Bacterial inhibition of plasma was determined by broth dilution assay[16]. Bacterial strains were inoculated into nutrient broth (liquid nutrient medium) and held at the logarithmic growth phase. Bacteria cells containing 10^6 colony forming units (CFU/ml) were added into a 96-well plate. After that, different concentrations of sample were added into each well. Then bacterial samples were co-incubated for 24 h at 37 °C. The percentage of bacterial inhibition was detected by microplate reader for OD_{600nm} and calculated by comparison with a negative control. Streptomycin was used as a positive control in this experiment. Experiments were done in triplicate.

Antioxidant assay

Free radical scavenging was performed by ABTS radical cation ($ABTS^{+}$) reduction[13]. The oxidant reactions were prepared via ABTS radical production by incubating mixture solution, including 7 mM ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) and 140 mM $K_2S_2O_8$ ratio for 16 h at room temperature and adjusting the absorbance at 734 nm to 0.7 by using phosphate buffer saline pH 7.0. Thereby 10 µl of sample at different concentrations were mixed with 100 µl of mixture solution for 6 minutes at room temperature. The total free radical was detected for optical density at 734 nm. The experiment was performed in triplicates.

Statistical analysis

Statistical values of all results were calculated by using the analysis of variance. The differences between mean values of all data were compared using the least significant difference test. Statistical significance was set at $p < 0.05$.

Results and Discussion

Plasma components were separated from whole blood using anticoagulant (EDTA) and centrifugation. By liquid growth inhibition assay, *C. siamensis* and human plasma were antibacterial at concentrations of 62.5, 250 and 1000 µg/ml against *S. aureus* ATCC 25923 and drug-resistant *S. aureus* DMST 20652 methicillin-resistant (MRSA). The percentage of bacterial inhibition of *C. siamensis* plasma was significantly more effective than that of human plasma. *C. siamensis* plasma at the highest concentration of 1,000 mg/ml showed percentage inhibition on *S. aureus* ATCC 25923 of about 97%, together with inhibition in drug-resistant *S. aureus* DMST 20652 growth by 99% (Figure 1). Interestingly, *C. siamensis* plasma (highest percent inhibition: 99%) was statistically significantly more effective at inhibiting drug-resistant *S. aureus* DMST 20652 growth than human plasma (highest percent inhibition: 66%) by 1.5-fold (Figure 2).

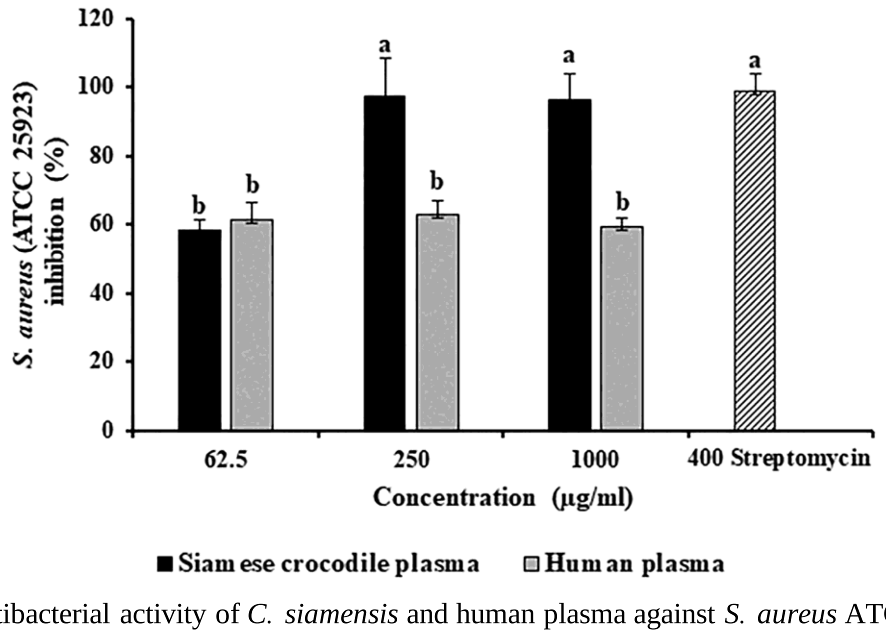

Figure 1. Antibacterial activity of *C. siamensis* and human plasma against *S. aureus* ATCC 25923 by broth dilution assay. Results are shown as mean ± SD. Different letters (a, b) on the top of individual bars indicate statistically significant differences at $p < 0.05$.

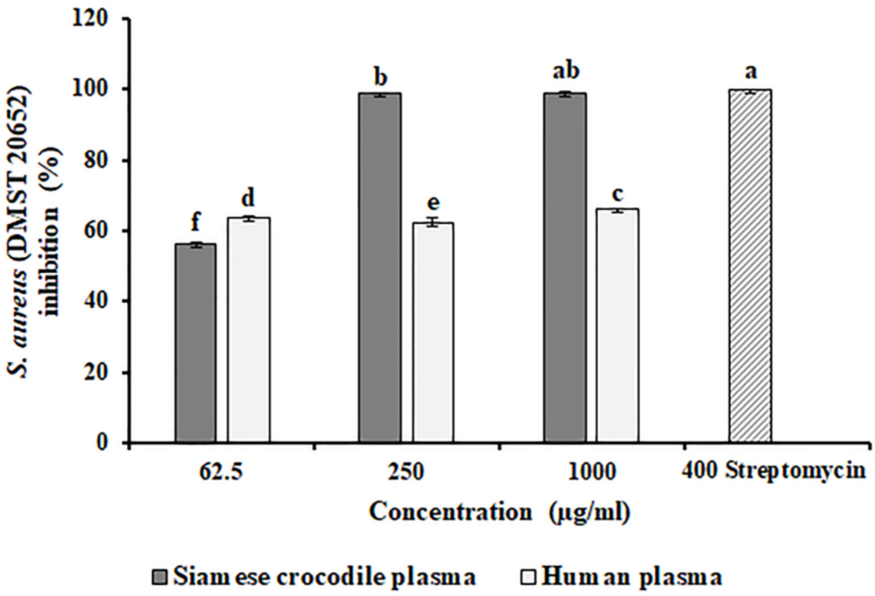

Figure 2. Antibacterial activity of *C. siamensis* and human plasma against the drug-resistant strain methicillin-resistant *S. aureus* DMST 20652 (MRSA) by broth dilution assay. Results are shown as mean ± SD. Different letters (a-f) on the top of individual bars indicate statistically significant differences at $p < 0.05$.

The production of free radicals is known to be involved in sepsis[17]. Thus, the ability of *C. siamensis* plasma to scavenge radicals was investigated. The results indicate that *C. siamensis* plasma could scavenge ABTS radicals significantly better than human plasma in a dose-dependent manner (Figure 3). At concentrations of 16.63, 62.5, 250, and 1,000 µg/ml *C. siamensis* plasma scavenged ABTS radicals at 19.3%, 52.6%, 79.1%, and 77.1%, while human plasma displayed ABTS radical scavenging activity for 0.6%, 15.3%, 49.2%, and 78.7%, respectively. The equal ABTS radical scavenging activity of *C. siamensis* plasma to standard Trolox was observed at concentrations of 250 and 1,000 µg/ml, while human plasma showed equal activity to Trolox only at 1,000 µg/ml.

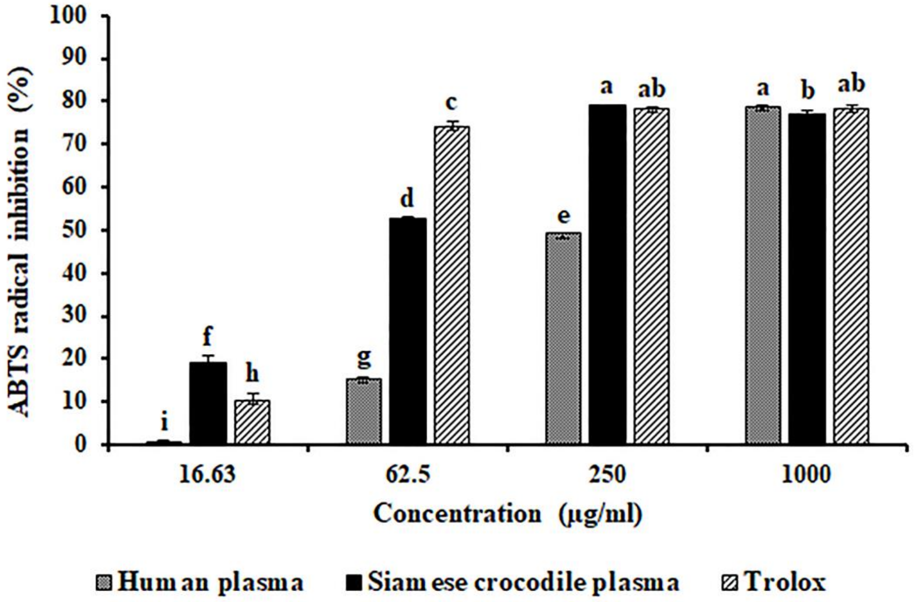

Figure 3. Antioxidant activity of *C. siamensis* and human plasma using ABTS assay. Results are shown as mean ± SD. Different letters (a-i) on the top of individual bars indicate statistically significant differences at $p < 0.05$.

Normally bacterial substances should inhibit or kill bacterial cells via some mechanism against an infection such a biochemical inhibition. *C. siamensis* plasma contains many strong proteins involved with the immune system that display antimicrobial activity, and these proteins can stand an unsuitable environment such as against proteinase digestion or heat as a short peptide[10,18]. The mechanisms of short peptides to inhibit bacteria were to directly destroy the bacterial cell membrane by enhancing permeabilization[19]. However, methicillin-resistant *S. aureus* DMST 20652 contains plasmid encoded β-lactamase, a bacterial substance produced by *S. aureus* (MRAS)[2]. Bacterial cells can recover by blocking methicillin activity, which can protect cell membrane production[2]. Therefore the inhibition efficiency of plasma against both *S. aureus* strains have identical activity via their non-specific mechanisms. Thus the results show similar data for inhibition of *S. aureus* and *S. aureus* (MRSA) (Figure 1 and Figure 2).

Moreover, bacterial infection can affect oxidative stress by causing a high level of free radical production and foreign matter inside the cell[3]. Bacterial infection can generate free radicals inside the host cell, causing an oxidative stress response[20]. In addition, phagocytic processes to inhibit bacterial infections generate two very potent toxic substances, H_2O_2 and O_2^- [3]. The bacterial cell can remove H_2O_2 by catalase inside the cell, but it affects the host cell. In a previous study, Preecharram et al. reported antimicrobial peptide in *C. siamensis* plasma. The amino acid composition of this antimicrobial peptide revealed high amounts of free amino acid, glutamic acid, glycine, serine, alanine, aspartic acid and lysine[10]. The basic amino acids (lysine) and polar amino acids (serine), of this peptide are comprised, might cause antioxidant activity. These amino acids are thought to be proton donors to stabilize oxidative molecules[21]. Moreover, the antioxidant activity of proteins might be exerted by specific domains or functional groups in each subunit that could act as electron donors, eventually leading to the termination of radical chain reactions. Examples include aliphatic and aromatic hydrophobic amino acid residues (valine, leucine, proline, histidine or tyrosine)[22]. The proteins from crocodile plasma still inhibited the level of free radicals more than human plasma by relating with the electron donor to ABTS radical cation[13,23]. Hence, *C. siamensis* plasma is an important ingredient that can be used in bacterial infection therapies.

Conclusion

Taken together, blood plasma separated from crocodiles (*Crocodylus siamensis*) was significantly effective against both types of *S. aureus*, and it also reduced total free radicals more via ABTS production than human plasma.

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