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RESEARCH PAPER

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Assessment of Some Immune Components from The Bioactive Crude Extract Derived from The Epidermal Mucus of Climbing Perch *Anabas testudines*

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Abstract

Fish and other members of the aquatic habitats are usually in constant interaction with their habitat and potentially harmful pathogens. This study was undertaken to evaluate some of the immune components in the bioactive crude obtained from epidermal mucus of climbing perch. The enzyme activities were assessed using the spectrophotometer and turbidometric assay for the lysozyme activity, and while antimicrobial peptides were determined via AKTA Purifier 10 FPLC with a Superdex™ peptide 10/300 GL column and Q-TOF LC/MS for characterization. Likewise, the antibacterial activity against *P. aeruginosa* ATCC 10451 was achieved using chromatography fractions. Results showed that higher activities of lysozyme and protease were present in the crude extract. While values for alkaline phosphatase and esterase activities varied. The data analysis of Q-TOF LC/MS showed apolipoprotein A-1, haemoglobin subunit beta (Fragment), elongation factor 1-alpha, cytochrome C oxidase subunit1 (Fragment), Beta-actin (Fragment) and potent antimicrobial peptides including de novo AAGPKGPLGPR peptide in crude. This result emphasizes their role in protecting to climbing perch from foreign invaders as a part of the innate immune system throughout the epidermal mucus. The bioactive crude has a high potential to be used as a natural antimicrobial agent for pharmaceutical applications.

Introduction

The innate immune components in fishes are comprised of the mucus layer secreted on the skin, gills, and gastrointestinal tract, as well as the non-specific immune cells (phagocytes and natural killer cells) found in the blood(Subramanian et al., 2007). The skin mucus is an important indicator of the immune competence of fish (Al-Rasheed et al., 2018; Brinchmann, 2016). This is because fish mucus has numerous humoral immune activities(Cordero et al., 2016; Guardiola et al., 2014). Mucus protects against pathogens and injuriously suspended particles through inhibiting pathogen adherence by sloughing off due to the continuous secretion of the mucus or by providing a repository of many piscine antibacterial proteins and peptides (AMPs) (Subramanian et al., 2007; Uthayakumar et al., 2012). The majority of the antimicrobial peptides are found in specialized peptides with somewhat distinct structures (Rajanbabu & Chen, 2011). Nevertheless, a large number of peptides occur as larger proteins or protein fragments (Conceição et al., 2012). They include AMP-

independent anti-infectious substances that ensure the integrity of the skin such as cathepsin B, alkaline phosphatase, complement, transferrin, lysozyme, and C-reactive protein (Cole et al., 1997; X. Zhao et al., 2008).

Among small indigenous fishes, *Anabas testudines (*Bloch, 1972) is an economically important fish in majority of Southern Asian countries (Abdul Halim et al., 2017). The fish is characterized by a thick coat of slime containing mucopolysaccharides, lipids, and proteins, which are essential in keeping the skin moist and this is thought to also enhance the survivability of the fish especially outside the aquatic environment(Agarwal et al., 1980). Anabas is considered to be among the toughest fish with the ability to tolerate very turbid and brackish water conditions(Roberts, 1989; Sakurai et al., 1993). Despite the economic importance of this fish species, literatures on the innate immune components as well as the antibacterial properties of the epidermal mucus have not been fully investigated. Therefore, this study was carried out to assess some innate immune components in fish mucus by evaluating the activity of lysozyme (LYZ), protease, alkaline phosphatase, and esterase enzymes.

Materials and Methods

Preparation of Bioactive Crude Extract

The epidermal mucus from climbing perch *Anabas testudines* (Bloch, 1972) was prepared according to our previous method (Al-Rasheed et al., 2018). Briefly, 15 apparently healthy climbing perch (18-20 cm) were purchased from local wet market in Seri Kembangan, Serdang, Malaysia and kept in an aerated glass aquarium filled with room temperature pond water for seven days to acclimatize. The epidermal mucus was obtained by subjecting the fish to hypothermic stress as we previously reported (Al-Rasheed et al., 2018). The mucus produced was then scraped and lyophilized at -80^{o}C under vacuum at 0.018 pressure in a lyophilizer. 200 mg of the lyophilized epidermal mucus was mixed with 1% acetic acid (HAc) in a ratio of 1:4, and heated in a boiling water bath for 3 mins to inhibit proteolytic enzymes activity and solubilize the low-molecular-weight peptides (Conlon, 2007). The heated mixture was completely homogenized with polytron homogenizer on dry ice for 5 min. The resultant homogenate was then centrifuged in a refrigerated centrifuge at 15,000 rpm, for 35 min, at 4^{o}C and part of the supernatants of the homogenate was filtered with a Whatman No.1 filter paper size 0.45 µm and stored at 4^{o}C.

Antimicrobial Susceptibility Screening

The antibacterial activity of the acidic extract was determined by evenly streaking 0.1 ml of bacterial inoculum (10^5 CFU/ml) on Mueller Hinton agar plate. This is then followed by punching a 6 mm well in the solid media and 50 µl of the aqueous extract was used to fill up the wells. The plates were then incubated overnight at 37^{o}C. The antibacterial activity was determined based on the diameter of zone of inhibition. Ciprofloxacin (15 µg/ml) was used as positive control. The antibacterial activity was evaluated against *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Salmonella choleraesuis* reference strains as well as local isolates *Bacillus subtilis* UPMC 1175 and *Serratia marcencen* S381 (obtained from Institute of Bioscience, Universiti Putra Malaysia).

Enzymes Activity in Bioactive Crude

The activity of protease, lysozyme, alkaline phosphatase, and esterase were estimated in the bioactive crude (10 mg/ml) derived from the mucus of the climbing perch.

Protease

The protease was determined using the substrate azocasein (Sigma) as a sensitive and convenient non-specific substrate used to quantify the proteolytic activity in bioactive crude. Measurement was done by the release of red colored azopeptides as described by Peres et al (Peres et al., 2014). Briefly, 50 ml of azocasein (5 mg/mL) prepared in 50 mM trisHCl buffer (pH 8.0) containing 2 mM CaCl2, and 50 mL of the corresponding bioactive crude was incubated at 37^{o}C for 1 h. The reaction was stopped by the addition of 100 ml of 5% (w/v) TCA solution. The mixture was then centrifuged at 10, 000 g for 5 min after 10 min incubation at room temperature. Fifty (50) mL of the supernatant was mixed with 75 ml of 0.5 M NaOH, and the absorbance was measured at 450 nm against a blank. One unit (U) of protease activity was defined as a 0.001 increase in absorbance of the assay solution.

Lysozyme (LYZ)

The turbidimetric assay method described by Ng et al (Ng et al., 2013) was employed to determine the lysozyme (LYZ) activity in the crude extract by lysis of Gram-positive *Micrococcus lysodeikticus*. A total of 10 µl of bioactive crude and standards of egg white lysozyme (Sigma) respectively, were added in dry cuvettes containing 1.0 mL of the bacteria. The assay was set to run for 5 min at 31^{o}C and a change in optical density OD at 450 nm was recorded at 30 s intervals, with stirring for 10s after each measurement. Range of concentration solutions (0.1, 0.08, 0.06, 0.04, 0.02, 0.01, 0.008, 0.006) mg/ml were prepared from stock solution of 1 mg/ ml which were plotted against the lysozyme activity in OD/min (Dash et al., 2014).

Alkaline Phosphatase (ALP)

Alkaline phosphatase activity in the bioactive crude was determined spectrophotometrically by the

dimension clinical chemistry system using the ALP Flex® reagent cartridge (Siemens) according to the manufacturer's instructions. The assay is an in vitro diagnostic test used for the quantitative determination of alkaline phosphatase activity in serum and plasma. The assay uses p-nitrophenylphosphate (p-NNP) as a substrate, which will then become catalyzed into p-nitrophenol (p-NP) by all alkaline phosphatase isoenzymes.

Esterase

Spectrophotometric assay was also used to detect esterase activity following the hydrolysis of p-nitrophenyl myristate (Sigma, St. Louis, MO) at 410 nm (Guardiola et al., 2014; Ross et al., 2000). For the reaction, 100 µl of substrate solution (25 mM p-nitrophenyl myristate in DMSO) was mixed with 880 µl of 50 mM Tris/HCL buffer (pH 7.0) and 20 µl of bioactive crude. The mixture was incubated for 10 min at 30°C in a shaking water bath at 100 rpm and then 100 µl of ethanol was added to stop the reaction. The OD of liberated p-nitrophenol was evaluated at 410 nm. One unit of activity corresponds to the amount of enzyme required to release one mM of p-nitrophenol product in 1 min, and the activity was expressed as Umg-1 mucus proteins. Esterase activity was determined by referring to the standard curve of p-nitrophenol which was plotted using p-nitrophenol standard solution in the range of 0.01-0.3 mM prepared from stock solution of p-nitrophenol (Sigma-Aldrich) 10 mm (Chen et al., 2017).

Isolation of AMPPs by Size Exclusion Chromatography (SEC)

The isolation of antimicrobial proteins and peptides were conducted by a two-step chromatographic process and *P. aeruginosa* ATCC 10451(Thermo Fisher Scientific, R4607065 Cheng, Melaka, Malaysia) was selected as testing bacteria. In the initial step, a total of 300 mL crude extract was lyophilized and suspended in 30 mL sterile distilled water and centrifuged at 15,000 rpm for 15 min at 4°C. The suspension was subjected manually to reversed-phase Sep-Pak Vac 1 g C18 (125 Å, 55–105 µm; Waters Corporation, WAT036905 MA, USA) by 20-mL plastic syringe as described by Conlon (Conlon, 2007). The cartridge eluate consisting 70% acetonitrile with 0.1% Triflouroacetic acid was concentrated to approximately 1.5 ml at room temperature for 60 min. In addition, reducing elute was filtered out using ultrafiltration (UF) advance centrifugal device (MWCO 30kD with Omega Membrane). The filtrates < 30 kDa was loaded onto GE AKTA Purifier 10 FPLC that is equipped with a Superdex 75 10/300 GL column [57]. The column was equilibrated with 50 mM of Sodium phosphate, pH 5.8 in 150 mM/L of NaCl buffer at a flow rate of 0.5 mL/min. The elution profile was monitored at 280 nm(Song et al., 2012). The inhibitory activity against *P. aeruginosa* ATCC 10145 was accomplished for fractions at three peaks.

Protein and Peptides Profile

To estimate the profile and molecular weight (MW) of the antimicrobial proteins and peptides, 15% SDS-PAGE was performed as previously described (Laemmli & Favre, 1973). In this experiment, 20 µg of the total protein sample was diluted in a ratio of 1:1 with sample buffer containing (4% (w/v) SDS, 50 mM Tris-HCl, 2% mercaptoethanol (v/v), 12% (v/v) glycerol, and 0.5% (w/v) bromophenol blue adjusted with HCl to pH 6.8, and loaded onto a separating gel of 15% SDS-under reducing conditions and run in an electrophoresis apparatus for 4 h at 80V. Gel stained with Coomassie Brilliant Blue and protein bands were viewed against the protein standard (BioRad) (Hames, 1998).

Identification of Antimicrobial Proteins and Peptides

The LC-MS/MS technique was carried out to identify the antimicrobial proteins and peptides of climbing perch. The sample was subjected to trypsin digestion, and the identification of proteins and peptides was carried out using Accurate Mass quadruple time-of-flight (Q-TOF) Liquid Chromatography (Agilent 6520, USA) (LC-MS/MS Laboratory/Monash University/Malaysia). Trypsinized sample was injected into (Q-TOF) LC/MSMS to identify the sequence, and the running solvents were (A); 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The freeze-dried sample was diluted in 0.75 ml of solvent A. Acquired data were analyzed with PEAKS Studio 8.5, and database search done on the UniProt Anabantaria fasta Database.

Statistical Analysis

The results were analyzed using SPSS statistical software (version 23). One way analysis of variance used to deduce variability and level of significance ($P < 0.05$). All values were expressed as mean±standard error (S.E).

Results

Antibacterial Activity of Extracts Against Bacteria Isolates

Evaluation of the antibacterial activity of the acidic mucus extract revealed variable degree of inhibition (Figure 1). The extract prevented the growth of *S. aureus*, *P. aeruginosa, S. choleraesuis*, *B. subtilis* UPMC 1175 and *S. marcencen* S381, however, the difference was only statistically significant for *P. aeruginosa*. Based on the result obtained, the highest zone of inhibition was observed in the plate inoculated with *P. aeruginosa* with a diameter of inhibition (12.65±0.47). Similarly, *S. aureus, S. choleraesuis, B. subtilis* UPMC 1175, and *S. marcencen* S381 had 8.5±0.577, 6.75±0.95, 7.25±1.25, 6.5±1.753, respectively.

Enzymatic Activities of Bioactive Crude

The level activities of immune-related enzymes involving protease, lysozyme, alkaline phosphatase, and esterase in the bioactive crude activities are presented in Table 1. The activity of protease was 213.3±1.365 U/mg, and the concentration and activity level of bioactive crude lysozyme were 0.13±0.0054 µg/mL; 263.25±18.135 U/mg, respectively. Similarly, alkaline phosphatase and esterase activities had 0.013±1.35 U/mg; and 1.43±0.033 U/mg, respectively.

Isolation of Antibacterial Fractions by Size-Exclusion Chromatography

A total of four peaks represented the fractions including peak 1 (fractions: B2-B4, B6-B8, B10-B12), peak 2 (fractions C2- C4), peak 3 (fractions: C6-C8), and peak 4 (fractions: D2 - D4) (Figure 1). Protein profile of SepPak C18 and Ultrafiltration 30 kDa fraction showed bands with Mw (25 to 10 kDa) at 15% SDS PAGE (Figure 2). However, fractions at peak 1of Superdex™ peptide 10/300 GL did not show any visible bands on the gel. Antimicrobial activity was sequentially screened for each fraction by using well diffusion agar, and antimicrobial activity against *P. aeruginosa* ATCC10145 has detected at peak 1 fractions eluted between 12.5 to 17.5 mins. Additionally, individual fractions showed equal IZD (9mm). Though, IZD of elution fraction of SepPak C18 and Ultrafiltration 30 kDa fraction was 18 mm (Figure 2).

Q-TOF LC/MS Data Analysis

Proteins and peptides in the bioactive fractions at peak 1 were identified using Q-TOF LC/MS. Trypsin

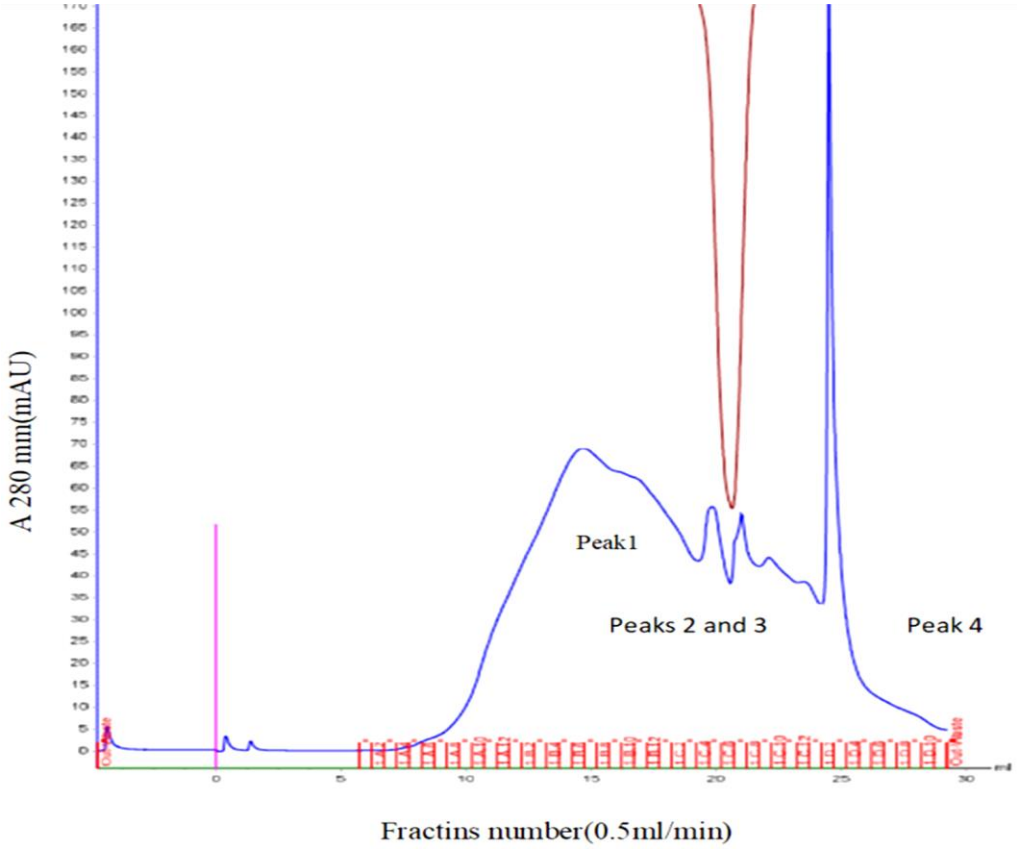

Figure 1. Size-exclusion chromatography of antibacterial proteins and peptides using Superdex™ peptide 10/300 GL on AKTA Purifier 10 FPLC. Four chromatogram peaks; peak 1 (A12–B12), peak 2(C3–C6), peak 3 (C8–C10), and peak 4 (D2–D6) detected by absorbance at 280 mm (mAU).

Table 1. Enzyme hydrolysate activity of crude of bioactive proteins and peptides

Parameters	U/mg**
Protease	213.33±1.365
Lysozyme	263.25±18.135
Alkaline phosphatase	0.013±1.35
Esterase	1.43±0.033

**U/mg unit of enzyme per mg in crude cationic proteins and peptides (Mean ± S.D)

digestion was applied to reduce the influence of ion suppression effects in Q-TOF LC/MS measurements to obtain more peptide peaks. Subsequently, Q-TOF LC/MS data was analysed using PEAKS Studio 8.5. The analysis results are shown in Table 2.

Proteins in the bioactive crude extract are identified by accession numbers that match with the identified proteins. The arranged and aligned amino acid sequences of apolipoprotein A-1 and haemoglobin subunit beta are shown in Figure 4 a, b. Furthermore, aligned amino acid sequences of proteins, elongation factor alpha, beta -actin and cytochrome c oxidase subunit 1 are shown in Figure 5.

The physical parameters of *de novo* peptides which were anionic and cationic peptides with mass ranging from 1020.25 to 1902.06 Da with different hydrophobicity ratios, and residues of amino acids are shown in Table 3. Similarly, the MS/MS spectrum of *de novo* peptide AAGPKGPLGPR is shown in Figure 3 and the MS/MS spectra of other peptides detected are presented in Figure 6**.** All proteins and peptides were analysed from UniProt database.

Discussion

The present study was able to demonstrate the potentials of the bioactive crude obtained from climbing perch for use as an alternative antimicrobial agent especially in the current situation where most infectious disease pathogens are developing resistance to the conventional drugs.Although, in this study the extract was able to exert considerable antibacterial activity on all the bacterial organisms tested by preventing their growth, statistically significance was observed only for *P. aeroginosa.* The fact that the acidic mucus extract was able to prevent the growth of the bacterial organisms

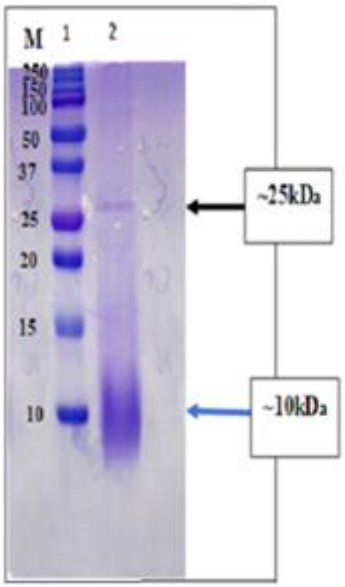

Figure 2. SDS-PAGE (15%) analysis of elution fraction form SepPak C18 and ultrafiltration 30 kDa. Line 1 is the calibrated high protein marker (BioRad), and lane 2 represents the protein profile of fraction; arrows indicate the bands of fraction proteins.

Table 2. Q-TOF LC/MS analysis details, accession number, and physical parameters of identified proteins in the bioactive fraction of Superdex™ peptide 10/300 GL

Protein name	Accession No.	Peptides	Unique peptides	% protein coverage
Apolipoprotein A-1 *Channa striata*	A0A077H3P6	22	22	44
Beta-actin (Fragment) Channa argus x Channa maculata	A0A0U2K0I0	6	0	34
Beta-actin (Fragment) *Channa punctata*	F2YRP1	6	0	34
Hemoglobin subunit beta *Channa striata*	V9QM19	5	5	20
Elongation factor 1-alpha (Fragment) *Channa maculata*	A0A1W5YI2	7	5	12
Cytochrome c oxidase subunit1 (Fragment) *Anabas testudineus*	A0A159YN20	2	0	5
Cytochrome c oxidase subunit 1 (Fragment) *Anabas testudineus*	A0A1B1RVD3	2	0	5

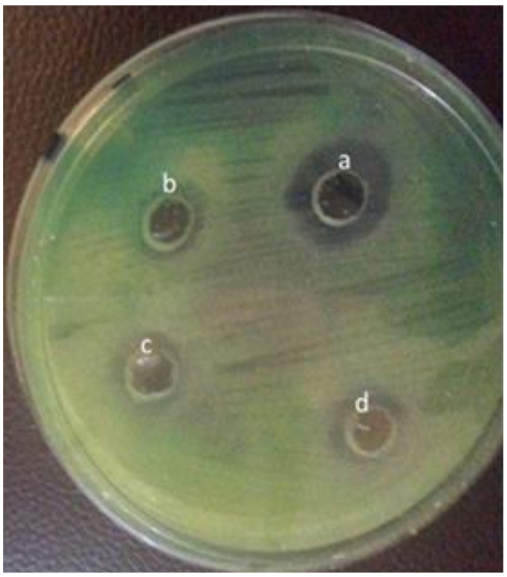

Figure 3. Mueller-Hinton agar plate showing well diffusion assay (zone of inhibition measured in mm) against P. aeruginosa ATCC 10145 for (a) fraction for SepPak C18 and ultrafiltration 30 kDa and fractions at peak 1 (b) B2–B4, (c) B6–B8, and (d) B10–B12 for Superdex™ peptide 10/300 G.

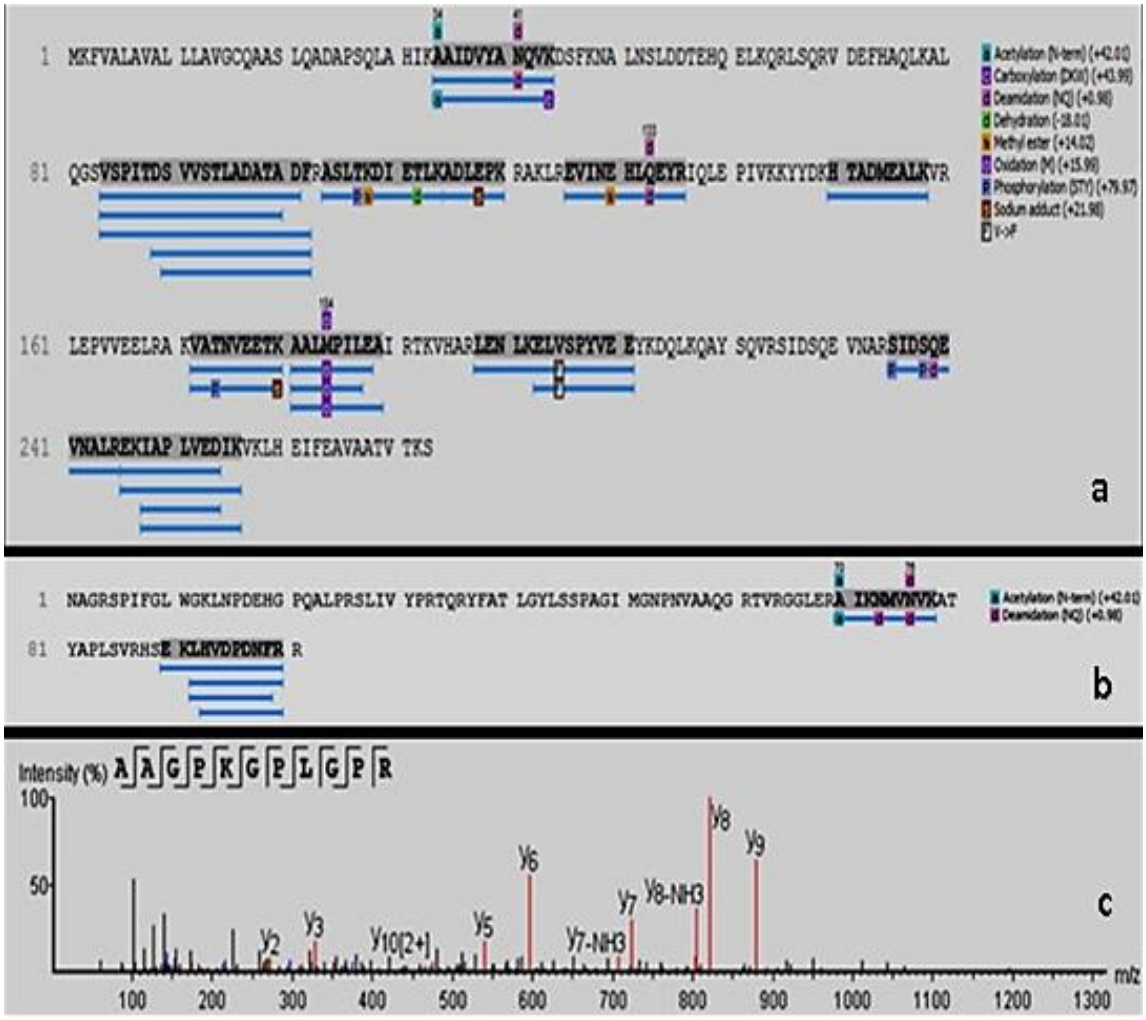

Figure 4. (a) Aligned amino acid sequences (blue) coverage of apolipoprotein A-1 of bioactive fraction, matched with identified apolipoprotein A-1 from *Channa striata*; (b) Haemoglobin subunit beta of bioactive fractions matched with peptide sequence of identified haemoglobin subunit beta from of bioactive fractions matched with peptide sequence of identified haemoglobin subunit beta from *Channa argus* and *Channa maculata*; (c) MS/MS spectrum of de novo peptide AAGPKGPLGPR showing the fragmentation pattern of the peptide with a parent mass of 1020.25 and doubly charged.

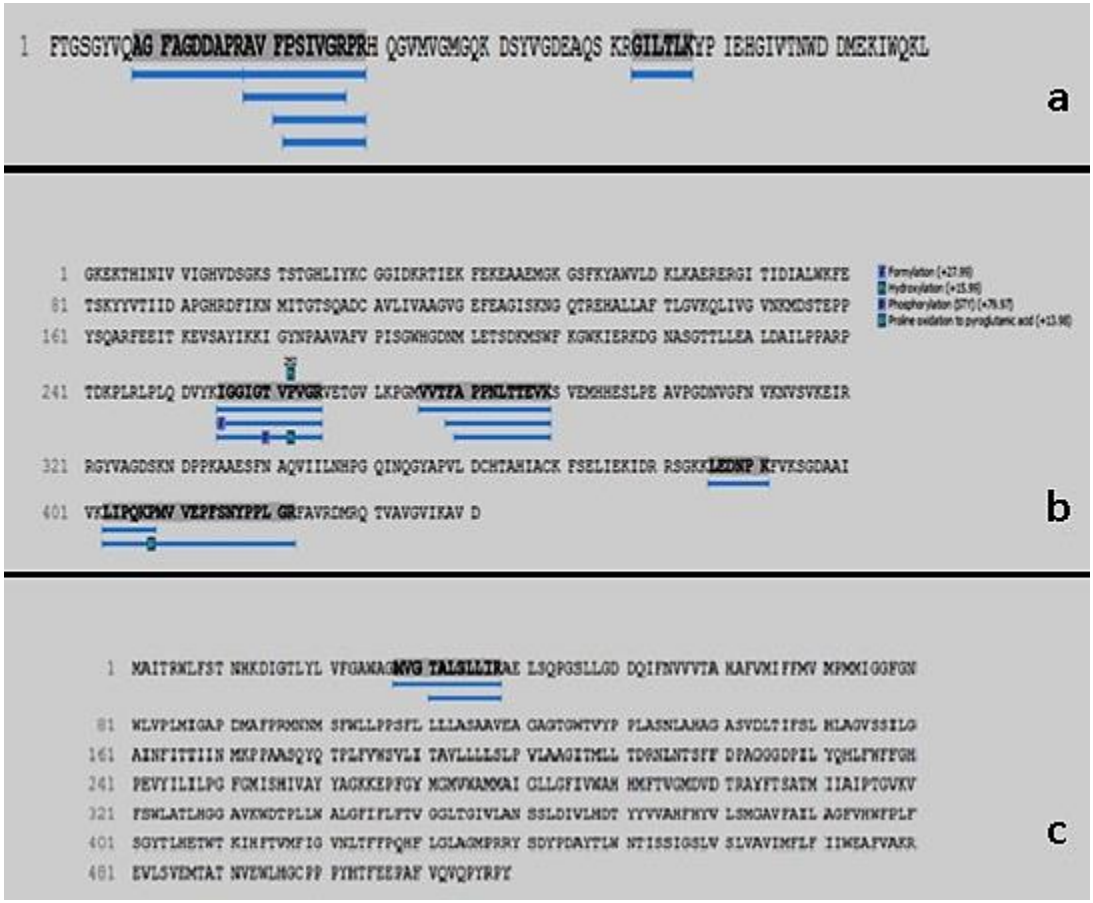

Figure 5. (a) Aligned amino acid sequences (blue) coverage of elongation factor alpha of bioactive fraction, matched with identified elongation factor from *Channa mulculata*; (b) Beta-actin of bioactive fraction matched with beta-actine from *Channa punctata;* (c) Cytochrome C oxidase subunit 1 of bioactive fraction matched with cytochrome C oxidase of bioactive of *Channa punctata*.

Table 3. Sequences of De novo peptides in the biological active fraction

Peptide sequence	Residues	Hydrophobicity ratio	Net charge	M.W (Dalton)
WTGPSGLTGFLQQR	14	28.2	+1	1547.71
LLLNGGGSADSVDTPASALK	20	40	-1	1886
LNGGGSADSVDTPASALK	18	33.3	-1	1659.75
SVPQQQLKSSVDEEVTK	17	23.53	-1	1902.06
AAGPKGPLGPR	11	27	+2	1020.25

The data of de novo peptides were analyzed using APD3: Antimicrobial Peptide Predictor at http://aps.unmc.edu/AP/prediction/prediction_main.php

tested is an indication that they possess some antibacterial properties. The inhibitory effect of acidity crude extract may be due to the ability of the cationic antibacterial peptides and proteins to penetrate host tissues via pores created by pathogenic bacteria (Bragadeeswaran et al., 2011). This suggests that fish secrete antibacterial proteins and peptides that are able to permeabilize the membrane of the target cell and thus acts (same word repeated) as a defense barrier (Ravichandran et al., 2010). Moreover, the extraction using mild acid solution of 1% of acetic acid may have increased the soluble cationic peptides and proteins in acidic medium which have broad-spectrum bactericidal activity against a wide range of pathogens (Hancock & Sahl, 2006). The findings in this study showed that; protease activity in the bioactive crude extract of climbing perch was three-fold higher than the activity of protease in skin mucus extract of olive flounder (*Paralichthys olivaceus*) (Jung et al., 2012; Palaksha et al., 2008). The high activity of protease indicates a potential role in the prevention of colonization by pathogenic organisms on the skin of climbing perch, which is similar to an earlier report by Marcos-López et al. (Marcos-López et al., 2017). Similarly, the presence of lysozyme with activity 263.25 U/mg was found to be high compared to the epidermal mucus of freshwater fish *Caspian kutum* (Rutilus frisii kutum) and snakehead fish *Channa striatus* as well as hagfish *Myxine glutinosa* (Arulprakash et al., 2013; Bragadeeswaran et al., 2011; Heidari & Farzadfar, 2017). While the concentration of lysozyme in bioactive crude was lower than skin mucus LYZ activity of Indian major carps *C. catla* (Dash et al., 2014). The high (LYZ) activity in seawater species was similar to LYZ activity in the bioactive crude as previously

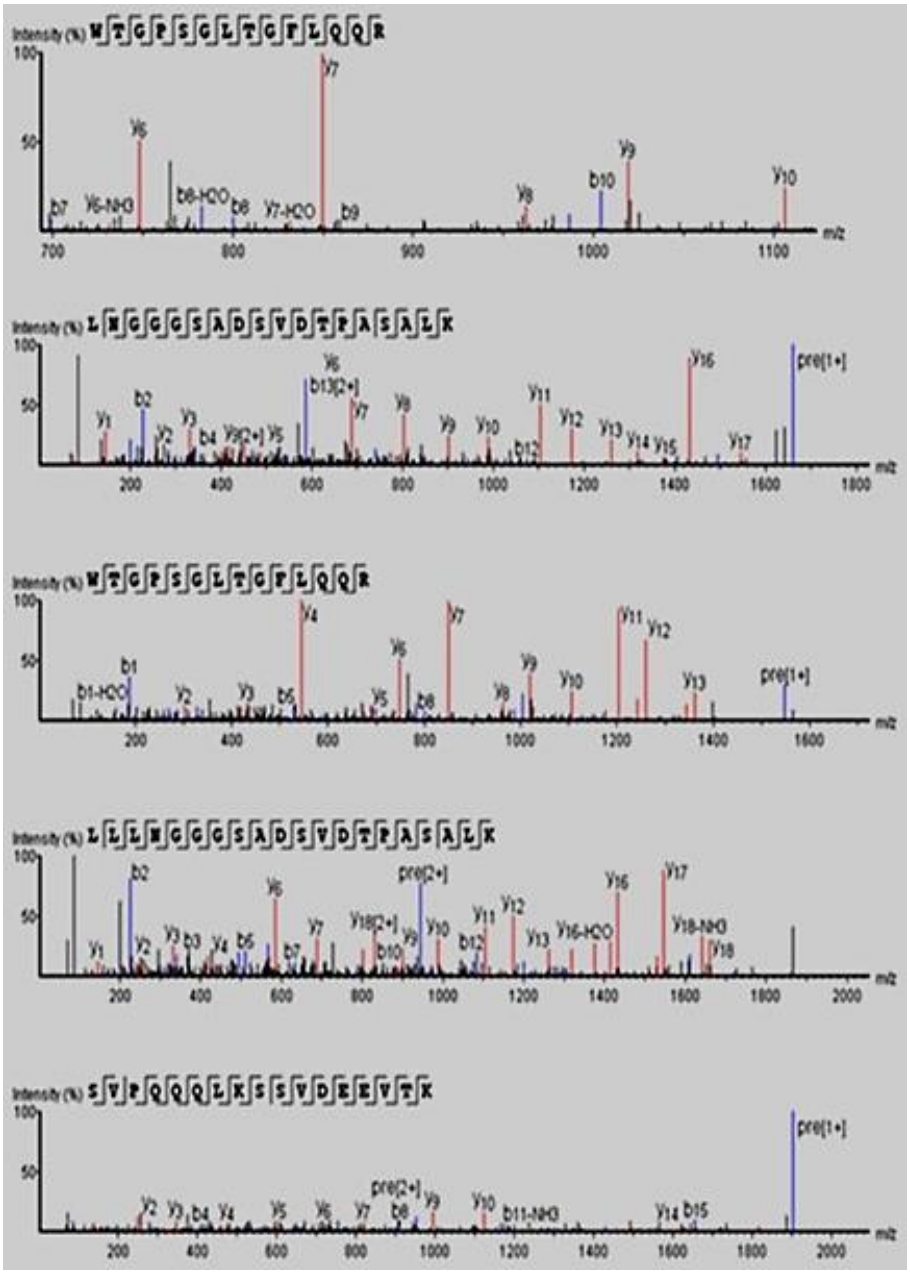

Figure 6. MS/MS spectrum of de novo peptides showing the fragmentation pattern of peptide WTGPSGLTGFLQQR, LLLNGGGSADSVDTPASALK, LNGGGSADSVDTPASALK and SVPQQQLKSSVDEEVTK

mentioned based on their species-specific evolutionary adaptation to different environmental conditions (Dash et al., 2014). This is likely because the fish an obligate air-breathing fish with the ability to walk on the ground for long periods, thus indicates skin resistance to external invasion (Kasi Marimuthu, Jeevanand Arumugam, 2009).

The precise function of ALP is that it act as a potential stress indicator. Elevation in the specific APL activity had been observed in fish epidermal mucus following the physical, chemical and microbial stress (Bezerra et al., 2014; Iger & Abraham, 1990, 1997). However, the activity of ALP in bioactive crude in this present study was lower than ALP in the skin mucus of freshwater snakehead fish *Channa striatus*. The disparity may be attributed to the characteristic pH optimum condition, broad substrate specificity or the storage effect (Cordero et al., 2016). Esterase in the skin mucus of fish may play a significant role in the innate immune response against pathogenic microorganisms (Bragadeeswaran et al., 2011). Bioactive crude extract obtained from climbing perch showed higher esterase activity than epidermal mucus of freshwater snakehead fish *Channa striatus* (Arulprakash et al., 2013). With regards to the antimicrobial proteins and peptides, elution fraction from Ultrafiltration 30 kDa exhibited more antibacterial activity on *P. aeruginosa* ATCC 10145 in comparison to the Superdex™ peptide 10/300 GL chromatography fraction, indicating possible synergistic action between protein components.

The LC-MS/MS in recent advances of proteomics research methods have been used for the identification and quantification of proteins in the mucus of fish (Cordero et al., 2016). Digestion fraction of Superdex™ peptide 10/300 GL was run through Q-TOF LC/MS, and a high number of suitable peptides were generated. Although, only the fragmented and matched information in the database of parameters UniProt, Anabantaria fasta sequence was selected. There is limited information about epidermal mucus and the epidermal protein of climbing perch (*Anabas testudenius*). This study was able to demonstrate for the first time that apolipoprotein A-1 was derived from epidermal mucus of climbing perch. It was matched with apolipoprotein A-1 of *Channa striata*. Previous reports indicate that the protein is up-regulated in the skin mucus of sea lice infected Atlantic salmon (Easy & Ross, 2009). Moreover, apolipoprotein A1 has also been identified in the skin mucus of naïve European sea bass (Rajan et al., 2011). Furthermore, proteomic analysis of the mucosal proteins of lumpsucker (*Cyclopterus lumpus*) revealed the presence of apolipoprotein A-1 and are thought to be involved in immune or stress responses (Patel & Brinchmann, 2017). In channel catfish, this protein also showed lytic activities against Gram-positive *Micrococcus lysodeikticus* and Gram-negative *Aeromonas hydrophila* (Pridgeon & Klesius, 2013). Additionally, studies have also reported a possible role of apolipoprotein A-I in enhancing the neutralization of other toxic bacterial products, such as lipoteichoic acid (Johnston et al., 2008). Other components of the crude mucus detected in this study are; hemoglobin subunit beta (Fragment) which was found to match with hemoglobin subunit beta (Fragment) of Channa *striata*. The identification of this fragment in the bioactive crude was novel and been reported for the first time. The protein corresponds to the haemoglobin B-chain was identified previously from *Monopterus albus*. haemoglobin, which is a cationic protein, which is made up of twin α and β globin subunits (α2 & β2) (Yi et al., 2017). In addition to their oxygen-carrying capacities, they also have immunological properties. However, many proteolytically generated hemoglobin-derived bioactive peptides have shown potent activities toward many pathogenic microorganisms (Yi et al., 2017). This study for the first time demonstrated Elongation factor 1-alpha in bioactive crude from epidermal mucus of climbing perch (*Anabas testudineus*). It is a member of the G protein family, and one of the four subunits that compose the eukaryotic elongation factor 1 involved in the regulation of peptide synthesis identified from European sea bass (*Dicentrarchus labrax*) (Infante et al., 2008). Moreover, cytochrome C oxidase subunit1 (Fragment) which has been previously recorded in the complete mitochondrial genome of the *Anabas testudineus,* including cytochrome c oxidase was also identified (H. Zhao et al., 2016). However, this was the first time to identify cytochrome C oxidase subunit1 in bioactive crude derived from epidermal mucus of climbing perch (*Anabas testudineus*). However, cytochrome C oxidase subunit1 (Fragment) has been earlier identified in the skin mucus of gilthead seabream (*Sparus aurata*) (Pérez-Sánchez et al., 2017).

β-actin is a dynamic and structural protein that plays important roles during phagocytosis and cell motility cytokinesis and cytoplasmic streaming (Brinchmann, 2016; Patel & Brinchmann, 2017). This study also demonstrated Beta-actin (Fragment) in bioactive crude from epidermal mucus of climbing perch (*Anabas testudineus*) and the protein matched with Beta-actin (Fragment) of *Channa argus* x *Channa maculate* and *Channa punctate*. Similarly, previous studies have shown that actin fragments have been identified in the skin mucus of sea lice (*Lepeophtheirus salmonis*), infected Atlantic salmon (*Salmo salar*) and in the skin mucus of lumpsucker (*Cyclopterus lumpus*).

Peptides with a negative charge are potentially anionic antimicrobial peptides as reported by Harris *et al*. (Harris et al., 2009). Some of these peptides were used as metal ions to form cationic salt bridges to interact with the negative charge of bacteria. The *de novo* peptide AAGPKGPLGPR identified in this study was selected among other peptides predicted to be antibacterial peptides depending on its net charge (+2) and low residues as well as its hydrophobic ratio (27%). Most of the identified AMPs are relatively short comprising of about 10-50 amino acid residues. They generally have an overall positive charge ranging from +2 to +11 and contain a substantial proportion of hydrophobic residues (Hancock & Sahl, 2006; Pasupuleti et al., 2012; Yeaman & Yount, 2003). Amino acid composition of AAGPKGPLGPR peptide was low - Ala - Gly - Pro - Lys - Gly - Pro - Leu - Gly - Pro - Arg) rich in Proline 27% and Gly 27%. Unlike other forms of AMPs, Proline-rich antimicrobial peptides (PR-AMPs) act differently via a distinctive 'non-lytic' mechanisms. They penetrate microbial membranes without disrupting the membrane integrity, and then bind to and interact with the specific intracellular targets (Cao et al., 2015).

Similar to the findings in this study, antimicrobial peptides have been demonstrated previously from *Misgurnus anguillicaudatus*, *Pardachirus marmoratus* and *Pardachirus pavoninus* (Lazarovici et al., 1986; Liang et al., 2011). This may be because they are all teleost fish characterized by a labyrinth organ that allows them to breath air while outside their aquatic environment as well as the adaptation to travel across land especially when their habitat becomes inhospitable. The mucus layers of these fishes are composed of many enzymes and antimicrobial proteins, which are thought to be involved in the innate immunity of the fish (Haniffa et al., 2014). The components of the mucus are also thought to function in the cytoplasm against intracellular pathogens in addition to many proteins that exert strong resistance to invading pathogens, including the lipoproteins A-1 observed in the present study (Concha et al., 2003).

Conclusion

This study provides for first time proteomic analysis of bioactive crude derived from epidermal mucus of climbing perch. Apolipoprotein A-1, and haemoglobin subunit beta were the major proteins detected from the mucus extract, in addition to de novo peptides derived from crude were detected. Other identified proteins, including Beta-actin, Elongation factor 1-alpha and cytochrome c-oxidase subunit1. These results emphasize their role in protecting climbing perch from foreign invaders as a part of the innate immune system throughout the epidermal mucus. A high-resolution mass spectrometry-based proteomic approach was able to identify proteins in the crude extract. These outcomes may serve an opening to further investigations to determine the potential medical application of bioactive proteins and peptides in crude in the development of protection assays as well as recombinant protein fractions that may be suitable in developing diagnostic kits for future use.

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