

Purification and Antimicrobial Activity of Antimicrobial Protein from Brown-spotted Grouper, *Epinephelus fario*

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Abstract: Antimicrobial proteins and peptides had been found from a wide variety of organisms in the last few years. These molecules have attracted much research interest because of their biochemical diversity, broad specificity on anti-viral, anti-bacterial, anti-fungi, anti-protozoan parasites, anti-tumoural, and wound-healing effects. Antimicrobial proteins and peptides play key roles in innate immunity. They interact directly with bacteria and kill them. The brown-spotted grouper, *Epinephelus fario*, is an important marine fish cultured in southern China. Recently, bacteria and virus have caused high mortality in *E. fario* cultures, but its endogenous antimicrobial peptides and proteins have not been explored. An antimicrobial component was found from the skin homogenate of *E. fario*. After the skin homogenate was digested with trypsin, its antimicrobial activity was lost, which showed that the antimicrobial component is a protein. The antimicrobial protein (*Efap*) was purified from the skin homogenate of *E. fario* by successive ion-exchange and gel filtration chromatography. *Efap* was demonstrated to be single protein band by SDS-PAGE, with the apparent molecular weight of 41 kD. *Efap* exhibited antimicrobial activity both for the Gram-positive bacteria, *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis*, and for the Gram-negative bacteria, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Pasteurella multocida*, *Aeromonas hydrophila*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Except *A. hydrophila*, *P. aeruginosa*, and *E. coli* (MIC>20 mol/L), most of the tested Gram-negative bacteria were sensitive to *Efap* (MIC<20 mol/L). Interestingly, *Efap* showed potent antimicrobial activity against Gram-positive bacteria *S. aureus* (MIC 5-10 mol/L) but comparatively weak antimicrobial activity against *M. luteus* and *B. subtilis*. The broad antimicrobial activities of *Efap* suggest that it contributes to the innate host defence of *E. fario*.

Key words: *Epinephelus fario*; Antimicrobial proteins and peptides; Purification; Gram-positive bacteria; Gram-negative bacteria

鲑点石斑鱼皮肤抗菌活性蛋白的纯化及抗菌活性

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摘要: 近年来在多种生物体中都发现有抗菌活性蛋白和多肽。由于其具有生物化学多样性, 抗病毒、微生物、真菌、原生动物、肿瘤, 促进伤口愈合等生物学活性, 而引起研究者的极大兴趣。抗菌活性蛋白和多肽在动物的先天免疫中具有重要作用, 它们直接作用于细菌, 并将其杀死。鲑点石斑鱼(*Epinephelus fario*)是中国南方水产养殖中重要的海水鱼。近年来, 由于细菌和病毒引发的病害造成鲑点石斑鱼大量死亡, 但其抗菌活性蛋白及多肽目前还未见报道。本研究发现鲑点石斑鱼皮肤具有抗菌活性成分, 鲑点石斑鱼皮肤匀浆物经胰蛋白酶水解后抗菌活性丧失, 说明该活性是由蛋白质引起的。经离子交换层析及凝胶过滤层析, 从鲑点石斑鱼皮肤中分离纯化到抗菌活性蛋白(*Efap*)。SDS-PAGE显示, *Efap*为单链蛋白, 分子量约41 kD。该成分能同时抑制革兰氏阳性菌, 如金黄色葡萄球菌、膝黄微球菌、枯草芽孢杆菌和革兰氏阴性菌, 如溶藻弧菌、副溶血弧菌、河流弧菌、多杀性巴氏杆菌、嗜水气单胞菌、大肠杆菌和铜绿假单胞菌。革兰氏阴性菌中, 溶藻弧菌、副溶血弧菌、河流弧菌和多杀性

Received date: 2008-09-28; Accepted date: 2008-11-17

Foundation items: Key Research Program for International Cooperation (2005DFA30610); Program for New Century Excellent Talents in University (NCET-05-0755); National Natural Science Foundation (30700128); Natural Science Foundation of Hainan Province (80623); Research Foundation of Education Department of Hainan Province (Hj200731)

收稿日期: 2008-09-28; 接受日期: 2008-11-17

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巴氏杆菌对 *Efab* 较敏感, MIC<20 mol/L, 其他 3 种菌敏感性较差, MIC>20 mol/L。另外, *Efab* 显示出较强的抗金黄色葡萄球菌的活性, MIC 为 5—10 mol/L。*Efab* 的广谱抗菌性, 说明其在鲑点石斑鱼免疫防御中具有一定的作用。

关键词: 鲑点石斑鱼; 抗菌蛋白及多肽; 纯化; 革兰氏阳性菌; 革兰氏阴性菌

中图分类号: Q176; S917.4; Q71 **文献标识码:** A

文章编号: 0254-5853-(2008)06-0627-06

Antimicrobial proteins and peptides were obtained from a wide variety of organisms. These molecules have attracted much research interest because of their biochemical diversity, broad specificity on anti-viral, anti-bacterial, anti-fungi, anti-protozoan parasites, and even anti-tumoural or wound-healing effects (Zasloff, 2002). Antimicrobial proteins and peptides play key roles in innate immunity. They interact directly with bacteria and kill them (Lauth et al, 2002). First identified in frogs and insects, antimicrobial proteins and peptides are now known to be widespread throughout the animal kingdom. Fish live in a microbe-rich environment and are vulnerable to be invaded by pathogenic or opportunistic micro-organisms. Compounds with broad antimicrobial activities are thought to be especially important for fish, as their adaptive immune system is structurally simpler than that of mammals or amphibians, and is not fully effective in young fry or at low environmental temperature (Silphaduang & Noga, 2001). The skin epithelium and other mucosal surfaces of fish are rich in anti-microbial peptides (Bergsson et al, 2005).

Fish are of great economic importance in aquaculture throughout the world. Intensive culture, the recent techniques for fish culture, has increased the production but accompanied with the outbreak of many infectious diseases in fish farms. The bacterial and viral diseases have caused huge losses in fish culture in the past few years. To prevent the outbreak of these diseases, large amounts of antibiotics have been used in the fish farms and thus bacterial resistance to conventional antibiotics is becoming more prevalent (Lalumera et al, 2004). In order to avoid drug resistance for bacterial pathogens, many attempts were made to find effective replacements for antibiotics at present. Antimicrobial proteins and peptides are important components of the innate host defences and represent a source of potential useful natural antibiotics for pharmaceutical application.

The brown-spotted grouper, *Epinephelus fario*, is an important marine fish cultured in southern China. Its production reaches 10,000 tons annually. Recently, bacteria and virus have caused high mortality in *E. fario* cultures, but its endogenous antimicrobial peptides and proteins have not been explored. In the present study, the

antimicrobial protein from *E. fario* skin (*Efab*) was purified and its activities against bacterial pathogens were tested.

1 Materials and methods

1.1 Materials

Sephedax G-75, DEAE-Sephadex A-50 and Q-Sepharose (high performance) were from Amersham Pharmacia. Trypsin and bovine serum albumin (BSA) were purchased from Sigma. Different bacterial strains were selected for this study, Gram-negative bacteria including *Vibrio alginolyticus* (ATCC 17749), *Vibrio parahaemolyticus* (ATCC 17802), *Pasteurella multocida* (ATCC 12947), *Vibrio fluvialis* (ATCC 33812), *Aeromonas hydrophila* (ATCC 35654), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 35032), and Gram-positive bacteria including *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 49732), and *Bacillus subtilis* (ATCC 9372). All laboratory chemicals used were of reagent grade.

1.2 Preparation of grouper skin homogenate

Healthy brown-spotted grouper (weight range 500–600g) were obtained in August 2007 from cultured net pens in the Dongao Bay, Wanning, Hainan. Then, they were injected intraperitoneally with live *M. luteus* and *E. coli* mixture (50 μ L of each organism from an overnight culture, 10^9 CFU/mL). After challenged, the groupers were returned to the sea water. The challenged groupers were killed by stabbing the brain with a knife, after 12h. Their scales were scraped gently and skins were peeled and washed in 50mmol/L Tris-HCl buffer (pH 7.8, containing 5 mmol/L EDTA and 0.1 mol/L NaCl). The skins were immersed immediately in liquid nitrogen. Then, the frozen skins were ground into powder with a mortar and pestle under liquid nitrogen, and homogenized in the same buffer. The homogenate was centrifuged at 15,000g for 30min. The supernatant was collected, lyophilized and stored in -80°C until use.

1.3 Proteolytic digestion of homogenate

Susceptibility of the antimicrobial activity of crude skin homogenate to proteolytic digestion was determined by incubation of 4.5mg skin homogenate (containing 1 mg proteins) with 250 μ g/L crystalline trypsin for 60min

at 37°C. Antimicrobial activities of the skin homogenate before and after protease treatment against *E. coli* were determined.

1.4 Purification of antimicrobial protein from skin of *E. fario* (*Efap*)

The skin homogenate was purified by ion exchange chromatogram and gel filtration chromatogram. Briefly, the lyophilized skin homogenate was dissolved in 20mL 50mmol/L Tris-HCl (pH 7.8, containing 5mmol/L EDTA) and dialyzed against the same buffer for 24h at 4°C (The molecular weight cut-off of the dialysis tubing was 3,500 D). Then, the sample was loaded in DEAE-Sephadex A-50 ion exchange column (2.6cm×30cm). The elution was performed at a flow rate of 30mL/h with a linear NaCl gradient, collecting fractions with antimicrobial activity of 5mL per tube. The fractions were lyophilized and applied to a Sephadex G-75 (superfine) column (2.6 cm ×100cm) equilibrated with 50mmol/L Tris-HCl (pH 7.5, containing 5 mmol/L EDTA and 0.1 mol/L NaCl). Elution was achieved with the same buffer at a flow rate of 9mL/h, collecting fractions of 3mL per tube. The peak with antimicrobial activity was collected, dialyzed against 50 mmol/L Tris-HCl (pH 8.8, containing 5 mmol/L EDTA) for 24 h at 4°C, and loaded on a Q-Sepharose (high performance) column (2.6cm ×30cm) pre-equilibrated with the same buffer. A linear NaCl gradient of 0–0.5 mol/L was employed to elute the proteins. The elution was performed at a flow rate of 30 mL/h with a linear NaCl gradient, collecting fractions with antimicrobial activity of 5 mL per tube.

1.5 Protein concentration

The protein concentration was determined by staining with Coomassie brilliant blue G-250 with BSA as a standard.

1.6 Electrophoretic Studies

SDS-PAGE was performed as reported by Laemmli (1970). For SDS-PAGE, samples were pretreated in 2.5% SDS alone (nonreducing conditions) or in 2.5% SDS and 5% β -mercaptoethanol (reducing conditions) for 5 min at 100 °C. Gels were stained with 0.1% Coomassie brilliant blue R-250.

1.7 Antimicrobial assays

Different bacterial strains were selected for this study, Gram-negative bacteria including *V. alginolyticus*, *V. parahaemolyticus*, *P. multocida*, *V. fluvialis*, *A. hydrophila*, *E. coli* and *P. aeruginosa*, and Gram-positive bacteria including *S. aureus*, *M. luteus* and *B. subtilis*. Strains were grown on broth nutrient medium.

Antimicrobial activity was monitored by a liquid growth inhibition assay. Briefly, logarithmic phase bacterial cultures were diluted in the broth [1% (w/v) bactotryptone, 0.9% (w/v) NaCl] to an A_{600} of 0.001, which is approximately equivalent to 10^5 CFU/mL. Diluted bacteria (90μL) were mixed with 10μL of either water (control) or *Efap* in wells of a microtitration plate. After overnight incubation at 25°C, the bacterial growth was monitored by measuring the change in the absorbance of the culture with a microplate reader at 600 nm (Casteels et al, 1993).

1.8 Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined as follows. Briefly, bacteria were incubated in Todd Hewitt broth [50% (w/v) beef heart infusion; 2% (w/v) peptic digest of animal tissue; 0.2% (w/v) dextrose; 0.2% (w/v) NaCl; 0.04% (w/v) Na_2HPO_4 ; 0.25 (w/v) Na_2CO_3] in the presence of 2-fold serial dilutions of sample (final concentration 1.25–80μmol/L). Bacterial growth was monitored by a liquid growth inhibition assay. MIC was expressed as a range of the highest concentration of *Efap* at which bacteria were able to grow and the lowest concentration that the bacterial growth was completely inhibited. All assays were performed in duplicate (Lauth et al, 2002).

1.9 Hemolytic assay

Freshly collected fish blood were washed with phosphate-buffered saline (pH 7.4) until the supernatant was colorless and resuspended in phosphate-buffered saline supplemented with glucose (0.2%, w/v). *Efap* (10 μL of 200mmol/L, serially diluted in phosphate buffered saline) was added to 90 μL of erythrocyte suspension (5×10^6 cells/mL) in microcentrifuge tubes. *Efap* were incubated for 30min at 37°C and centrifuged for 5min at 2,000 g at room temperature. The absorbance of the supernatant was determined at 415 nm in a UV-VIS spectrophotometer UV-2550 (Sahimadzu Corporation, Japan). The percentage of hemolysis was defined relative to the hemolysis obtained with the erythrocyte suspension treated with 0.1% SDS (100% hemolysis)(Liu et al, 2008).

2 Results

2.1 Proteolytic digestion of skin homogenate

Initial screening of skin homogenate of *E. fario* showed activity against Gram-positive bacteria (*S. aureus*, *M. luteus* and *B. subtilis*) and Gram-negative

bacteria (*V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, *P. multocida*, *E. coli*, *A. hydrophila* and *P. aeruginosa*). Then, the homogenate was treated with trypsin for 90min at 37°C and its antimicrobial activity against *E. coli* (other bacteria were not tested) was completely abolished. The results suggested that the skin homogenate contained a proteinaceous antibiotic.

2.2 Purification and characterization of *Efap*

One gram of lyophilized *E. fario* skin homogenate (containing 220 mg proteins) was purified by DEAE-Sephadex A-50 column (pH 7.8) and five protein peaks were obtained. The antimicrobial activities against *E. coli* were detected in peak V (Fig. 1). Peak V of DEAE-Sephadex A-50 column was collected, lyophilized, and then applied to a Sephadex G-75 gel

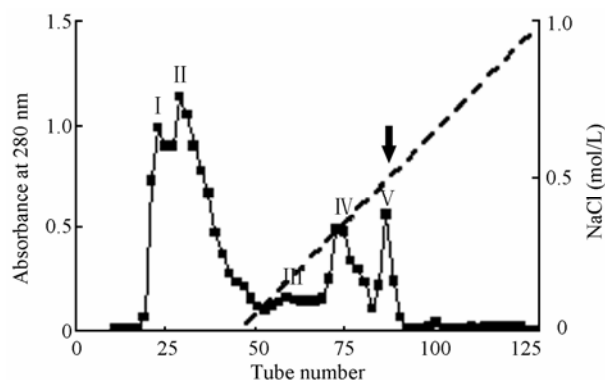


Fig. 1 Ion exchange chromatography of *Epinephelus fario* skin homogenate on a DEAE-Sephadex A-50 column (2.6cm ×30cm)

The protein concentration was estimated from the absorbance at 280nm (■).

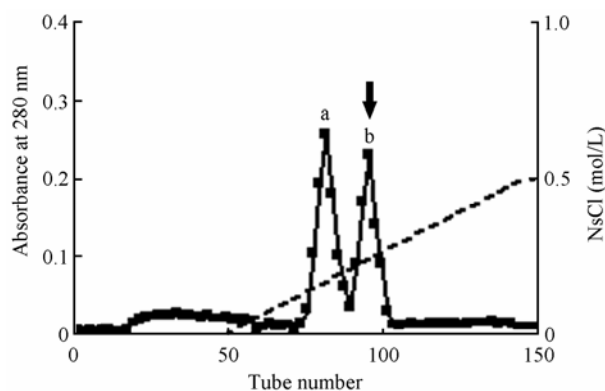


Fig. 3 Peak C of Sephadex G-75 column was finally loaded on a Q-Sepharose column (2.6cm ×30cm)

The protein concentration was estimated from the absorbance at 280 nm (■).

2.3 Antimicrobial spectrum and hemolytic activity

Efap was tested against three Gram-positive and seven Gram-negative bacteria for antimicrobial activity

filtration column equilibrated with 50mmol/L Tris-HCl (pH 7.8, containing 5 mmol/L EDTA and 0.1 mol/L NaCl). This purification step resulted in the separation of three protein peaks, in which antimicrobial activity was found in peak C (Fig. 2). Peak C of Sephadex G-75 gel filtration column was collected and loaded on Q-Sepharose column and two protein peaks were obtained, in which antimicrobial activity was found in peak b (Fig. 3). Totally 5 mg product was obtained and the antimicrobial component was termed as *Efap*. The results of SDS-PAGE under reducing and non-reducing conditions showed that the purified *Efap* was a single chain protein and its molecular weight was about 41kD (Fig. 4).

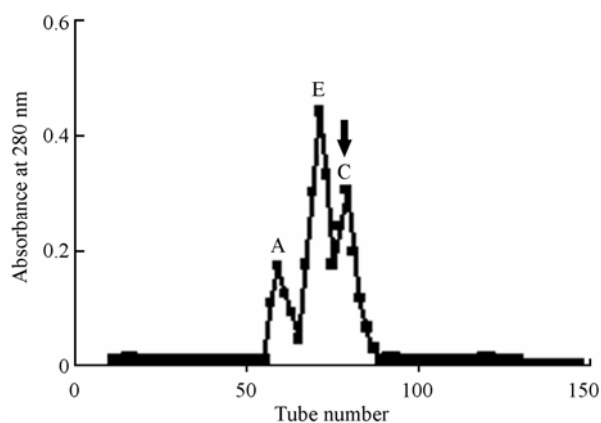


Fig. 2 Gel filtration of peak V of DEAE-Sephadex A-50 column on a Sephadex G-75 gel filtration column (2.6cm ×100cm)

The protein concentration was estimated from the absorbance at 280 nm (■).

(Tab. 1). The results showed that *Efap* was active against all tested Gram-positive bacteria and Gram-negative bacteria. Except *A. hydrophila*, *P. aeruginosa*, and *E. coli* (MIC>20 mol/L), most of the tested Gram-negative bacteria were sensitive to *Efap* with MIC less than 20 mol/L. Interestingly, *Efap* showed strong activities against Gram-positive strain, *S. aureus* (MIC 5-10 mol/L), but *B. subtilis* are most resistant to *Efap*. In general, *Efap* was highly active against the Gram-negative bacteria, such as *V. parahaemolyticus*, *V. alginolyticus*, *P. multocida*, *V. fluvialis*, and *A. hydrophila*, which are the main pathogens of aquaculture fish diseases.

Efap was not hemolytic for fish red blood cells at concentrations up to 2.5mmol/L, at which *Efap* showed antimicrobial activities against many tested micro-organisms. Hemolytic activity was observed

above 5 mmol/L in a dose-dependent manner (data not shown).

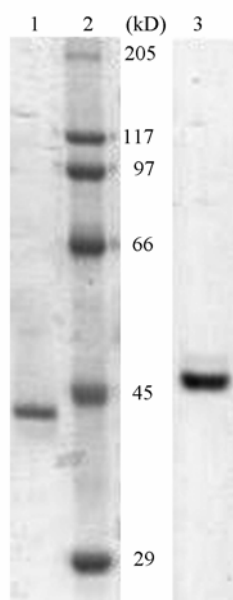


Fig. 4 SDS-PAGE of *Efaf*

Lane 1, *Efaf* (reducing conditions); lane 2, Protein Marker; lane 3, *Efaf* (non-reducing conditions).

Tab. 1 MIC of *Efaf* against different bacteria

Stain (Gram+/-)	MIC (mol/L)
<i>Staphylococcus aureus</i> (+)	5–10
<i>Micrococcus luteus</i> (+)	20–40
<i>Bacillus subtilis</i> (+)	40–80
<i>Vibrio parahaemolyticus</i> (-)	5–10
<i>Vibrio alginolyticus</i> (-)	10–20
<i>Pasteurella multocida</i> (-)	5–10
<i>Vibrio fluvialis</i> (-)	5–10
<i>Aeromonas hydrophila</i> (-)	20–40
<i>Pseudomonas aeruginosa</i> (-)	20–40
<i>Escherichia coli</i> (-)	20–40

3 Discussion

The production of antimicrobial peptides has been identified as a major defense mechanism against infections in lower organisms as well as an important component of the innate immune response of mammals, including humans. Fish have evolved to thrive in an aqueous environment rich in microbial flora and are presumed to use their innate immune system as the first line of defense against microbial invasion. In the last decade, many species-specific antimicrobial peptides have been isolated from fish (misgurin, pleurocidin, paradaxins, hagfish intestinal antimicrobial peptides, and parasin I), some of them showed a broad spectrum of

activity against Gram-positive and Gram-negative bacteria (Zasloff, 2002; Silphaduang & Noga, 2001). Compared with largely found antimicrobial peptides from aquatic animals, less antimicrobial proteins from aquatic species are reported. Carp antimicrobial proteins, 27 kD and 31 kD proteins, had potent microbicidal activities (0.018–0.18 mol/L) against different strains of Gram-negative and Gram-positive bacteria (Lemaitre et al, 1996). Furthermore, it has been shown that larger antimicrobial proteins, such as aplysianin A from the sea hare (Takamatsu et al, 1995) and achacin from the giant African snail (Obara et al, 1992), have strong antimicrobial activities. Those proteins purportedly form large ion channels in the bacterial membrane in a manner similar to insect defensins.

Although brown-spotted groupers are susceptible to many infectious agents including bacteria such as vibrios (Herrera et al, 2006), antimicrobial proteins and peptides have not been previously reported. In this study, we report an antimicrobial protein from *E. fario* skin. The purification was accomplished by ion-exchange and gel filtration chromatography. The purified protein, named *Efaf*, is a 41 kD protein. In general, *Efaf* was highly active against tested Gram-negative bacteria, some of which are the main pathogens of aquaculture fish diseases, such as *V. parahaemolyticus*, *V. alginolyticus*, *Vibrio fluvialis*, *P. multocida*, and *A. hydrophila*. *Efaf* showed high antimicrobial activity against Gram-positive bacteria, *S. aureus* (MIC 5–10 mol/L) too. The results suggest that *Efaf* have a quite broad antimicrobial spectrum. Most of reported antimicrobial peptides typically have strong antimicrobial activity against a wide range of Gram-positive bacteria but very weak or no activity against Gram-negative bacteria, like mytimycin (Mitta et al, 2000). However, *Efaf* seems has higher antimicrobial activity against Gram-negative bacteria but weaker antimicrobial activity against Gram-positive bacteria. *Efaf* has weak hemolytic activity for fish red blood cells.

In conclusion, we isolated an antimicrobial protein from *E. fario* and the antimicrobial protein has activity against both Gram-positive and Gram-negative bacteria. This study might be helpful in selecting disease resistant groupers for aquaculture and enhance research to protect groupers from important microbial infections. Further work should be carried out on its proteomic and genomic studies.

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