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Analysis of enzyme activity, antibacterial activity, antiparasitic activity and physico-chemical stability of skin mucus derived from *Amphiprion clarkii*

Huan Wang[a], Wei Tang[a], Rui Zhang[a], Shaoxiong Ding[a,b,*]

[a] State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, 361102, Fujian Province, China
[b] Marine Biodiversity and Global Change Research Center, College of Ocean and Earth Sciences, Xiamen University, Xiamen, 361102, Fujian Province, China

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ABSTRACT

Recently, mucosal surfaces, especially fish skin and its secreted mucus, have attracted significant interest from immunologists. *Amphiprion clarkii*, a member of the family Pomacentridae, lives symbiosis with sea anemones and has a good resistance to common seawater bacterial diseases and parasites owing to the protection from its abundant skin mucus. In the present work, the activity of immune-related enzymes (lysozyme, protease, antiprotease, cathepsin B, alkaline phosphatase and peroxidase), the antibacterial activity against two Gram-positive bacteria and five Gram-negative bacteria, the antiparasitic activity against the pathogen of marine white spot disease (*Cryptocaryon irritans* theronts) and the physico-chemical stability (to pH and heat) of the skin mucus of *A. clarkii* were analysed. The results showed that the levels of lysozyme and peroxidase were very similar (from 2 to 4 U mg^{-1} protein). However, cathepsin B was detected of 63.32 U mg^{-1} protein and alkaline phosphatase was only 0.12 U mg^{-1} protein. Moreover, protease showed a higher percentage of activity than antiprotease. *A. clarkii* skin mucus showed a strong antibacterial activity against Gram-negative bacteria, particularly against *Aeromonas hydrophila* and *Vibrio parahaemolyticus* but showed no effect on Gram-positive bacteria at the tested concentrations. The bactericidal activity functioned within a short time in a distinct time- and dose-dependent manner. SEM showed that after treated with *A. clarkii* skin mucus, the *V. parahaemolyticus* cells distorted and piled together, and the filaments appeared and became into cotton-shaped or quasi-honeycomb texture to adhere cells. Meanwhile, *A. clarkii* skin mucus showed an apparent antiparasitic activity against *C. irritans* theronts with a distinct dose- and time-dependent relationship. LM and SEM observation showed that after treated with skin mucus, the theronts quickly stopped their swimming and cilia movement, cells became rounded, cilia shed, small bubbles formed on the surface, cell nucleolus enlarged, cytoskeleton deformed, cell membranes ruptured and cell content leaked out. Antibacterial activity was not affected by 30–90 °C heat treatment but was slightly suppressed by 100 °C. In the pH treatment groups, antibacterial activity was not affected by the moderate pH treatment of 5.0–8.0, but slightly suppressed by weak acid and weak base. Therefore, we speculated that the skin mucus of *A. clarkii* might be a potential source of novel antibacterial and antiparasitic components for fish or human health-related applications. This study broadened our understanding of the role of skin mucus in the innate immune system and provided a basis for the further isolation and purification of active substances.

1. Introduction

In the aquaculture industry, the abuse of antibiotics has led to the emergence of drug resistance and has posed a major threat to global human and animal healthcare [1]. The development of effective substitutes with strong antipathogenic microbial effects but little or no toxicity to the host cell is urgently required [2]. The abundant skin mucus of fish may be an alternative source of antimicrobial agents. Fish are exposed to a complex aquatic environment that often contain different types of pathogenic and nonpathogenic microorganisms. The mucus layer, as a key part of the innate immune defences in fish, not only prevents the pathogen from adhering to underlying tissues but also provides a medium within which anti-pathogenic mechanisms may act [3,4]. The antimicrobial properties of fish skin mucus have attracted the attention of researchers in the field over the past few decades. To date, several potent antibacterial peptides/proteins (AMPs) have been isolated from fish with thick skin mucus layer, including myxinidin from hagfish [5], SSAP from rockfish [6], pelteobagrin from yellow

* Corresponding author. State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, 361102, Fujian Province, China.
E-mail address: sxding@xmu.edu.cn (S. Ding).

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catfish [7], AJN-10 from Japanese eel [8], and hipposin from Atlantic halibut [9]. The AMP species and activity in the mucus varies greatly among these fish species. Moreover, to some extent, the activity of AMPs could be reflected by the anti-pathogenic microbial activity of fish skin mucus. Therefore, the exploration of disease resistance of fish skin mucus has significant implications for the screening of new AMPs.

Anemonefishes, as major members of the family Pomacentridae, are an important part of the coral reef ecosystem. Furthermore, due to their bright body colour and petite body shape, anemonefishes have become one of the most popular ornamental marine species among hobbyists, and there is a well-developed artificial propagation industry. Many studies of these fish have been conducted to understand the maintenance of their social structure, the mutualistic symbiosis with their host sea anemones and the mechanism involved in their sex change [10–16]. However, research on their immunity is lacking. Unlike other fish, anemonefishes are symbiotic with sea anemones, and their ability to avoid being stung by sea anemones is attributed to protection via abundant skin mucus [17,18]. In addition, in our long-term experience with breeding in the laboratory, we have found that anemonefishes have a good resistance to common seawater bacterial diseases and parasites, even in the case of individuals with a wound on the skin, which suggests that skin mucus might also play an important role in innate immunity. Therefore, the aim of the present study was to evaluate the antibacterial and antiparasitic activity of skin mucus derived from *Amphiprion clarkii*; this investigation could broaden our understanding of the biology and function of skin mucus in the innate immune system and provide a basis for the further isolation and purification of new active substances.

2. Materials and methods

2.1. Fish care and maintenance

Ninety adult specimens of *A. clarkii* (mean body weight 7.25 ± 2.68 g) were maintained at Xiamen University in seawater recirculating aquaculture systems (750 L) with PVC pipe replacing the anemone. The seawater was maintained at 27 ± 2 °C with a flow rate of 800 L h^{-1} and 30‰ salinity. The photoperiod was 12 h light:12 h dark, and the fish were fed a commercial pellet diet. The health conditions of the fish were monitored during their entire life to ensure that no infections by pathogenic bacteria or parasites occurred. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Xiamen University.

2.2. Skin mucus collection

Skin mucus samples were collected according to the method of Subramanian et al. [19] with minor modifications. The fish were starved for 24 h to avoid contamination from intestinal excretions; the mucus was collected non-lethally by anaesthetizing the fish with 100 mg L^{-1} MS-222 (Sigma, America). After being washed with sterile seawater, the fish were transferred into sterile polyethylene bags containing 10 mL of 100 mM NaCl and gently moved front and back for approximately 2–3 min to slough off the skin mucus. The fish were then returned to the tanks to recover, and the mucus samples were pooled (3 pools of 30 fish each). The fresh mucus samples were immediately transferred to 50 mL sterile centrifuge tubes and lyophilized following freezing at −80 °C. The lyophilized skin mucus powder was dissolved in Milli-Q water; the undissolved portion was separated by centrifugation at 12,000 rpm/min for 30 min at 4 °C and discarded. After the supernatant was filtered through a 0.22 μm Millipore filter, the total protein concentration was determined by the BCA method (Sangon Biotech, China) and adjusted to 1 mg protein mL^{-1}. The samples were stored in 1.5 mL centrifuge tubes and held at −80 °C until further analysis.

2.3. Skin mucus enzyme activities

2.3.1. Lysozyme activity

The lysozyme activity in the mucus samples was measured according to the method described by Ellis [20] with minor modifications. Briefly, 200 μL of skin mucus samples were placed in flat-bottomed 24-well plates in triplicate. Two millilitres of freeze-dried lysozyme-sensitive bacteria (*Micrococcus lysodeikticus*, 0.25 mg mL^{-1}, Sigma) were added to each well as the lysozyme substrate. The absorbance at 450 nm was read by an automatic microplate reader after incubation for 15 min at 37 °C. The lysozyme units were determined from a standard curve made using hen egg white lysozyme, and the results were expressed as U mg^{-1} mucus protein.

2.3.2. Protease activity

The protease activity in the skin mucus was quantified by an azocasein hydrolysis assay according to the method of Ross et al. [21]. Briefly, the skin mucus was incubated with an equal volume of azocasein (7 mg mL^{-1} dissolved in 100 mM ammonium bicarbonate buffer, Sigma) on a shaker at 30 °C for 19 h. The reaction was stopped by adding trichloroacetic acid (4.6% final concentration). The mixture was cooled on ice, centrifuged at 10,000 × *g* for 10 min, and 100 μL of supernatant was transferred to a 96-well plate containing 100 μL of 0.5 N NaOH. The OD was measured at 450 nm using an automatic microplate reader. Trypsin solution (5 mg mL^{-1}, Sigma) replaced the skin mucus as positive control (100% protease activity), and buffer served as negative control (0% protease activity).

2.3.3. Antiprotease activity

A modification of the method described by Ellis [22] was used according to the ability of skin mucus to inhibit trypsin activity. Briefly, 20 μL of skin mucus samples were incubated with the same volume of trypsin solution (5 mg mL^{-1}, Sigma) at 22 °C for 10 min. Then, 200 μL of 100 mM ammonium bicarbonate buffer and 250 μL of 0.7% azocasein were added and incubated at 30 °C for 2 h, following the addition of 500 μL of 4.6% TCA, a new incubation at 30 °C for 30 min was carried out. After centrifuged at 10,000 rpm for 10 min, the supernatant was transferred to the 96-well plate containing 100 μL of 0.5 N NaOH, and the OD was measured at 450 nm using an automatic microplate reader. Buffer replaced the skin mucus as positive control (100% protease activity), and buffer replaced the trypsin as negative control (100% antiprotease activity).

2.3.4. Cathepsin B activity

Cathepsin B activity in the skin mucus was assayed according to the method of Barrett et al. [23] with slight modifications. Briefly, after reconstituted in 0.1 M sodium phosphate buffer (pH 6.0), 50 μL of skin mucus samples was incubated with 20 μL of 1 mM dithiothreitol, 20 μL of buffer (1 mM EDTA, 0.1 M sodium phosphate, 0.08% (w/v) Brij, pH 6.0) and 60 μL of milli-Q water in a 96-well plate at 30 °C for 5 min. Then, 50 μL of 25 μM carbobenzoxy-L-phenylalanyl-L-arginyl-4-methylcoumaryl-7-amide was added to each well as the cathepsin B substrate. After incubation for 30 min, the fluorescence of AMC was measured continuously at 30 °C for 30 min at excitation wavelength of 380 nm and emission wavelength of 405 nm. The enzyme activity was calculated according to the initial rate of reaction and the amount of enzyme required to release 1 μmol of substrate in 1 min was defined as 1 U of activity.

2.3.5. Alkaline phosphatase activity

The alkaline phosphatase activity in the skin mucus was measured according to the method described by Ross et al. [21]. Briefly, 50 μL of skin mucus reconstituted in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8) at 30 °C for 15 min. Then equal volume of 4 mM *p*-nitrophenol phosphate was added as substrate and the OD was continuously measured at 405 nm over 3 h at 1 min intervals.

The enzyme activity was calculated according to the initial rate of reaction and the amount of enzyme required to release 1 mmol of *p*-nitrophenol in 1 min was defined as 1 U of activity.

2.3.6. Peroxidase activity

The peroxidase activity was determined according Quade et al. [24]. Briefly, 30 μL of skin mucus samples were diluted with 120 μL of Hank's buffer in a 96-well plate. Then, 50 μL of 20 mM TMB and 5 mM H_2O_2 were added as substrates. After 2 min, the colour change reaction was terminated by adding 50 μL of 2 M sulfuric acid. The OD was measured at 450 nm using an automatic microplate reader. The samples without skin mucus were used as blanks. The one unit peroxidase activity was defined as the amount that produces a change in absorbance of 1, and the activity was expressed as U mg^{-1} mucus proteins.

2.4. Skin mucus antibacterial activity

2.4.1. Antibacterial spectrum detection

The antibacterial activity of *A. clarkii* mucus was evaluated against seven bacterial strains, including two Gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus lysodeikticus*) and five Gram-negative bacteria (*Aeromonas hydrophila*, *Vibrio harveyi*, *V. alginolyticus*, *V. parahaemolyticus* and *Pseudomonas fluorescens*) (Table 2). *S. aureus*, *M. lysodeikticus* and *P. fluorescens* were purchased from the China General Microbiological Culture Collection Center (CGMCC). Other strains were gifted from Fisheries college, Jimei University, Xiamen, China. All strains have been confirmed as pathogenic by artificial infection. Marine bacteria were grown in saline peptone water (3% tryptone and 3% NaCl, w/v, pH 7.0) at 28 °C, and non-marine bacteria were grown in Mueller-Hinton broth/agar medium (MHB/MHA, pH 7.3) at 37 °C.

The antimicrobial activity of skin mucus was determined by evaluating the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the method of Wang [25] with minor modifications. Briefly, the logarithmic phase of the bacterial cultures were adjusted to a final concentration of 2×10^6 CFU/mL and co-incubated with a 2-fold serial dilution of skin mucus (final concentrations 0.0156–0.5 mg protein mL^{-1}). The microbial growth was examined after 24 h of incubation at 28 °C or 37 °C. The MIC was expressed as the range from the highest protein concentration at which microbial growth occurred to the lowest protein concentration at which microbial growth was completely inhibited.

To determine the MBC, the contents of the wells without visible microbial growth were plated onto corresponding agar plates and incubated overnight at 28 or 37 °C to assess viability. The criteria for the MBC were the same as those for the MIC. All tests were repeated at least three times. For all series of experiments, the two controls (no skin mucus or no bacteria) were included.

2.4.2. Kill-curve studies

A kinetic study was performed to determine the bactericidal effect of skin mucus using *V. parahaemolyticus* as described by Supungul et al. [26]. Briefly, the logarithmic phase culture of *V. parahaemolyticus* was incubated with skin mucus (0.25 and 0.5 mg protein mL^{-1}, twice the MBC) as described above. At various time points (0–360 min), 5 μL of the mixture was removed and diluted in NaPB; after plating on nutrient broth agar and incubating at 28 °C overnight, the colonies were counted. The percent CFU was defined relative to the CFU obtained in the control (100% CFU at 0 min). The data at each time point were expressed as the mean ± SD of 3 replicates, and the presence of a significant difference was determined using a two-way ANOVA with a Tukey post hoc test ($P < 0.05$).

Table 1
Enzyme activities in skin mucus of *A.clarkii*. The data are shown as the mean ± SD.

Enzyme	Activity
Lysozyme (U mg^{-1} protein)	2.96 ± 0.31
Protease (%)	18.74 ± 0.42
Antiprotease (%)	4.57 ± 0.24
Cathepsin B (U mg^{-1} protein)	63.32 ± 1.18
Alkaline phosphatase (U mg^{-1} protein)	0.12 ± 0.01
Peroxidase (U mg^{-1} protein)	3.45 ± 0.26

Table 2
Antibacterial activity of skin mucus from *A.clarkii*.

Bacteria	CGMCC no.[a]	MIC(mg protein mL^{-1})	MBC(mg protein mL^{-1})
Gram-positive bacteria			
Staphylococcus aureus	1.2465	> 0.5	> 0.5
Micrococcus lysodeikticus	1.634	> 0.5	> 0.5
Gram-negative bacteria			
Aeromonas hydrophila	J[b]	0.0625–0.125	0.125–0.25
Vibrio harveyi	J[b]	0.25–0.5	> 0.5
Vibrio alginolyticus	J[b]	0.25–0.5	0.25–0.5
Vibrio parahaemolyticus	J[b]	0.0625–0.125	0.125–0.25
Pseudomonas fluorescens	1.3202	0.125–0.25	0.25–0.5

[a] CGMCC no. indicates China General Microbiological Culture Collection number.
[b] "J" indicates Fisheries college, Jimei University, Xiamen, China.

2.4.3. Scanning electron microscope (SEM) of bacteria surface ultrastructure following exposure to skin mucus

After incubated with skin mucus (1 mg protein mL^{-1}) for 8 min, 30 min and 1 h, the morphological changes of *V. parahaemolyticus* were assessed under SEM. Collected *V. parahaemolyticus* samples were fixed in 2.5% glutaraldehyde at 4 °C for 24 h, washed in 0.1 M phosphate buffer three times for 20 min and dehydrated in a graded series of ethanol solutions for critical point drying in carbon dioxide. The samples were placed on aluminium stubs and coated with gold for observation under a Jeol JSM-6390LV SEM.

2.5. Skin mucus antiparasitic activity

2.5.1. Parasiticidal effect of skin mucus

In our aquaria, we have observed that *A. clarkii* was not infected with the marine white spot disease caused by *C. irritans*. Therefore, we explored whether skin mucus possessed antiparasitic activity.

Seawater (1.2 mL) containing ~200 theronts hatched within 2 h was transferred to a 24-well cell culture plate, and a different volume of skin mucus was added to each well to a final available protein concentration of 0.05, 0.1, 0.2 or 0.3 mg ml^{-1}. Negative and blank controls were created by adding the same volumes of seawater or Milli-Q water, respectively, to the theront solutions and incubating at room temperature. At each time point (5, 15, 30, 45 and 60 min), the mortality of the theronts was quantified based on swimming ability and cilia oscillation.

2.5.2. Light microscopy (LM) and SEM of theront surface ultrastructure following exposure to skin mucus

After 10 min of incubation with skin mucus (0.3 mg protein mL^{-1}), changes in the morphology of the parasitic theronts were assessed using LM and SEM. Sample preparation for SEM was conducted as described in section 2.4.3.

2.6. Skin mucus physico-chemical stability

V. parahaemolyticus was selected as the test strain for the analysis of the physico-chemical stability of skin mucus. The MIC was determined as described in section 2.4.1 under different treatments to determine

whether pH or heat could affect skin mucus activity.

To assess pH tolerance, the skin mucus power was treated with different pH buffers (pH 2–3: 0.2 M glycine-HCl buffer; pH 4–5: 0.2 M acetate buffer; pH 6–8: 0.2 M phosphate buffer; pH 9–11: 0.2 M glycine-NaOH buffer) for 1 h at 4 °C.

To assess heat stability, the skin mucus was heat-treated for 20 min at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C, and 100 °C.

3. Results

3.1. Skin mucus enzyme activities

The six immune-related enzymes, lysozyme, protease, antiprotease, cathepsin B, alkaline phosphatase and peroxidase were detected in the 1 mg protein mL^{-1} samples of skin mucus (Table 1). While levels of lysozyme and peroxidase were very similar, cathepsin B showed a higher activity and alkaline phosphatase showed a lower level. Moreover, protease showed a higher percentage of activity than antiprotease. Both lysozyme, protease, antiprotease and alkaline phosphatase showed a lower activity level in *A. clarkii* than have been reported in other fish [27].

3.2. Skin mucus antibacterial activity

3.2.1. MIC and MBC

The antibacterial activity of skin mucus from *A. clarkii* against both Gram-positive and Gram-negative bacteria was determined using MIC and MBC assays (Table 2). The results showed that the skin mucus exhibited strong activity against the Gram-negative bacteria tested. In particular, the important marine pathogen, *V. parahaemolyticus* (0.0625–0.125 mg protein mL^{-1}), and the non-marine pathogen, *A. hydrophila* (0.0625–0.125 mg protein mL^{-1}), were the most susceptible to the skin mucus. However, the skin mucus had no effect on Gram-positive bacteria under the tested concentrations. For all tested bacteria, the MBC was either equal to or twice the MIC.

3.2.2. Kinetics study

In the kinetics study, a highly sensitive strain of *V. parahaemolyticus* was used to evaluate the bactericidal activity of the skin mucus from *A. clarkii* (Fig. 1). In the groups treated with 0.5 mg protein mL^{-1} of skin mucus, more than 90% of *V. parahaemolyticus* were killed within 8 min, and nearly all the bacteria were killed after treated for 30 min. Treatment with 0.25 mg protein mL^{-1} of skin mucus also killed over 90% of the bacteria within 30 min and nearly all the bacteria after 45 min. In the short term experiments, both the 0.5 and 0.25 mg protein mL^{-1} skin mucus treatment groups showed the strongest bactericidal activity within the first 8 min followed by a slight decrease in the sterilization efficiency. Thus, the kinetics study indicated that the skin mucus of *A. clarkii* could kill most bacteria within a short time in a distinct time- and dose-dependent manner.

3.2.3. Morphological changes in V. parahaemolyticus using SEM

To further characterize the antibacterial effect of *A. clarkii* skin mucus, scanning electron micrographs of the effects of *A. clarkii* skin mucus on morphological changes in *V. parahaemolyticus* were shown in Fig. 2. The untreated *V. parahaemolyticus* were rod-shaped, plump and round with a smooth surface with no any adhesion between the individuals (Fig. 2A). After treated by skin mucus, the *V. parahaemolyticus* became distorted with grooves and corrugations, a few strange filaments hold some cells together, and a few cotton-shaped substances appeared at 8 min (Fig. 2B); with time extension, the filaments disappeared and more cotton-shaped substances clustered more cells together at 30 min (Fig. 2C); the aggregation degree continued to be worse, the cotton-shaped substance became quasi-honeycomb texture to cover on the *V. parahaemolyticus* at 1 h (Fig. 2D). Therefore, we speclated that the skin mucus treatment caused the cell membrane of *V. parahaemolyticus* to rupture and the cell contents to flow out.

3.3. Skin mucus antiparasitic activity

3.3.1. Antiparasitic effect of skin mucus

The antiparasitic activity of skin mucus from *A. clarkii* was assessed by studying the acute toxicity to *C. irritans* theronts (Fig. 3). The results showed that the skin mucus was highly active against theronts at a low concentration. In the groups treated with 0.3 mg protein mL^{-1} of skin mucus, almost 100% of theronts were killed within 5 min of incubation. Treatment with 0.2 mg protein mL^{-1} of skin mucus killed over 70% of theronts within 5 min, and nearly all of the theronts were dead at 60 min. In the 0.1 mg protein mL^{-1} treatment group, skin mucus showed a mild antiparasitic effect, and the mortality of theronts was approximately 50% after 60 min of incubation. In the 0.05 mg protein mL^{-1} treatment group, there was little significant antiparasitic effect. Thus, the range of skin mucus concentrations against *C. irritans* theronts demonstrated a distinct dose- and time-dependent relationship.

3.3.2. Observation of C. irritans theronts morphological changes

When treated with 0.3 mg protein mL^{-1} of skin mucus for 10 min, almost all *C. irritans* theronts have quickly stopped their swimming and cilia movement, some changed from an elliptical to a round shape (Figs. 4B, C and 5B), some theronts shed their cilia and small bubbles formed on their surface (Figs. 4E–H and 5B-I), some theronts exhibited an enlarged cell nucleolus and a deformed cytoskeleton (Figs. 4C–F and 5C and D), and some cell membranes were ruptured and cell content leakage occurred (Figs. 4G–I and 5E-I).

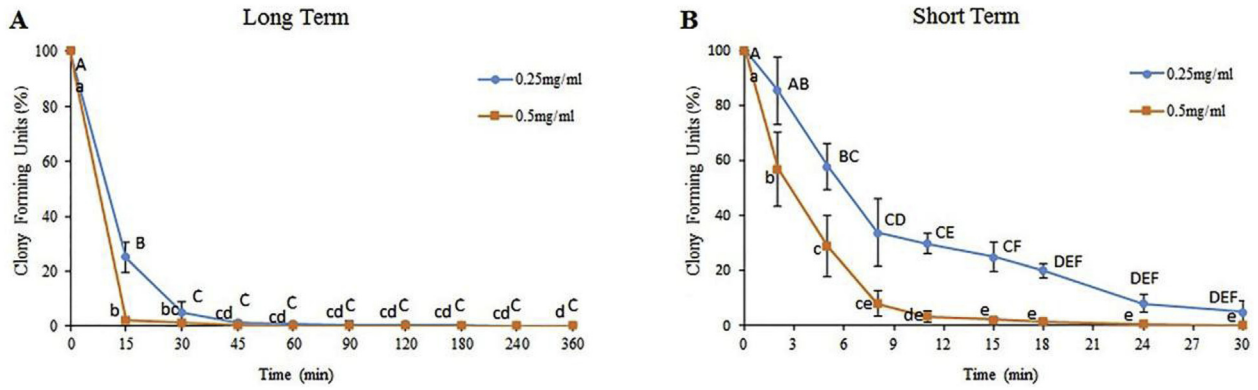

Fig. 1. Kinetics of skin mucus from *A. clarkii* against *V. parahaemolyticus*. The data at each time point are shown as the mean ± SD (n = 3). Different letters, (A through E) or (a through e), indicate significant differences ($P < 0.05$) between time points.

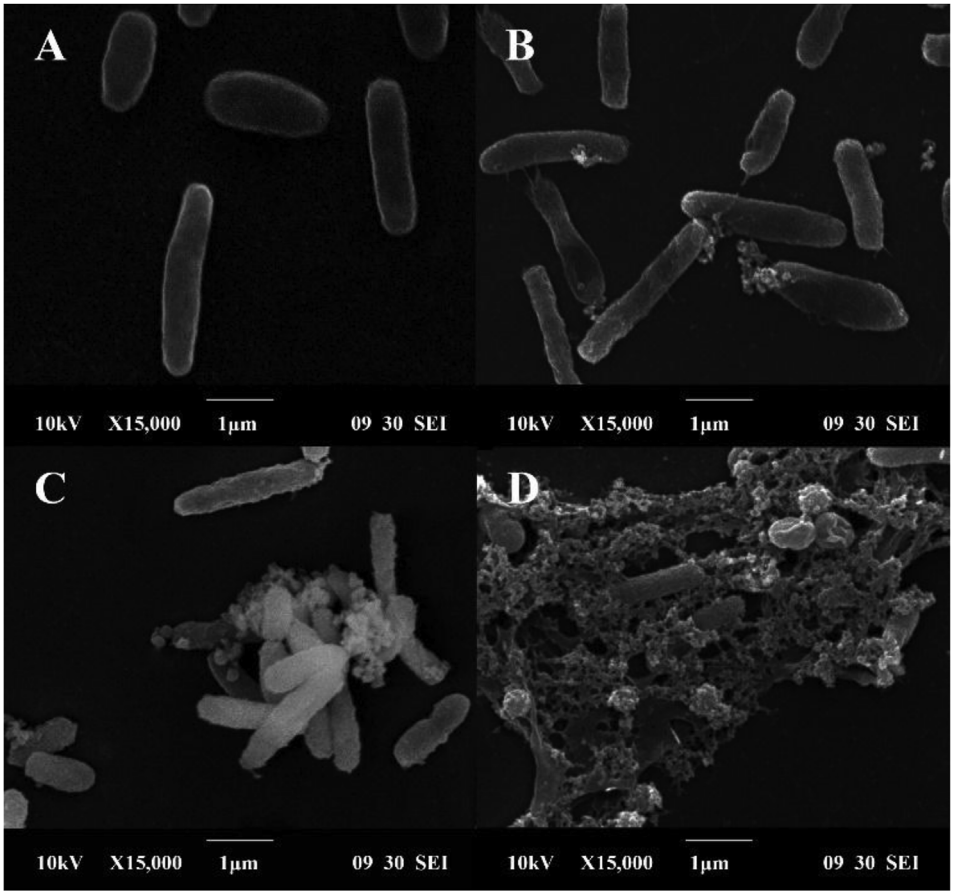

Fig. 2. SEM examination of morphological changes in *V. parahaemolyticus* after treatment with *A. clarkii* skin mucus. A. Untreated (control). B through D. Treated with 1 mg protein mL^{-1} skin mucus for 8 min (B), for 30 min (C) and for 1 h (D).

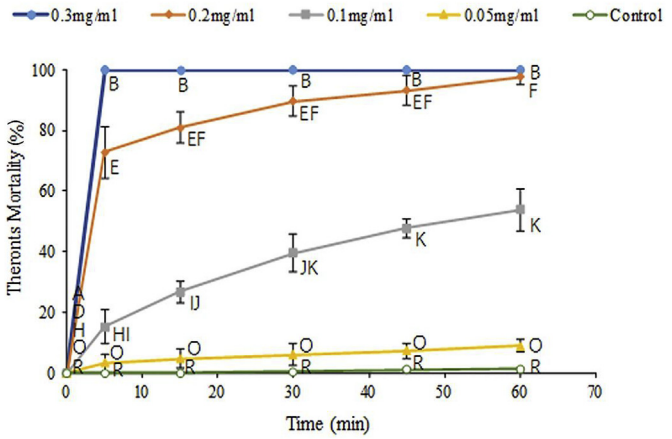

Fig. 3. Effect of different concentrations of *A. clarkii* skin mucus on *C. irritans* therons. The data at each time point are shown as the mean ± SD (n = 3). Different letters (A and B, D through F, H through K, O and Q, R and T) indicate significant differences ($P < 0.05$) between time points.

3.4. Skin mucus physico-chemical stability analysis

3.4.1. pH tolerance

The pH tolerance of *A. clarkii* skin mucus was determined by studying the MIC against *V. parahaemolyticus* after different pH buffer treatments (Table 3). After the moderate pH treatment (pH 5.0–8.0), the antibacterial activity was not affected by acids or bases. As the acidity and alkalinity increase (pH 4.0 and pH 9.0), the antibacterial activity decreased. In the environment of strong acid and alkali (pH < 4.0 and pH > 9.0), the acid and alkali could also kill *V. parahaemolyticus*, so we conld not judge the change of antibacterial activity. Therefore, the *A. clarkii* skin mucus has a pH stability range of 5.0–8.0.

3.4.2. Heat stability

To detect the heat stability of *A. clarkii* skin mucus, the MIC against *V. parahaemolyticus* was determined after treatment at different temperatures (Table 4). In the 30–90 °C treatment groups, antibacterial activity was not affected by the heat treatment. In the 100 °C treatment group, antibacterial activity was suppressed to 25%. Thus, the *A. clarkii* skin mucus has an ideal heat stability range of 30–90 °C.

4. Discussion

The fish skin mucus layer, which is the biological interface between fish and their aqueous environment, constitutes the first line of defence against potential pathogens [28]. Fish mucus forms an effective physico-chemical barrier that contains numerous innate immune factors such as lysozyme, protease, glycoproteins, immunoglobulins and AMPs [4]. Previous studies have demonstrated that the protective effect of mucus and the activity of its various components vary among fish species, and therefore the skin mucus might be a potential source of novel antibacterial components [27,29–32]. Thus, the study of skin mucus in more teleost fish species and a deeper characterization would enhance the understanding of fish mucosal immunity and the interaction with pathogens and disease processes. Given the importance of skin mucus in the immune system and the lack of knowledge about the skin mucus of coral reef fish, we conducted this study.

The enzymes in epidermal mucus play an important role in the immune function of fish, and protease, antiprotease, lysozyme, alkaline

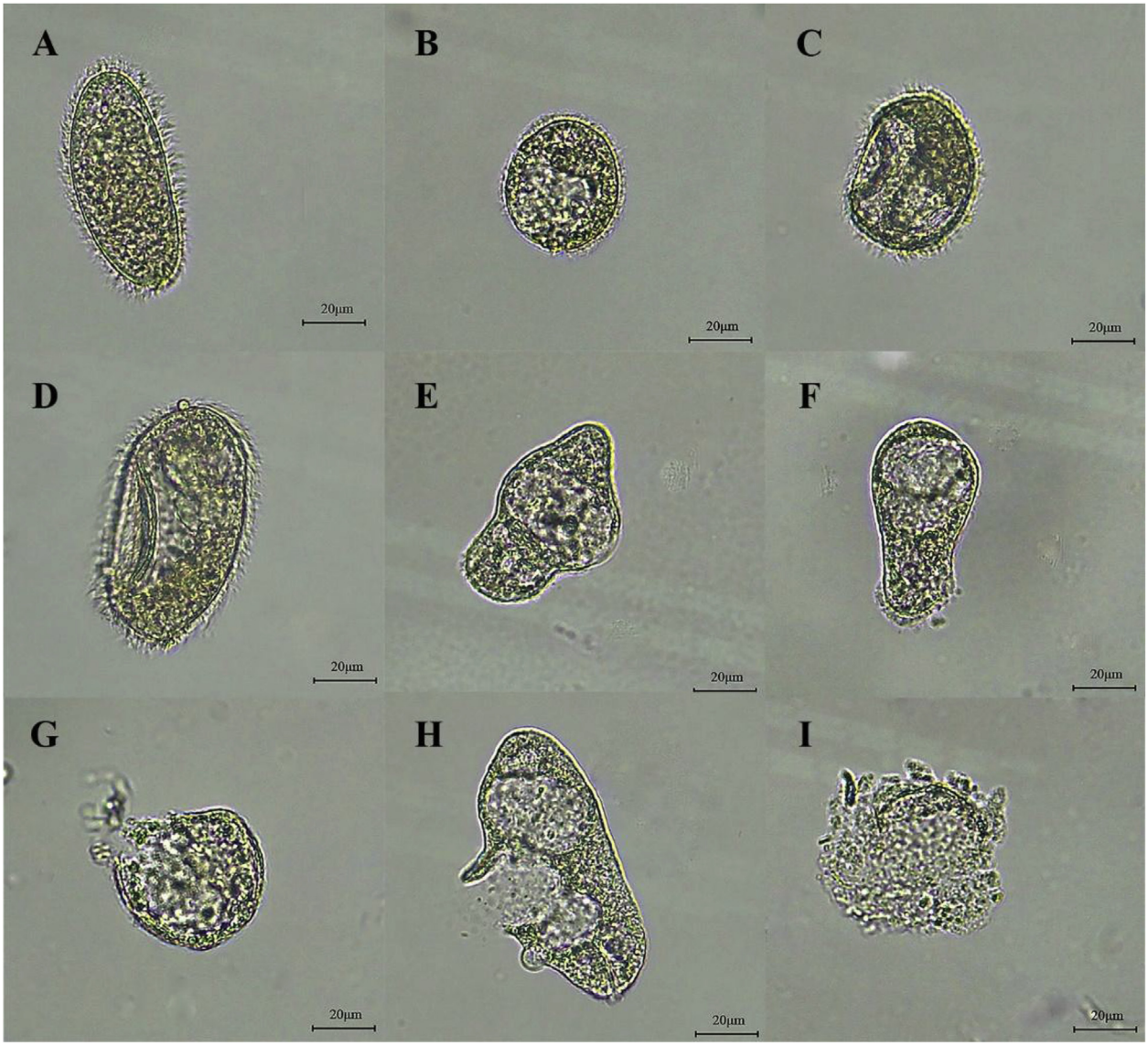

Fig. 4. LM of morphological changes in *C. irritans* theronts after treatment with *A. clarkii* skin mucus. The results of the light microscopy showed that the skin mucus could quickly induce the theronts stopped ciliary movements, deformation, cilia fell off, cell nucleolus enlargement, cell membrane rupture and cell content leakage. A. Untreated theronts. B through I. Theronts treated with 0.3 mg protein mL^{-1} of skin mucus for 10 min.

phosphatase, cathepsin B and peroxidase have been identified in many fish species [27,33–35]. Among them, lysozyme and protease are the two most important antibacterial enzymes. Interestingly, the values obtained for lysozyme and protease activity in the skin mucus of *A. clarkii* are significantly lower than those reported for other marine fish species such as *Sparus aurata*, *Dicentrarchus labrax*, *Umbrina cirrosa*, *Dentex dentex* and *Epinephelus marginatus* [27]. The difference of enzyme activity could be associated with the habitat and evolutionary or genetic adaptation to environmental factors, as *A. clarkii*, which inhabits coral reef ecosystem, has evolved a special mucus layer that allows a mutualistic symbiosis with sea anemones [18]. Nigam et al. reported that enzyme activities were significantly higher in bottom-dwelling species than in species inhabiting clear water [36]. In addition, variation in the enzyme activity of mucus could also be related to factors such as the stage of growth and maturity, diet [37], handling stress, and seasonal changes [38].

The prevention of invasion by aquatic microorganisms is mediated by a variety of immune compounds; not only are enzymes at work, but AMPs also play a significant role. Thus, evaluating the overall anti-pathogenic microbial activity might be more important than evaluating the activity of individual enzymes. Many studies have demonstrated the antimicrobial activity of fish skin mucus extracts against a broad range of microbial pathogens [27,32,39–43]. Consistent with this, the skin mucus of *A. clarkii* also showed significant bactericidal activity against Gram-negative bacteria, whereas the viability of Gram-positive bacteria was not significantly affected at the tested concentrations. The tested Gram-negative bacteria, *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, and *A. hydrophila*, are the main pathogens of fish and invertebrates; for example, *V. parahaemolyticus* is an enteric pathogen that is widely distributed in the marine environment and is typically responsible for acute human gastroenteritis [44], *V. harveyi* is a serious pathogen of marine fish and invertebrates [45], and *A. hydrophila* is one of the most common pathogens in freshwater habitats throughout the world [46]. The selectivity of bactericidal activity may be explained by the nature of the active molecules present in the skin mucus. Lysozyme possesses lytic activity against both Gram-positive bacteria and Gram-negative bacteria by directly or indirectly hydrolysing N-acetylmuramic acid and N-acetylglucosamine, which are constituents of the peptidoglycan layer of bacterial cell walls [47,48]. Protease may play a protective role by degrading pathogens or directly hampering their invasion and

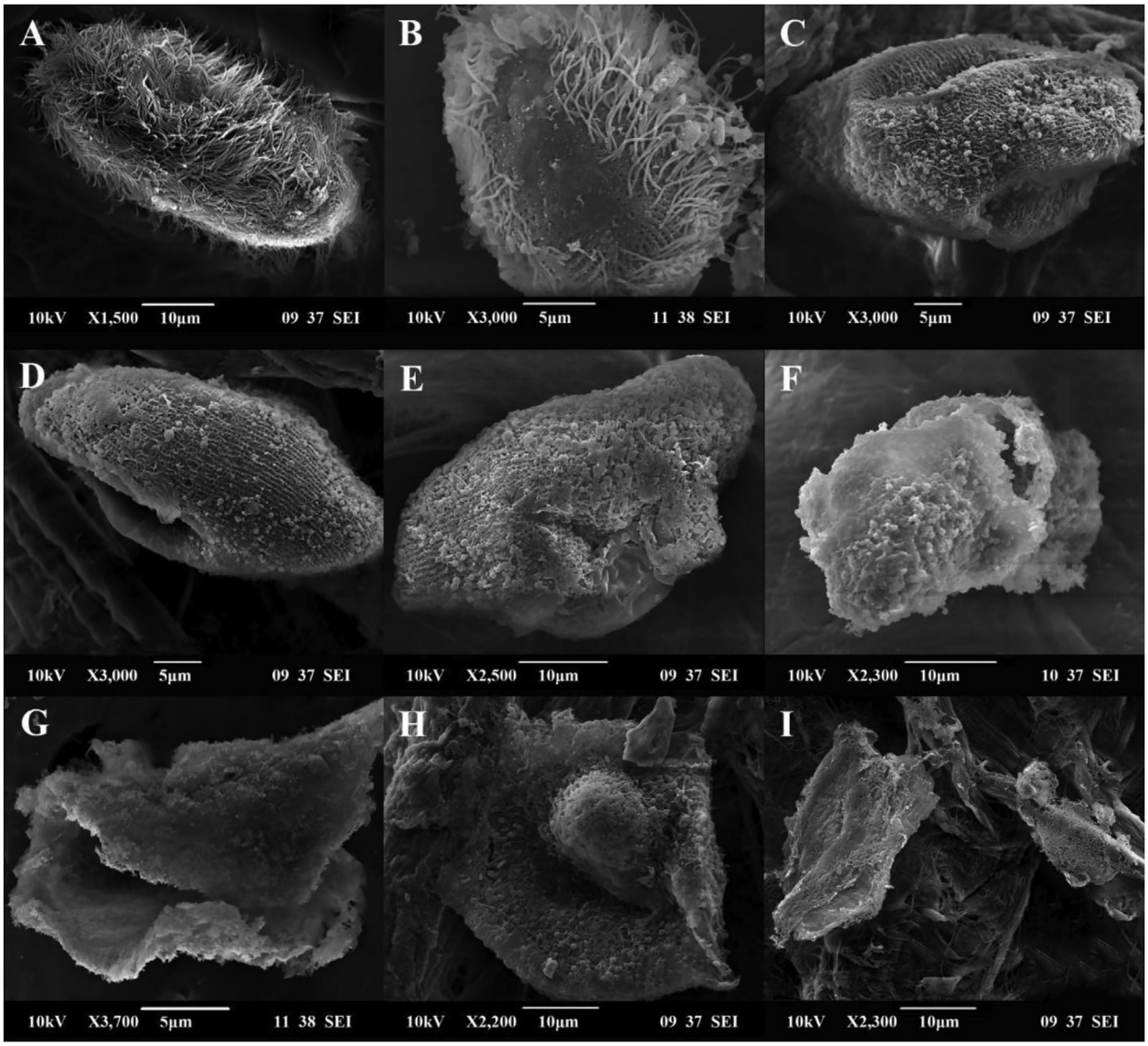

Fig. 5. SEM of morphological changes in *C. irritans* theronts after treatment with *A. clarkii* skin mucus. The results of the SEM showed that the skin mucus quickly induced theront deformation, cilia fell off, cell membrane rupture and cell content leakage. A. Untreated theronts. B through I. Theronts treated with 0.3 mg protein mL^{-1} of skin mucus for 10 min.

Table 3
Effect of pH on activity of skin mucus against *V. parahaemolyticus*.

pH	MIC(mg protein ml^{-1})
2.0	–
3.0	–
4.0	0.125–0.25
5.0	0.0625–0.125
6.0	0.0625–0.125
7.0	0.0625–0.125
8.0	0.0625–0.125
9.0	0.125–0.25
10.0	–
11.0	–

"—" indicates no macroscopic bacterial growth was observed in either the test group or the blank control group.

Table 4
Effect of heat on activity of skin mucus against *V. parahaemolyticus*.

Temperature (°C)	MIC(mg protein mL^{-1})
30	0.0625–0.125
40	0.0625–0.125
50	0.0625–0.125
60	0.0625–0.125
70	0.0625–0.125
80	0.0625–0.125
90	0.0625–0.125
100	0.25–0.5

colonisation [35,49]. Therefore, together with the data obtained for the two most important immune-related enzymes (lysozyme and protease) activity, we speculate that other active components such as cationic AMPs may play a major role in bactericidal action [50]. The cationic AMPs have two common physical features: a cationic charge and a significant proportion of hydrophobic residues, the former property promotes the preferential binding of AMPs to the negatively charged bacterial cytoplasmic membrane instead of the positive charged

whereas the latter property makes AMPs have an amphiphilic conformation, so when the AMPs reach the lipid membrane interface of the target microorganism, it can be integrated into or through the membrane [51]. AMPs not only destroy cell membranes, but also pass through membranes to target intracellular processes such as DNA, RNA and protein synthesis [52,53]. The greater effect of *A. clarkii* skin mucus on Gram-negative strains than Gram-positive ones may be due to the strong interaction between the active molecules present in the skin mucus and the outer membrane (present only in the Gram-negative strains) [54]. Futhermore, the results of kinetic studies showed that the antibacterial effect was time- and dose-dependent, and it exerted its antimicrobial effects within 30 min of exposure to *V. parahaemolyticus*, which suggests that the antimicrobial effects of *A. clarkii* skin mucus were most effective at the onset of the pathogen infection. Similar antimicrobial effects against bacteria were observed for many antipeptides such as Pc-hepcidin [25] and Pc-piscidin [55]. The SEM results further reveal the mechanism of antibacterial activity of *A. clarkii* skin mucus. After treated with *A. clarkii* skin mucus, the *V. parahaemolyticus* cells distorted and piled together, and the filaments appeared and became into cotton-shaped or quasi-honeycomb texture to adhere cells, all the changes emerged a time-dependent correlation. Therefore, we speclated that the cell membrane may have been ruptured and the cotton-shaped or quasi-honeycomb texture may be cell content. The cell membrane is an important structural component of the bacterium. When the cell membrane is damaged, contents such as proteins, nucleic acids and other substances in the cell can leak out, which may kill the bacterium [56].

To investigate the immune response of skin mucus from *A. clarkii* against the ectoparasite *C. irritans*, the acute toxicity of skin mucus to *C. irritans* theronts was examined at different concentrations. The results showed that the skin mucus was effective against *C. irritans* theronts in a distinct dose- and time-dependent manner. The skin mucus induced the cilia falling off, the formation of small globular bubbles, cell membrane rupture and content leakage within the first 10 min of exposure. These results indicate that the skin mucus of *A. clarkii* could directly affect theront survival to prevent *C. irritans* infection. Although other AMPs such as SR-LAAO/APP [57], HbβP-1 [58] and piscidin [55] showed similar activity against *C. irritans* theronts, the morphological changes in treated *C. irritans* theronts were not the same; most AMPs induced the loss of cilia and caused the cell membrane to rupture in *C. irritans* theronts without the formation of small globular bubbles. The unique result in the present study may indicate the presence of a new AMP in *A. clarkii* skin mucus. Although further research is required to elucidate the mechanism of *A. clarkii* skin mucus action on *C. irritans* theronts, on account for the results of LM and SEM, one possible scenario is that when the *C. irritans* theront adheres to *A. clarkii*, the active substance of skin mucus first contacts the cell membrane and causes the cilia to fall off; the active substance then enters the cell by an unknown way and simultaneously acts on the cell nucleus and cell membrane of the theront, causing the formation of globular bubbles and cell nucleus enlargement through changes in osmotic pressure, which ultimately leads to cell rupture.

The antibacterial activity of skin mucus was not affected by pH treatment within the moderate environment of 5.0–8.0, and slightly suppressed by weak acid and weak base. In the environment of strong acid and alkali, the acid and alkali could also kill *V. parahaemolyticus*, so we conld not judge the change of antibacterial activity. Therefore, the skin mucus contains a pH stable active constituent at pH 5.0–8.0. With regard to thermal stability, the antibacterial activity of skin mucus was not significantly affected by heat treatment within the range of 30–100 °C for 20 min. This result indicates that the skin mucus contains a thermally stable active constituent. These physico-chemical properties of skin mucus are similar those of the antimicrobial peptides that have been found in other fish such as piscidin 1 and SJGAP [59].

5. Conclusions

In conclusion, several physico-chemical and biological parameters of the skin mucus of *A. clarkii* were determined to obtain more knowledge about the biology and function of this defensive barrier. In this study, several enzyme activity were analysed and both lysozyme, protease, antiprotease and alkaline phosphatase showed a lower level than has been reported for most other marine fish. However, *A. clarkii* skin mucus showed strong antibacterial and antiparasitic activities. The antibacterial activity was stable under heat treatment and moderate pH treatment. Therefore, we speculate that the skin mucus of *A. clarkii* might be a potential source of novel antibacterial and antiparasitic components for fish or human health-related applications. This study broadened our understanding of the biology and function of skin mucus in the innate immune system and provided a basis for the further isolation and purification of active substances.

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