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Purification and characterization of antimicrobial material, a 40S ribosomal protein S30 from the epidermal mucus of rock bream, *Oplegnathus fasciatus*

by

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The Graduate School

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(돌돔의 상피 점액질로부터 항균 물질, 40S ribosomal protein S30의 정제 및 특성)

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돌돔(*Oplegnathus fasciatus*)의 상피 점액질로부터 항균 물질, 40S ribosomal protein S30의 정제 및 특성

주 효 진

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요 약

어류의 상피 조직 및 상피 점액질은 외부 환경에 존재하고 있는 병원체로부터 자신의 생체를 보호하기 위해서 다양한 항균물질을 분비한다고 알려져 있다. 본 연구에서, 돌돔의 상피 점액질의 산 추출물은 다양한 그람 양성균과 그람 음성균에 대해 항균활성을 나타냈으며, 어병세균인 *Aeromonas hydrophila*와 *Edwardsiella tarda*에 대해서도 항균반응을 나타냈다. 상피 점액질의 산 추출물은 Trypsin처리에 의해 항균활성이 대부분 소실되었지만 열, 산성 및 염기성 조건하에서는 활성이 유지되었다. 이러한 결과는 추출물에서 항균 활성을 나타내는 물질이 단백질 또는 폴리펩타이드 성분이라는 것을 의미한다. 돌돔의 상피 점액질 추출물을 사용하여 역상, 양이온 및 겔 크로마토그래피로 항균물질을 정제하였으며, 정제 과정에서 각각의 분획에 대한 항균 활성은 *Staphylococcus aureus*를 사용하여 확인하였다. 정제한 물질의 N-말단의 부분적인 아미노산 서열을 분석하기 위해서 Edman 분해법을 행한 결과, N-말단으로부터 25번째 아미노산 잔기까지 분석되었다. 이러한 부분적인 아미노산 서열의 상동성을 조사하기 위해서 기존에 알려진 단백질의 아미노산 서열과 비교 분석하였고, 그 결과 정제된 물질은 척추동물의 40S ribosomal protein S30 (RPS30)의 아미노산 서열과 높은 상동성을 가지는 것을 확인하였다. 그리고 정제한 물질의 완전한 아미노산 서열을 알아보기 위해서 cDNA 클로닝을 진행하였다. 한편, cDNA의 primer를 제작하기 위해서 돌돔의 아가미로부터 밝혀진 RPS30의 cDNA EST library를 사용하였다. 합성 primer와 돌돔 상피조직으로부터 추출한 total RNA

를 이용하여 제작된 cDNA로 클로닝한 결과, 정제된 단백질은 180개의 뉴클레오타이드로 구성된, 59개의 아미노산으로 이루졌고 전체 뉴클레오타이드 서열은 571개임을 확인하였다. 돌돔 RPS30의 이론적인 분자량은 6,673.84 Da이며, pI 값은 11.92이다. cDNA 클로닝에 의해 확인된 돌돔 RPS30의 일차 구조는 다른 어류들 (제브라 피쉬, 연어, 금붕어)의 RPS30 일차구조와 동일하였다. 그러나 다른 척추 동물의 RPS30 일차구조와는 총 59개의 아미노산들 중에서 2개의 아미노산 잔기가 치환(^{20}Asp→^{20}Ala, ^{36}Ile→^{36}Met)되어 있다. 굴토끼의 RPS30 삼차원 구조를 토대로 돌돔 RPS30을 homology modeling한 결과, 돌돔 RPS30은 N-말단부터 11-15번째 잔기에 짧은 α-helix와 30-41번째 잔기에 긴 α-helix를 포함하는 구조를 형성하고있다. 따라서 정제된 돌돔 RPS30은 높은 등전점과 많은 Arg과 Lys 잔기를 가지고 있는 염기성 단백질이며, 이러한 특징은 기존에 밝혀진 수많은 염기성 항균 펩타이드와 유사한 성질을 가질 것이라고 생각된다. 본 연구에서 돌돔 RPS30은 생체 내에서 주 기능 중 하나인 eukaryotic 40S ribosome subunit의 구성 역할을 하는 동시에 돌돔의 상피 점액질에서 선천성 면역 반응에 관여하는 중요한 항균 물질로 작용한다고 추측된다. 뿐만 아니라 수산 양식 산업에서 중요한 자원 중의 하나인 돌돔으로부터, 돌돔 RPS30과 같은 항균 활성 물질의 특성을 밝히는 연구는 천연항균물질의 선도물질 개발과 같은 고부가가치 산업을 비롯하여 수산 양식에 관련된 질병예방, 폐사율 감소 등 수산 양식 산업에 기여할 방안을 모색할 수 있는 계기가 될 것이라 생각된다.

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1. Introduction

Marine organisms live in aquatic environment and are constantly exposed to the infection of pathogenic microorganisms. Among marine organisms, especially, teleost have an effective immune system to defense against invading marine pathogens. In aquatic environment, teleost is directly in contact with various microorganisms. Teleost require immune system to act directly and quickly more than land creatures. Therefore epidermal mucus of teleost, there are materials with defense systems to respond effectively to disease pathogens present in the external environment. The epidermal mucus of teleost is produced in epidermal goblet or mucus cell, that are contains the components, such as a large amount of water and gel-forming macromolecules including mucins and other glycoproteins. The mucus layer on the fish surface performs a number of functions including disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication, feeding, and nest building [*Subramanian et al. 2008*].

The epidermal mucus of teleost contains a variety of bioactive materials such as immunoglobulins (IgM and IgT), lectins, glycoproteins, complement proteins, C-reactive protein, lysozyme, flavoenzymes,

proteolytic enzymes and antimicrobial peptides are among these materials are associated with innate immunity plays a first-line host defense against potential pathogens [*Ellis et al. 2001*]. Because of teleost live in the aquatic environment, we need to be noted about the disease resistance of the epidermal mucus of teleost.

Generally, antimicrobial properties associated with innate immunity have a broad-spectrum antimicrobial activity against many pathogen including bacteria, viruses, fungi, and metazoan parasites, and they have the advantage that an immediate, non-specific immune response [*Masso-Silva & Diamond 2014*]. Furthermore, antimicrobial materials are immediately synthesized at small metabolic cost, easily stored in large amounts and available after infection [*Oren & Shai 1996*]. A several AMPs have been identified from the primarily exposed tissues of teleost such as hagfish (*Myxine glutinosa L.*), coho salmon (*Oncorhynchus kisutch*), yellow catfish (*Pelteobagrus fulvidraco*), winter flounder (*Pleuronectes americanus*), hybrid striped bass (*M. chrysops x M. saxatilis*), atlantic halibut (*Hippoglossus hippoglssus*), red sea moses sole (*Pardachirus marmoratus*) and catfish (*Parasilurus asotus*). For example, Myxinidin have been isolated from skin mucus of the hagfish [*Subramanian et at. 2009*], HSDF from mucus and blood of the coho

salmon [*Patrzykat et al. 2001*], Pelteobagrin from skin mucus of yellow catfish [*Yueju et al. 2011*], Pleurocidin from the skin secretions of winter flounder [*Cole et al. 1997*], Piscidin from the mast cells of hybrid striped bass [*Silphaduang & Noga 2001*], Hipposin from skin mucus of the atlantic halibut [*Birkemo et al. 2003*], Pardaxin from skin mucus glands of the red sea moses sole [*Oren & Shai 1996*], Parasin I from skin mucus of catfish [*Park et al. 1998*] (Table 1). Through the identified AMPs as above, and were proven to be important innate immune functions tissues such as mucus, skin, gill, and intestine in teleost. AMPs are also derived through the proteolysis of precursor proteins such as histone-derived antimicrobial peptides and ribosomal antimicrobial peptides (Table. 2) [*Cho et al. 2009*].

The antimicrobial function of epidermal mucus on the fish surface is the first defense action from a variety of external invaders by physical, chemical and biological mechanisms. First, physically, it gives mucus covering the skin surface to prevent attachment of external pathogens, and the periodic replacement of the mucus layer to the surface attached to the pathogen. In addition, by using a variety of antimicrobial materials present in the epidermal cell and epidermal mucus prevent infection by biochemical action. Features because of innate immunity in teleost such

as the above are considered very important factor.

Antimicrobial peptides (AMPs) are one of the most common innate immune system factors, are comparatively short length and have amphipathic, cationic structure. The presence of cationic property suggests that might interact with bacterial membrane through electrostatic interaction. The research of teleost AMPs were started late compared to other species, such as mammals, amphibians, over 90 teleost AMPs have been identified grouped according to their structure. The innate immunity in epidermal mucus of teleost, it is a lot of research but is not yet completely understood. Therefore constant research for immune substances present in epidermal mucus of teleost would be needed.

The rock bream (*Oplegnathus fasciatus*) is an important food fish in the Northeast Asia, such as South Korea, Japan and China. Typical coastal province of the rock bream habitat is coastal South Korea and Japan, the East China Sea, South China Sea or the like. Countries in the Northeast have gained many economic benefits in the aquaculture of a rock bream. But, during process of aquaculture, a lot of economic losses due to mortality by disease, such as iridovirus, streptococcosis, edwardsiellosis, vibriosis, scuticociliatosis and white spot disease [*Jung et al. 2000*].

Although drug development to prevent infection of many diseases such as bacterial, viruses, fungi and parasites, whereby a resistant microbial and also frequent occurs. So it requires research and development of natural antibiotics such as antimicrobial peptides to prevent of infection and resistance [*Rajanbabu & Chen 2011*]. For these reasons, to study various antimicrobial substances present in the rock bream, the research and development to understand the characteristics of the immune system of the teleost of main aquaculture, it is has been proposed that further through the application that will help the aquaculture industry.

Moronecidin (Rbmoro) [*Bae et al. 2014*], piscidin (Rbpisc) [*Bae et al. 2016*] and glyrichin homologue (ofGlyrichin) [*Kasthuri et al. 2013*] have been found in the rock bream by cloning experiments. However, antimicrobial peptides by purification from rock bream have not been found yet. Evidence for the presence of at least one additional antimicrobial factor was obtained during that previous other study. The goals of the present study were identify and characterize the antimicrobial material in epidermal mucus of rock bream as a potentially important factor in the innate immunity. To obtain the results, the antimicrobial material was purified from extract of the epidermal mucus. Then, its antibacterial activity, amino acid sequence, and nucleotide

sequence were identified. The results in this study may be usefully provided to important source of future therapeutic antimicrobial drugs and biological information of AMPs in rock bream.

Table 1. Antimicrobial peptides isolated from mucus of fish.

AMP	Fish	Location	M.W. (amino acids)	References
Pleurocidins	Winter flounder	Skin secretion	2.7 kDa (25 aa)	*Cole et al. (1997)*
Hipposin	Atlantic halibut	Skin mucus	5.5 kDa (51 aa)	*Birkemo et al. (2003)*
Myxinidin	Hagfish	Skin mucus	1.3 kDa (12 aa)	*Subramanian et al. (2009)*
Pelteobagrin	Yellow catfish	Skin mucus	2.2 kDa (20 aa)	*Yueju et al. (2011)*
Parasin I	Catfish	Skin mucus	2.0 kDa (19 aa)	*Park et al. (1998)*
Pardaxin	Red sea moses sole	Mucus gland	3.3 kDa (33 aa)	*Oren, Shai (1996)*
Piscidin	Hybrid striped bass	Skin, Gill	2.5 kDa (22 aa)	*Silphaduang, Noga (2001)*

Table 2. Antimicrobial materials derived through the proteolysis of precursor proteins.

Antimicrobial materials	M.W. (amino acids)	pI
Histone derived AMP		
H2A - Buforin I	4.3 kDa (39 aa)	12.41
H2A - Buforin II	2.4 kDa (21 aa)	12.60
H2A - Parasin I	2.0 kDa (19 aa)	12.48
H2A - Hipposin	5.5 kDa (51 aa)	12.23
Ribosomal proteins		
40S ribosomal protein S19	16.0 kDa (145 aa)	10.31
40S ribosomal protein S30	6.7 kDa (59 aa)	11.92
60S ribosomal protein L30	12.8 kDa (115 aa)	9.65
60S ribosomal protein L39	6.3 kDa (51 aa)	12.55

2. Materials and methods

2.1. Fish and their maintenance

Rock bream, *Oplegnathus fasciatus* (220-250 g, 20-23 cm) were purchased from Namcheon beach market in Busan, South Korea. In total eighteen fish, each three of the fish were maintained in 50 L of glass tanks provided with 20 L of sterilized seawater at 20℃.

2.2. Bacterial strains and their culture conditions

Various gram positive bacteria and gram negative bacteria were chosen to test the antimicrobial activity of the epidermal mucus extract. Three species of gram positive bacteria, including *Bacillus subtilis* KCTC 1021, *Streptococcus iniae* FP 5229, *Staphylococcus aureus* RN 4220, and five species of Gram negative bacteria, including *Aeromonas hydrophila* KCTC 2358, *Edwardsiella tarda* KCTC 12267, *Escherichia coli* D 31, *Pseudomonas aeruginosa* KCTC 2004, *Listonella anguillarum* KCTC 2711. All Bacterial strains were grown overnight in tryptic soy broth (TSB, Merck, Darmstadt, Germany) at 37°C.

2.3. Epidermal mucus collection

Epidermal mucus was obtained from three healthy fish, and was anesthetized non-lethally with 100 mg/L tricaine methanesulfonate (MS-222). Epidermal mucus of rock bream was collected as described by *Ross et al. 2000* with slight modifications. Mucus was obtained from the epidermal surface of rock bream by scraping carefully using sterilized slide glass, avoiding contamination with scale, blood, feces. Collected mucus samples were transferred into 50 mL falcon tube, and frozen immediately using liquid nitrozen and stored at –80℃ until use.

2.4. Epidermal mucus extraction

Pooled epidermal mucus samples were boiled with 1 volume of 1% acetic acid (HAc) for 5 minutes and homogenized using homogenizer (Polytron PT1200C homogenizer, Kinematica AG, Switzerland) on ice box for 3 minutes. Samples were centrifuged at 20000 x g for 40 minutes at 4℃ and supernatant was pooled and passed through a Sep-Pak C18 cartridge (20 cc, 5 g, Waters associates). Column was activated with 40 mL of methanol in 0.1% trifluoroacetic acid (TFA) and washed with 40 mL of distilled water (D.W.) in 0.1% TFA, and the retentate was then

eluted with 60 mL of 60% methanol in 0.1% TFA. The total eluate (RM60) was concentrated to 5 mL by evaporation, and lyophilized following freezing at –80℃. Lyophilized sample powder was dissoloved in Milli-Q water in 0.01% HAc, and then subjected to high performance liquid chromatography (HPLC).

2.5. Ultrasensitive radial diffusion assay (URDA)

The antibacterial activity of the crude extract during the purification step and the purified material were determined by ultrasensitive radial diffusion assay (URDA) as previously described *Seo et al. 2005*. It examined the antimicrobial activity for a variety of bacteria to the material eluted from the Sep-Pak C18 cartridge with D.W., 60% methanol solvent and methanol solvent. The strains used in the experiment were four marine bacteria such as *Aeromonas hydrophila* KCTC 2358, *Edwardsiella tarda* KCTC12267, *Streptococcus iniae* FP5228, *Listonella anguillarum* KCTC2711 and four soil bacteria such as *Bacillus subtilis* KCTC1021, *Escherichia coli* D31, *Pseudomonas aeruginosa* KCTC2004, *Staphylococcus aureus* RN4220. Bacteria were precultured overnight for 16 hours in tryptic soy broth (TSB) at 37℃. After overnight incubation, the bacteria were diluted to a McFarland

turbidity standard of 0.5 (Vitek Colorimeter #52-1210, Hach, Loveland, Colorado) corresponding to ~10^8 CFU/mL for bacteria. One-half milliliter of diluted bacterial suspension containing 5 x 10^6 suspension containing 5 x 10^4 CFU/mL was added to 9.5 mL of underlay gel in 10 mM phosphate buffer (PB, pH 6.6) with 0.03% TSB and 1% Type I (low EEO) agarose. The crude material was serially diluted 2 fold in 5 μL of distilled water in 0.01% HAc and each diluted crude material was added to 2.5 mm diameter wells made in the 1 mm thick underlay gels. After 3 hours of incubation at 37℃, the bacterial suspension was overlaid with 10 mL of double-strength overlay gel containing 6% TSB with 10 mM phosphate buffer (PB, pH 6.6) in 1% agarose. The plates were incubated for an additional 18-24 hours, and then the diameters of the clear zones were measured using calliper.

2.6. Effects of pH, temperature, and proteolytic enzyme on the antimicrobial activity

To investigate the effect of pH (acidic, neutral, basic), temperature and proteolytic digestion on the crude extract of skin mucus, antimicrobial activity was tested by the URDA against *E. coli* D31, *B. subtilis* KCTC1021 and *S. aureus* RN4220, as described above. To determine the effect of pH on antimicrobial activity, the extract (1mg/g, w/w) was incubated with the following solutions: 10μl of 0.01% HAc (pH 3), D.W. (pH 7), and 0.01% sodium hydroxide (NaOH) (pH 10). After 1 hour of incubation at 37℃ in the appropriate solution, the extract was used for URDA. To determine the thermal stability, the extract was incubated at 100℃ for 10 minutes. After the heat treated, the extract was cooled and used for URDA. Susceptibility of the antibacterial activity of crude extract of skin mucus to proteolytic digestion was determined by incubation with 10μl (250 μg/mL) of crystalline trypsin (Fisher Scientific, Fairlawn, NJ) for 1 hour at 37℃. Antimicrobial testing of the extract before and after the protease treatment was done with the URDA against *E. coli* D 31, *B. subtilis* KCTC 1021 and *S. aureus* RN 4220.

2.7. Purification of antimicrobial material

The eluate of epidermal mucus from 60% methanol in 0.1% TFA was injected into a C18 reversed-phase column (RP-HPLC, CapCell-Pak C18, 5 um, 300 Å, 4.6 x 250 mm). The sample was eluted with a linear gradient of 5-65% acetonitrile (ACN) in 0.1% TFA for 60 minutes at a flow rate of 1 mL/min, and the eluate was monitored at 220 nm. Fractions were collected one by one per minute by using fraction collector and, dried under vacuum, dissolved in 0.01% HAc, and then tested for antibacterial activity against *S. aureus* by the URDA. Fractions having antibacterial activity with C18 column RP-HPLC were pooled, and injected into SP-5PW cation exchange column (TSK-Gel, 7.5 x 75 mm; Tosoh, Tokyo, Japan) equilibrated with 20 mM phosphate buffer (PB, pH 6.5). The sample was eluted with a linear gradient of buffer A (20 mM PB, pH 6.5) and buffer B (1.0 M NaCl in 20 mM PB, pH 6.5) from 0 to 1.0 M NaCl for 60 minutes at a flow rate of 1 mL/min, and the eluate was monitored at 220 nm. Fractions having antibacterial activity with SP-5PW column IE-HPLC were pooled, injected into a C18 reversed-phase column. The sample was eluted with a linear gradient of 10-40% ACN in 0.1% TFA for 60 minutes at a flow rate of 1 mL/min, and peaks were collected by hand. The peak having clearly antibacterial

activity with C18 column was injected into equal column. Peaks were eluted with isocratic flow of 22% ACN in 0.1% TFA for 40 minutes at 0.5 mL/min, and the eluate was monitored at 220 nm. Each sharp peaks were collected by hand and tested by URDA. Finally, the sample was eluted in the same manner as before, and obtained purified material. Purified material was tested for antibacterial activity against *S. aureus* by the URDA. The purified material injected into gel column (Superdex™ peptide 10/300 GL) identification of approximate molecular weight. Peaks were eluted with a isocratic flow of 18.5% ACN in 0.1% TFA for 40 minutes at 0.5 mL/min, and eluate was monitored at 220 nm. The finally purified material used for further characterization.

2.8. Determination of the partial amino acid sequence of purified protein

The partial amino acid sequence of the purified protein was analyzed by automated Edman degradation on a pulse liquid automatic sequencer (PPSQ-31A/33A protein sequencers, Shimadzu Co., Kyoto, Japan).

2.9. cDNA analysis of the purified protein

For sequence of 40S ribosomal protein S30 in open reading frame (ORF) analysis, total RNA was isolated from kidney and skin of rock bream, *Oplegnathus fasciatus*, using Hybrid-R kit (GeneAll, Seoul, Korea) according to the manufacturer's protocol. First stranded cDNA was synthesized using a GeneRacer Kit (Invitrogen, CA, USA) and GeneRacer™ Oligo dT. The gene specific primers were designed based on the partial N-terminal amino acid sequence (KVHGSLARAGKVRA QTPKVPKQGKG) of the purified protein obtained by Edman degradation, and the transcriptome data of rock bream (SRX1406648 Male gonad transcriptome for the rock bream, *Oplegnathus fasciatus*) by SRA (Sequence Read Archive) and expressed sequence tag (EST) data (EST name: AB710073) (Fig. 3). The gene specific primers used are as follows: Rb 180bp Forward primer: 5'-AAG GTG CAC GGT TCT CTG-3' and Rb 180bp Reverse primer: 5'-CCC AAC GCC AAC TCC TAA-3'. Polymerase chain reaction (PCR) was conducted using nTaq-HOT polymerase (Enzynomics, Daejeon, Korea). Used in a PCR the first stranded cDNA of the skins, kidneys as a template under the following conditions: initial denaturation at 94℃ for 10 min, 30 cycles of denaturation at 94℃ for 30 sec, annealing at 58℃ for 30 sec, and

extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min. The amplified fragments were purified using PCR purification kit (Nanohelix, Daejeon, Korea). Used in a PCR the first stranded cDNA of the first PCR product as a template under the following conditions: initial denaturation at 94℃ for 10 min, 30 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min, and subcloned into pTOP TA V2 Vector (Enzynomics, Daejeon, Korea) and were sequenced using the automatic DNA sequencer.

For rapid amplification of cDNA ends (RACE) PCR, total RNA was isolated from gill and skin of rock bream using Hybrid-R kit (GeneAll, Seoul, Korea) according to the manufacturer's protocol. First stranded cDNA was synthesized using a GeneRacer™ Kit (Invitrogen, CA, USA), GeneRacer™ RNA Oligo and GeneRacer™ Oligo dT. 5' RACE primers were designed based on partial ORF sequencing result. The gene specific primers used are as follows: GeneRacer™ 5' Forward primer: 5'-CGA CTG GAG CAC GAG GAC ACT GA-3' and 25 Reverse primer: 5'-GGC ACG GCC AGT CTT CTT CTT CTT C-3'. 5' RACE PCR was conducted using nTaq-HOT polymerase (Enzynomics, Daejeon, Korea). Used in a first 5' RACE PCR the first stranded cDNA

of gill and skin as a template under the following conditions: initial denaturation at 94℃ for 10 min, 35 cycles of denaturation at 94℃ for 30 sec, annealing at 69℃ for 30 sec, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min. The gene specific primers of second PCR used are as follows: GeneRacer™ 5' Nested Forward primer: 5'-GGA CAC TGA CAT GGA CTG AAG GAG TA-3' and 28 Reverse primer: 5'-CTC CTG CTT GTA ACC TTG GGT GTC TGT C-3'. Used in a second 5' RACE PCR first PCR products as a template under the following conditions: initial denaturation at 94℃ for 10 min, 35 cycles of denaturation at 94℃ for 30 sec, annealing at 71℃ for 30 sec, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min. Second PCR products were subcloned into pTOP TA V2 Vector (Enzynomics, Daejeon, Korea) and were sequenced using the automatic DNA sequencer.

3' RACE primers were designed based on partial ORF sequencing result. The gene specific primers used are as follows: 23 Forward primer: 5'-GGA GAA GAA GAA GAA GAC TG-3' and GeneRacer™ 3' Reverse primer: 5'-GCT GTC AAC GAT ACG CTA CGT AAC G-3'. 3' RACE PCR was conducted using nTaq-HOT polymerase (Enzynomics, Daejeon, Korea). Used in 3' RACE PCR the first stranded cDNA of the

gill as a template under the following conditions: initial denaturation at 94℃ for 10 min, 35 cycles of denaturation at 94℃ for 30 sec, annealing at 69℃ for 30 sec, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min. The gene specific primers of second PCR used are as follows: GeneRacer™ 5’ Nested Forward primer: 5’-GGA CAC TGA CAT GGA CTG AAG GAG TA-3’ and 28 Reverse primer: 5’-CTC CTG CTT GTA ACC TTG GGT GTC TGT C-3’. Used in a second 5’ RACE PCR first PCR products as a template under the following conditions: initial denaturation at 94℃ for 10 min, 35 cycles of denaturation at 94℃ for 30 sec, annealing at 61℃ for 30 sec, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min. PCR product was subcloned into pTOP TA V2 Vector (Enzynomics, Daejeon, Korea) and were sequenced using the automatic DNA sequencer.

2.10. Sequence analysis

The theoretical molecular weight and isoelectric point (pI) of purified protein was calculated by ExPASy (SIB Bioinformatics Resource Portal) (http://www.expasy.ch/tools/peptide-mass.html). The homology search for the purified protein was performed by TBLASTN and BLASTP in Basic Local Alignment Search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A sequence alignment was performed using BioEdit software.

2.11. Structural modeling

The structure of purified protein was built by homology modeling using the SWISS-MODEL server (http://swissmodel.expasy.org/). Three-dimensional structure of 40S ribosomal protein S30 in european rabbit, *Oryctolagus cuniculus* 40S HCV-IRES (Hepatitis C virus–internal ribosomal entry site) complex (PDB accession no. 5flx.1.5) was used as a template.

Fig. 1. Schematic diagram of acidified extraction process of epidermal mucus from rock bream. The eluate of 60% methanol in 0.1% TFA (RM60) was selected for purification process.

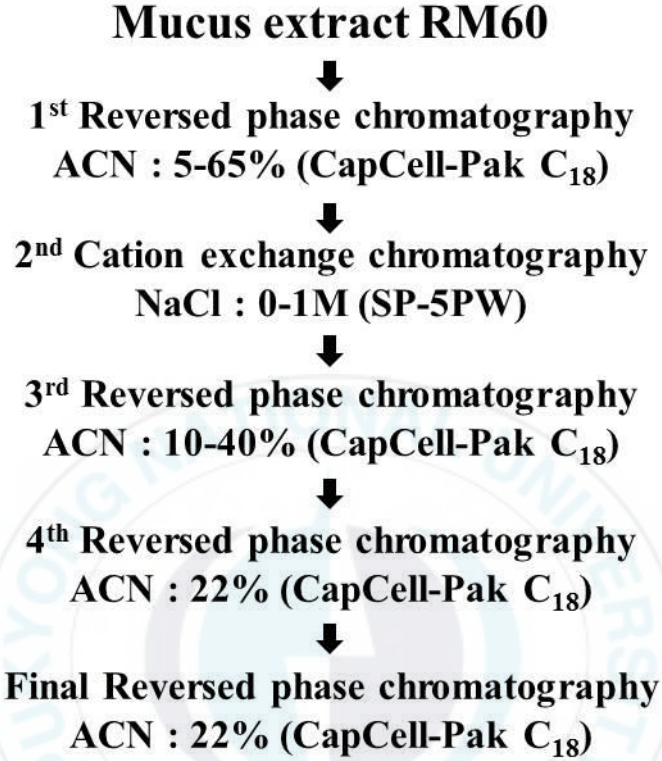

Fig. 2. Schematic diagram of purification process of epidermal mucus from rock bream. The gel chromatography was performed after the final purification process.

Fig. 3. mRNA sequence of 40S ribosomal protein S30 from rock bream obtained by expressed sequence tag (EST). AB710073 rock bream gills cDNA *Oplegnathus fasciatus* cDNA clone 005-14-H06, mRNA sequence (GenBank: AB710073.1). The gene specific primers were designed based on 40S ribosomal protein S30 nucleotide sequence (red color). Rb 180bp Forward primer and Rb 180bp Reverse primer are indicated by black arrow.

3. Results

3.1. Antimicrobial activity of crude extract from epidermal mucus

Preliminary screenings of acidifed epidermal mucus extracts of the rock bream using Sep-Pak C18 cartridge such as flow through (F.T), 60% methanol in 0.1% TFA (RM60) and 100% methanol in 0.1% TFA (RM100) exhibited potent antibacterial activity against *B. subtilis*, *E. coli* and *S. aureus* by the URDA (Fig. 4). Although all eluate with F.T, RM60 and RM100 showed strong antibacterial activity, RM60 was selected for their appropriate hydrophobicity. Then selected RM60 was confirmed for the antibacterial activity against four gram positive and four gram negative bacteria by URDA. RM60 showed antimicrobial activity against both gram negative and positive bacteria, except for *L. Anguillarum* (Table. 3).

3.2. Effects of protease, temperature, and pH on the antibacterial activity

To investigate the effect of proteolytic enzyme digestion, temperature, and pH on the antibacterial activity of the crude extract RM60 was tested by the URDA against *B. subtilis* KCTC1021, *E. coli* D31 and *S. aureus* RN4220 (data of *E. coli D31* not shown) (Fig. 5). Crude extract of skin mucus from rock bream was not affected by the pH conditions such as acidic, neutral and basic. These results indicate that the antibacterial activity of crude extract of skin mucus could be retained in the wide range of pH conditions. In the temperature stability investigation, antibacterial activity of the crude extract RM60 was also not affected by heat treatment at 100℃ for 10 minutes (Fig. 5). In the sensitivity of the extract to proteolytic digestion, the sample was predominantly lost its antibacterial activity after treated with trypsin (Fig. 5). Based on the result, the crude extract of skin mucus is heat-stable and is sensitive to serine protease, trypsin.

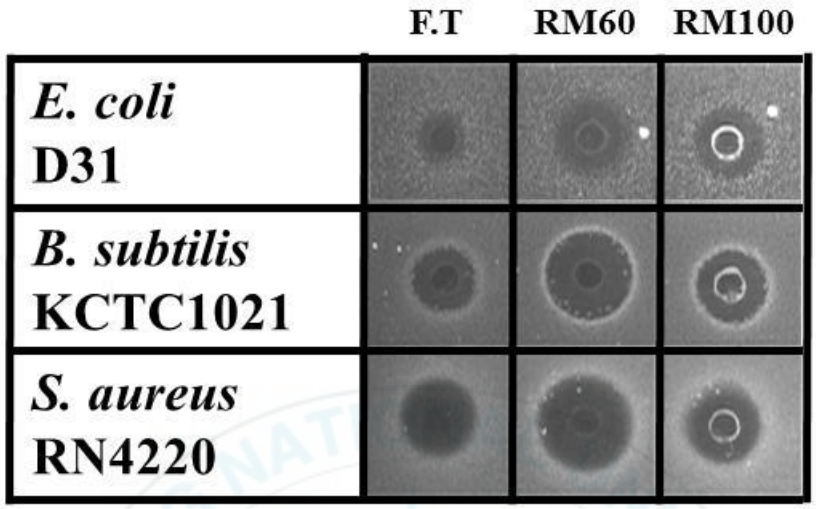

Fig. 4. Preliminary screenings of acidifed epidermal mucus extracts of the rock bream using Sep-Pak C18 cartridge. 10 μL of flow through (F.T), 60% methanol in 0.1% TFA (RM60) and 100% methanol in 0.1% TFA (RM100) exhibited antibacterial activity against *B. subtilis* KCTC1021, *E. coli* D31 and *S. aureus* RN4220 by the URDA

Table 3. Antibacterial activity of extract RM60 of epidermal mucus from rock bream.

Pathogen	Gram	Bacteria	Used amount (w/w)		
			1mg/g	2mg/g	4mg/g
Fish	+	*S. iniae*	++	++	+++
	-	*A. hydrophila*	-	+	++
	-	*E. tarda*	-	+	++
	-	*L. anguillarum*	-	-	-
Human	+	*B. subtilis*	++	+++	+++
	+	*S. aureus*	+++	+++	+++
	-	*E. coli*	+	++	++
	-	*P. aeruginosa*	-	+	+

Abbreviations : -, no antibacterial activity; +, inhibition zone ≤ 4.0 mm; ++, inhibition zone ≤ 7.0 mm; +++, inhibition zone ≥ 7.0 mm, total protein amount 1.03 g

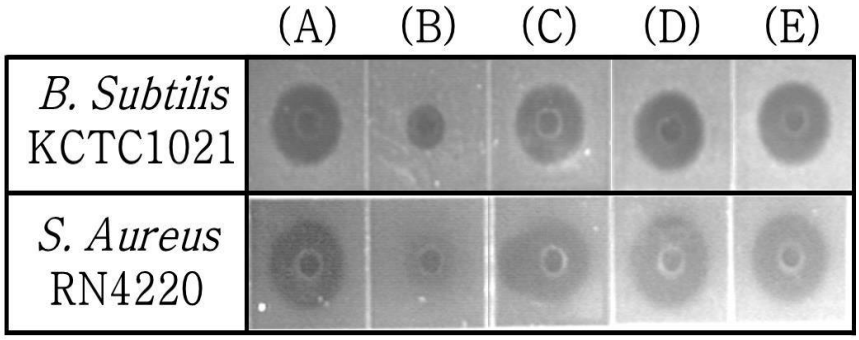

Fig. 5. Antibacterial activity of crude extract on various conditions. Antibacterial activity of crude extract RM60 (A), trypsin treated extract (B), heated treated extract (C), acidic treated (0.01% HAc) extract (D), and basic treated (0.01% NaOH) extract (E) against *B. subtilis* KCTC1021 and *S. aureus* RN4220. Used volume of sample for the assay was 10 μL (0.1 g/mL)

3.3. Isolation of antimicrobial protein from epidermal mucus

The antimicrobial peptide has been purified from the epidermal mucus of the rock bream. The selected eluate RM60 of Sep-Pak C18 cartridge, RM60 was first injected into a reversed phase HPLC using CapCell-Pak C18 column (Fig. 6). Each fraction was checked for the antibacterial activity against *S. aureus* and the fractions that were eluted between 15-40% ACN showed strong antibacterial activity. These fractions were pooled and separated further through cation exchange HPLC using SP-5PW column (Fig. 7). Fractions of 0 M NaCl (F.T) showed no antibacterial activity, but the fractions eluted between 0–0.9 M NaCl showed antibacterial activity. These fractions were pooled and desalted further through reversed phase HPLC using CapCell-Pak C18 column (Fig. 8). The peak eluted with 22% ACN in 0.1% TFA had strong antibacterial activity and then separated again through isocratic flow with 22% ACN in 0.1% TFA by equal column (Fig. 9). For a more pure separation, the peak eluted with 22% ACN in 0.1% TFA was injected into equal column (Fig. 10). Finally, a single peak was reluted again with 22% ACN isocratic flow, and then injected into gel chromatography using Superdex™ peptide 10/300 GL for confirmation of approximate molecular size. The approximate molecular weight of the purified

material is 5 to 10 KDa (Fig. 11).

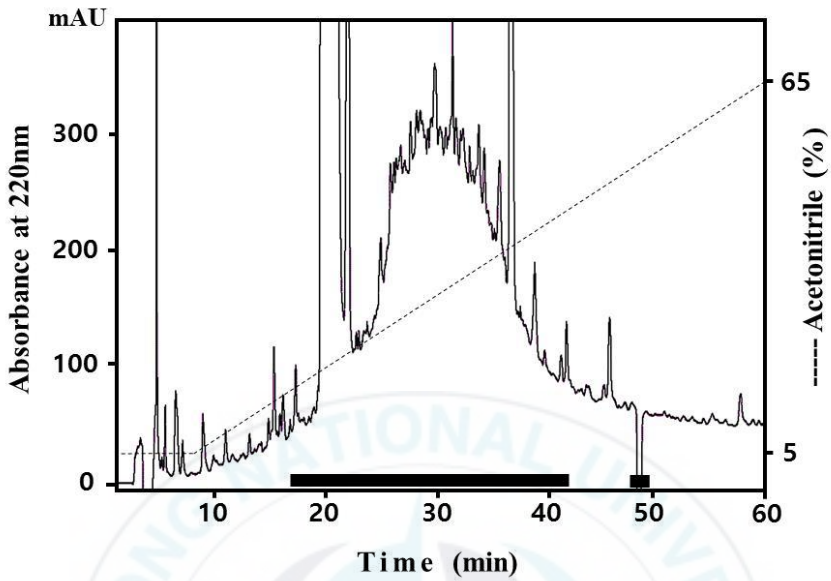

Fig. 6. The first HPLC chromatogram of the epidermal mucus extract from rock bream. The eluate of epidermal mucus from 60% methanol in 0.1% TFA elution using Sep-Pak C18 cartridge injected into a CapCell-Pak C18 reversed phase chromatography. The elution was performed with a linear gradient of 5-65% ACN in 0.1% TFA for 60 minutes at a flow rate of 1 mL/min, and eluate was monitored at 220 nm. Fractions (indicated by the black bar) showed antibacterial activity against *S. aureus* RN4220. Fractions of 15-40% ACN were pooled for the next purification process.

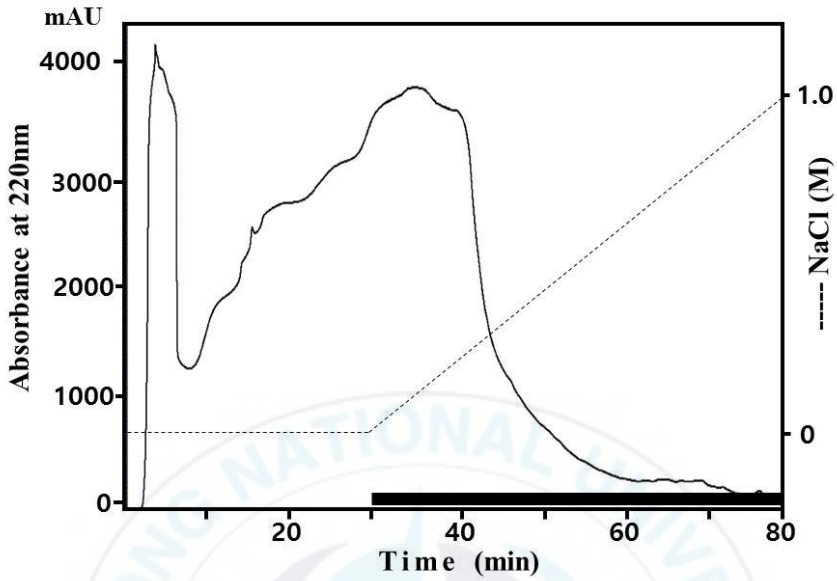

Fig. 7. The second HPLC chromatogram of the epidermal mucus extract from rock bream. Pooled fractions from previous step using a CapCell-Pak C18 reversed phase chromatography injected into SP-5PW cation exchange chromatography. The elution was performed with 0 M for 20 minutes and a linear gradient of 0-1.0 M NaCl in 20mM PB (pH 6.5) for 60 minutes at a flow rate of 1 mL/min, and eluate was monitored at 220 nm. Fractions (indicated by the black bar) showed antibacterial activity against *S. aureus* RN4220. Fractions of 0-0.9 M NaCl in 20 mM PB (pH 6.5) were pooled and desalt for the next purification process.

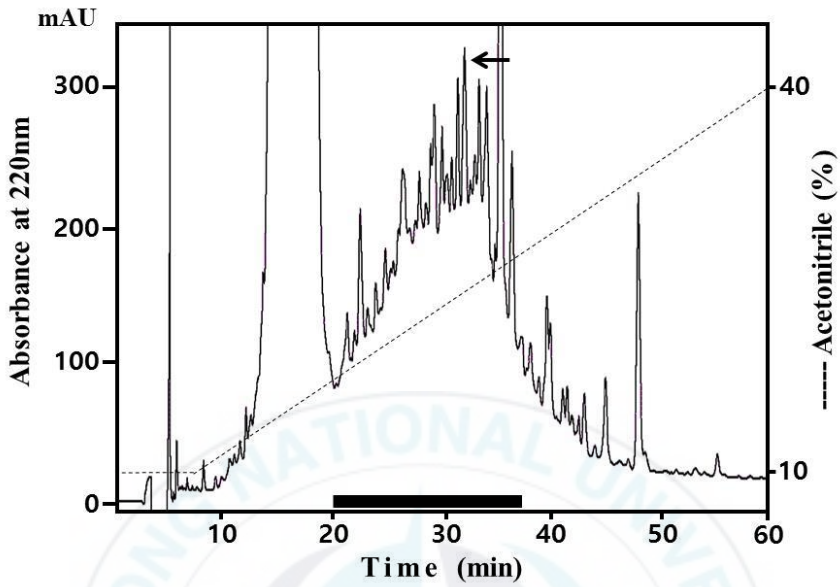

Fig. 8. The third HPLC chromatogram of the epidermal mucus extract from rock bream. The eluate of previous step from 0-0.9 M NaCl in 20 mM PB (pH 6.5) elution using a SP-5PW cation exchange chromatography injected into a CapCell-Pak C18 reversed phase chromatography. The elution was performed with a linear gradient of 10-40% ACN in 0.1% TFA for 60 minutes at a flow rate of 1 mL/min, and eluate was monitored at 220 nm. Each peak (indicated by the black bar) showed antibacterial activity against *S. aureus* RN4220. The elution point of activity peak (indicated by the arrow) was at 22% ACN, and selected for next purification process.

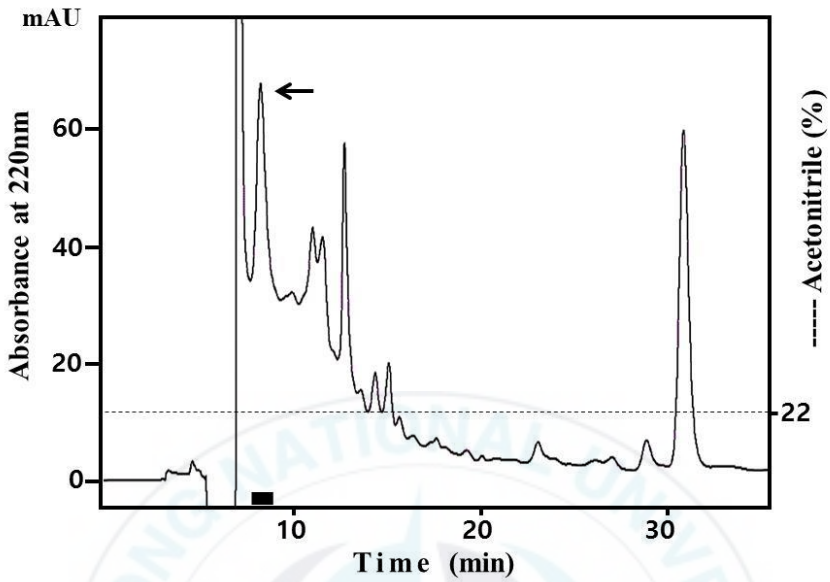

Fig. 9. The fourth HPLC chromatogram of the epidermal mucus extract from rock bream. The eluate of previous step from 22% ACN in 0.1% TFA elution using a CapCell-Pak C18 reversed phase chromatography injected into the equal column. The elution was performed with isocratically eluted at 22% ACN in 0.1% TFA for 35 minutes at a flow rate of 1 mL/min, and eluate was monitored at 220 nm. Peak (indicated by the black bar) showed antibacterial activity against *S. aureus* RN4220. The eluted peak (indicated by the arrow) for 8 minutes was selected for next purification process.

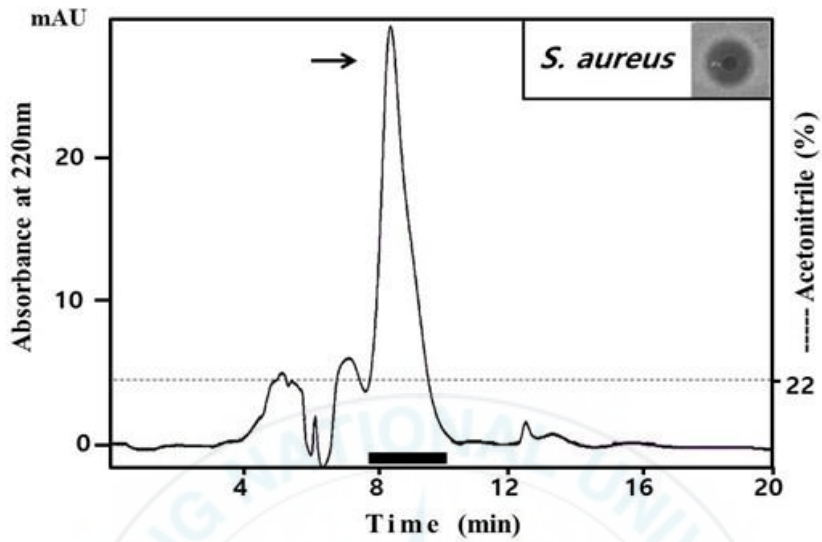

Fig. 10. The final HPLC chromatogram of the epidermal mucus extract from rock bream. The eluate of previous step from 22% ACN in 0.1% TFA elution using a CapCell-Pak C18 reversed phase chromatography injected into the equal column. The elution was performed with isocratically eluted at 22% ACN in 0.1% TFA for 20 minutes at a flow rate of 1 mL/min, and eluate was monitored at 220 nm. Peak (indicated by the black bar) showed antibacterial activity against *S. aureus* RN4220.

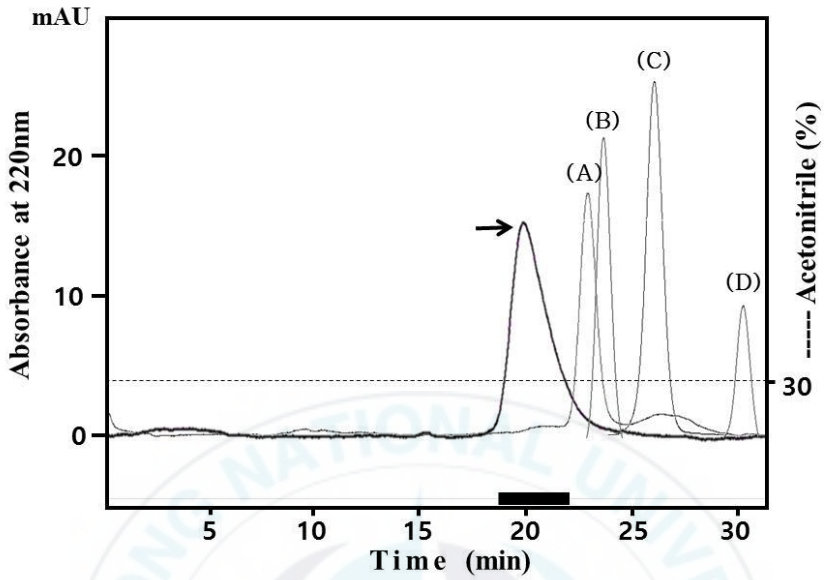

Fig. 11. The gel HPLC chromatogram of purified antimicrobial material and molecular size standards. Purified single peak of final purification step from 22% ACN in 0.1% TFA elution using a CapCell-Pak C18 reversed phase chromatography injected into the Superdex™ peptide 10/300 GL gel chromatography. The elution was performed with isocratically eluted at 30% ACN in 0.1% TFA for 40 minutes at a flow rate of 1 mL/min, and eluate was monitored at 220 nm. Single peak was purified antimicrobial material (indicated by the arrow), starfish AMP - ST-1 (3,715 Da unfolding standard) (A), sea anemone AMP - SAn4 (4,314 Da folding standard) (B), Piscidin (1,600 Da standard) (C), and PALAL (484 Da standard) (D).

3.4. Partial N-terminal amino acid sequence analysis

Partial N-terminal amino acid sequence of purified protein (residues 1-25) was K-V-H-G-S-L-A-R-A-G-K-V-R-A-Q-T-P-K-V-P-K-Q-G-K-G. BLAST homology searches showed that the identified 25 amino acids sequence of the purified protein were generally corresponds to that of 40S ribosomal protein S30 (RPS30) from other fish, such as zebrafish (*Danio rerio*), atlantic salmon (*Salmo salar*), goldfish (*Carassius auratus*), turbot (*Scophthalmus maximus*), and orange-spotted grouper (*Epinephelus coioides*) (Fig. 12). Based on these results, the purified protein was identified to be rock bream 40S ribosomal protein S30.

3.5. Sequence analysis

Based on EST data of rock bream gills mRNA sequence by BLAST (Fig. 3), and amino acid sequence by Edman degradation, the partial nucleotide sequence of the purified protein was determined through PCR. Identified nucleotide sequence was a part of fusion protein consisting of the ubiquitin-like protein fubi and 40S ribosomal protein S30, and that posttranslated into 40S ribosomal protein S30 (Fig. 13). The posttranslationally processed region of 180bp in open reading frame

(ORF) was translated into amino acid sequence containing 59 amino acids (Fig. 13). The full nucleotide sequence of the 40S ribosomal protein S30 precursor was determined through 3' and 5' RACE PCR. The full nucleotide sequence was comprised of 571 bp including 5' untranslated region (UTR) of 47 bp, open reading frame (ORF) of 402 bp (ubiquitin-like protein fubi of 222 bp and 40S ribosomal protein S30 of 180 bp), and 3' UTR of 122 bp. The ORF was translated into amino acid sequence containing 133 amino acids (ubiquitin-like protein fubi of 74 amino acids and 40S ribosomal protein S30 of 59 amino acids) (Fig. 14). Compute pI/Mw tool in ExPASy showed that the rock bream 40S RPS30 had a theoretical molecular weight of 6,673.84 Da, and a theoretical isoelectric point (pI) of 11.92.

Multiple alignment of the rock bream 40S RPS30 with other known 40S RPS30 amino acid sequences revealed that this protein was perfectly matched with other teleost RPS30 such as zebrafish (*Danio rerio*), atlantic salmon (*Salmo salar*), and goldfish (*Carassius auratus*) (Fig. 15).

3.6. Structural modeling

To obtain a structural model, rock bream RPS30 was applied to SWISS-MODEL, a fully automated protein structure homology-modeling server. Three-dimensional structure of 40S ribosomal protein S30 in european rabbit 40S HCV-IRES (Hepatitis C virus–internal ribosomal entry site) complex (PDB accession no. 5flx.1.5) was used as a template. The amino acid sequence of rock bream RPS30 showed 96.61% identity to that of rabbit 40S ribosomal protein S30. Three-dimensional structure of rock bream RPS30 was almost similar to that of rabbit 40S RPS30 containing two α-helices separated by three loop regions (Fig. 17).

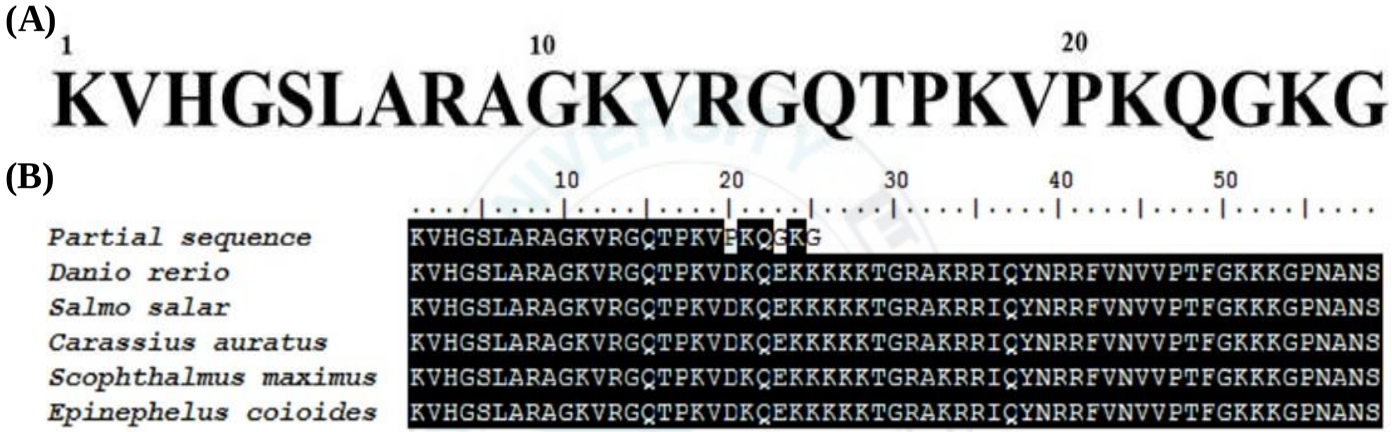

Fig. 12. Partial N-terminal amino acid sequence of purified peak acquired using Edman degradation and homology search results of the partial amino acid sequence with 40S ribosomal protein S30 from other teleost. (A) Partial N-terminal amino acid sequence (residue 1-25) obtained by Edman degradation. (B) 40S ribosomal protein S30 from other teleost: including zebrafish (*Danio rerio*) (NP_957031.1), atlantic salmon (*Salmo salar*) (NP_001133169.1), goldfish (*Carassius auratus*) (AGO58889.1), turbot (*Scophthalmus maximus*) (gi 116488152), and orange-spotted grouper (*Epinephelus coioides*) (HQ 441121.1).

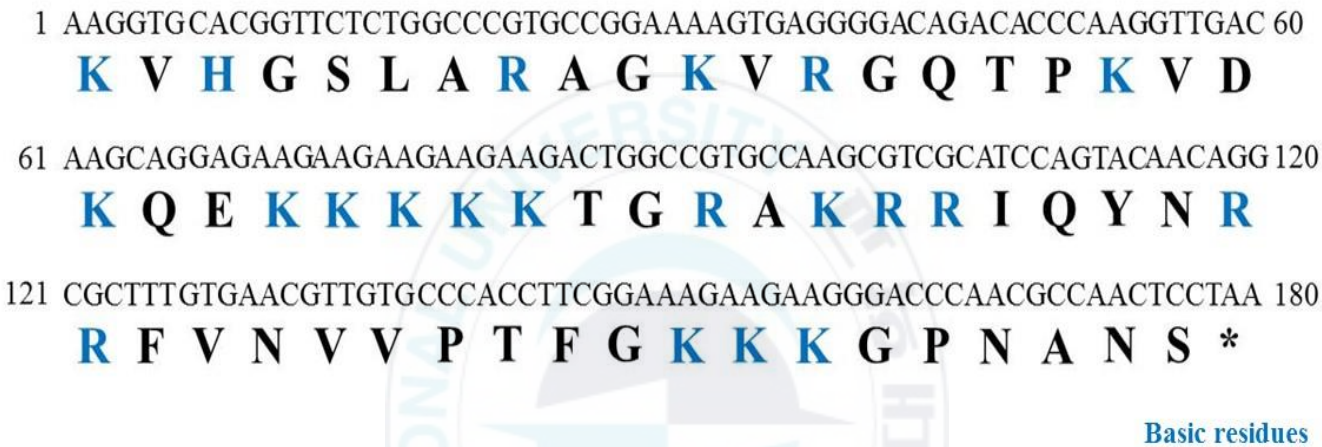

Fig. 13. The sequence of nucleotide and deduced amino acid sequence of the rock bream 40S ribosomal protein S30. Mature nucleotide sequence of 40S ribosomal protein S30 (180bp) in open reading frame (ORF) and deduced amino acid sequence (59 amino acids)**.** The basic residues (7 Arginine, 1 Histidine and 13 Lysine) are indicated in blue color.

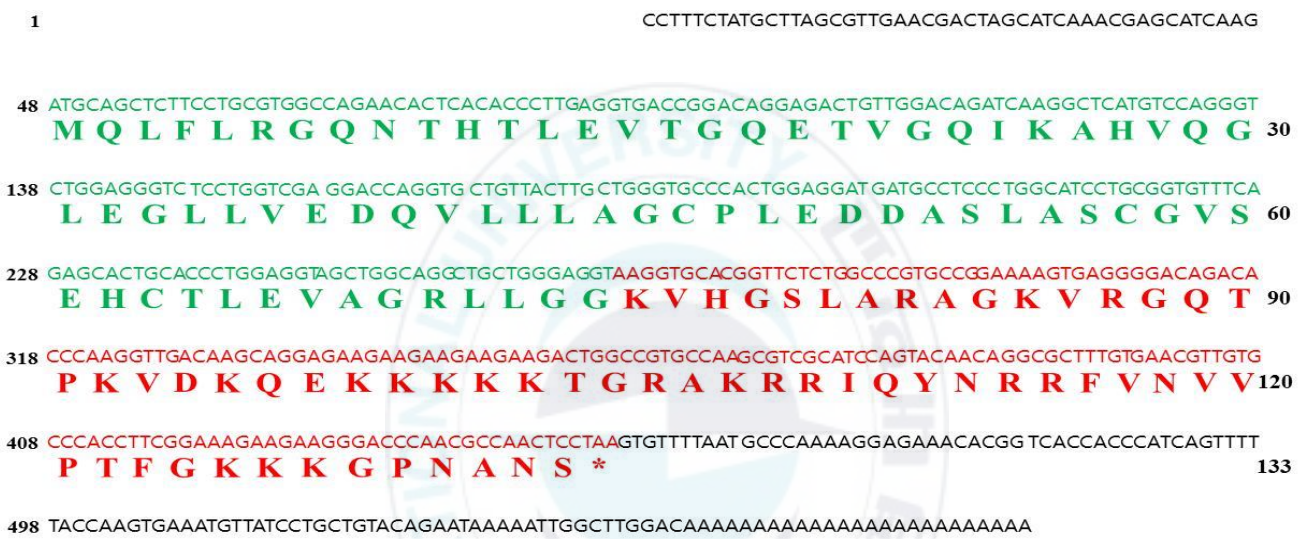

Fig. 14. The complete nucleotide sequence and deduced amino acid sequence of the rock bream 40S ribosomal protein S30 precursor. The nucleotide sequence (*Fau* gene) of ubiquitin-like protein (222bp, green color) and 40S ribosomal protein S30 (180bp, red color) in open reading frame (ORF) and deduced amino acid sequence (133 amino acids), 5' untranslated region (5'UTR) and 3' untranslated region (3'UTR).

Fig. 15. Alignment of amino acid sequence of 40S ribosomal protein S30 from rock bream *(Oplegnathus fasciatus)* with 40S ribosomal protein S30 from other species: including zebrafish (*Danio rerio*) (gi41152251), atlantic salmon (*Salmo salar*) (NP_001133169.1), goldfish (*Carassius auratus*) (gi514483207), western clawed frog (*Xenopus tropicalis*) (AGO58889.1), american alligator (*Alligatior mississippiensis*) (XP_006265026.2), american crow (*Corvus brachyrhynchos*) (gi676409897), house mouse (*Mus musculus*) (gi6679753) and human (*Homo sapiens*) (gi4503659), Conserved amino acids are indicated by black boxes.

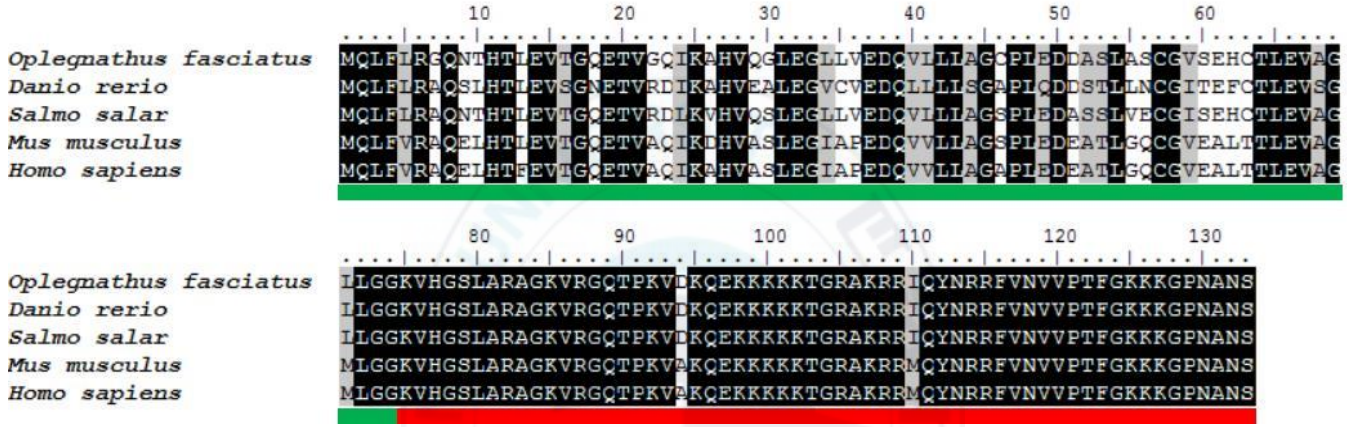

Fig. 16. Alignment of the complete amino acid sequence of 40S ribosomal protein S30 precursor from rock bream *(Oplegnathus fasciatus)* with 40S ribosomal protein S30 precursor from other species: including zebrafish (*Danio rerio*) (gi41152251), atlantic salmon (*Salmo salar*) (NP_001133169.1), house mouse (*Mus musculus*) (gi6679753), and human (*Homo sapiens*) (gi4503659). Conserved amino acids are indicated by black boxes. 133 amino acids are 40S ribosomal protein S30 precursor (fusion protein) consisting of the ubiquitin-like protein fubi (74 amino acids) at the N-terminus (indicated by the green line) and ribosomal protein S30 (59 amino acids) at the C-terminus (indicated by the red line).

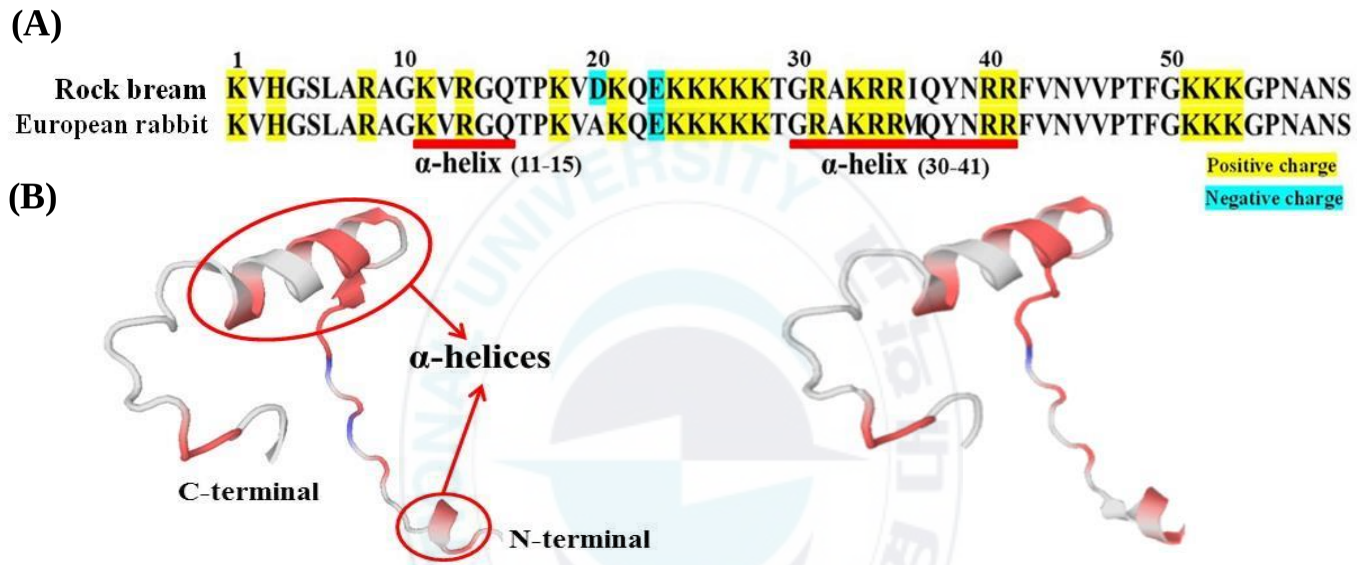

Fig. 17. Homology modeling of the rock bream 40S RPS30. (A) Alignment of the rock bream 40S RPS30 amino acid sequence with the rabbit 40S RPS30 in mammalian 40S HCV-IRES complex as template (Protein Data Bank [PDB] accession no. 5flx.1.5). The amino acid sequence of 40S ofRPS30 showed 96.61% identity to that of the rabbit 40S RPS30 (^{20}Asp → ^{20}Ala, ^{36}Ile → ^{36}Met). (B) The obtained modeling structure of rock bream 40S RPS30 (left) was almost similar to that of rabbit 40S RPS30 (right) containing two α-helices separated by three loop regions.

4. Discussion

Teleost live aquatic environment, also exist various pathogens. So, innate immunity and adaptive immunity is important to them. Although teleost have an effective adaptive immunity including antibody and specific cell-mediate response like land vertebrates, it may be constrained by the suboptimal environmental conditions such as low temperature and seasonal variants, and may need some time before working [*Oren & Shai 1996*]. Therefore, the teleost are required for quickly and non-specific immune system, and AMPs are an innate immune factor that are satisfied with these conditions. AMPs in epidermal mucus have been viewed as one of the key factors for the innate immune defense of teleost [*Narvaez et al. 2010*].

To find out the properties of the mucus extracts from rock bream used in this study, carried out several experiments such as measurement of antibacterial activity, pH conditions and heated treatment, and proteolytic enzyme digestion. First, in order to investigate the antibacterial activity, it carried out URDA. These results showed antimicrobial in a variety of gram positive and negative bacteria (Table 3 and Fig. 4). These results suggest that the extract possesses broad spectrum antimicrobial activity

against gram positive bacteria, gram negative bacteria, soil bacteria and marine bacteria. *S. aureus* RN4220, which showed high susceptibility to the extract, was selected for antibacterial assay during the purification. In addition, the antibacterial activity of the extract was mostly abolished by treatment with trypsin for 60 min at 37℃ (Fig. 5). Treatment of trypsin was cleaves peptide chains at the carboxyl side of the amino acid arginine or lysine. These results suggesting that the extract was proteinaceous and contained the positive charged residues such as Arginine or Lysine.

Generally, cationic AMPs contain several cationic residues such as arginine, lysine and histidine [*Bradshaw et al. 2003*]. Crude extract of epidermal mucus from rock bream was not affected by the pH conditions such as acidic, neutral and basic (Fig. 5). These results indicate that the antibacterial activity of crude extract of skin mucus could be retained in the wide range of pH conditions. In addition, crude extract of epidermal mucus showed no change in antibacterial activity to the heat treatment. This result indicates that antibacterial material in crude extract of skin mucus is heat-stable such as short polypeptide or small protein. Based on the screening results, in extract of epidermal mucus from rock bream will be having antimicrobial material such as AMPs.

The present study describes the purification and partial characterization of the antimicrobial protein from epidermal mucus of rock bream. The purified protein displays potent antibacterial activity against gram positive bacteria *S. aureus*. Unfortunately, due to the lack of purified protein it did not measure the antimicrobial activity against the other bacteria. After collecting the sample with recombinant protein or purification experiments will test the antimicrobial activity or several bacteria using MIC or MEC assay. The theoretical mass of purified 40S RPS30 of rock bream was calculated to be 6,673.84 Da, and the theoretical isoelectric point was 11.92, which was small protein and highly basic protein. Unfortunately, the sample was lost due to a malfunction during the analysis by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), and as a result the practical mass could not be measured. Partial primary structure of purified protein from rock bream is identical or highly homologous to 40S RPS30 from other species (Fig. 12). 40S RPS30, a component of the small subunit of eukaryotic ribosomes is one of the characterized polypeptides and now this active polypeptide has been purified from epidermal mucus of rock bream. Commonly, 40S RPS30 of 59 amino acids is the C-terminal cleavage product of fusion protein of 133 amino acids that is encoded by the *Fau* gene [*Kas et al 1992*]. The *Fau* gene encodes a fusion protein

consisting of the ubiquitin-like protein fubi (74 amino acids) at the N-terminus and 40S ribosomal protein S30 (59 amino acids) at the C-terminus (Fig. 14). The *Fau* gene has been reported to be expressed in variety of tissues in various animal species. Main function of 40S ribosomal protein S30 is a structural component of the small 40S subunit and RNA binding also additionally has the function of the antimicrobial activity [*Fernandes & Smith 2002*, *Hiemstra et al. 1999*, *Kas et al. 1992*, *Michiels et al. 1993*]. The function of ubiquitin-like protein fubi is not yet known.

Based on the analyzed deduced amino acid sequence compared to the sequence of 40S RPS30 of other fish such as zebrafish, goldfish, turbot and salmon that was a completely match, and the amino acid sequence of the other vertebrates such as mammals (human, *Homo sapiens* and house mouse, *Mus musculus*), avians (american crow, *Corvus brachyrhynchos*), reptilians (american alligator, *Alligator mississippiensis*) and amphibians (western clawed frog, *Xenopus tropicalis*) were matched to 96% (57/59 amino acids) (Fig. 15). The amino acid sequence of 40S RPS30 was found to be conserved among fish, and it was also highly conserved among vertebrates. Rock bream 40S RPS30 had twenty-one basic amino acids such as thirteen lysine, seven arginine and one histidine (Fig. 13).

Perhaps the antimicrobial activity of 40S RPS30 is expected to be due to its basic composition and cationic structure. Cationic antimicrobial material, such as 40S RPS30 can easily bind to membranes of bacteria with negative charges, thereby destroying or permeating through its membrane and killing or inhibiting the bacteria. The most valuable point in this study was that 40S RPS30 was found in the epidermal mucus of the rock beam, epidermis is one of the most important defense region for fish. Perhaps 40S RPS30 is normally present in the cell as part of the ribosome structure, but is secreted into the mucus during bacterial invasion or infection, which helps the immune response. To demonstrate this, we will conduct the immune challenge assay further.

In conclusion, we have purified a 40S RPS30 from an acidified epidermal mucus extract of rock bream that had potent antimicrobial activity against *S. aureus*. Small and cationic protein, 40S RPS30 had theoretical molecular weight of 6,673.84 Da, and a theoretical isoelectric point (pI) of 11.92. Full nucleotide sequence of rock bream 40S RPS30 precursor consisting of 571 nucleotides was obtained by cDNA cloning. 40S RPS30 was a part of fusion protein consisting of the ubiquitin-like protein fubi and 40S ribosomal protein S30, and that posttranslated into ribosomal protein S30. 40S RPS30 is a structural component of the 40S

ribosome also additionally has the function of the antimicrobial activity.

This study was performed overall purification and partial characterization of 40S RPS30 in epidermal mucus of rock bream. The results in this study may be usefully provided to important source of future therapeutic antimicrobial drugs and biological information of AMPs in rock bream.

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