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Research article

Mucus protein composition of wild *Channa striatus* (Bloch, 1793) (Perciformes: Channidae) from Peninsular Malaysia

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Abstract

Several wild Snakehead fish or *Channa striatus,* with total length and weight ranging between 24-36 cm and 130-210 g were collected from paddy field at Kampung Selinsing, Perak. From Bradford assay, the total protein concentration of epidermal mucus was determined as 2.45 mg/ml. Subsequently, the samples were fractionated and further analysed by using LC-MS/MS. From UniProt/SwissProt and UniProt/TrEMBL databases, 192 and 157 proteins were identified. The foremost protein detected by UniProt/SwissProt database was keratins (21%), followed by histones (16%), 14-3-3 proteins (13%), calmodulins (11%), ras-related proteins (10%), creatine kinases (8%), actins (6%) and other proteins (15%). On the other hand, the major protein perceived from UniProt/TrEMBL database was uncharacterized proteins (43%), followed by histones (21%), 60S ribosomal (8%), actins (6%), 40S ribosomal (5%), glycolysis related proteins (5%) and other proteins (12%). Based on the types of protein, structural proteins (38%) was the main protein identified by UniProt/SwissProt database, followed by regulatory (26%), enzymatic/catalytic (14%), calcium-binding/transport (11%), ribosomal (6%) and contractile proteins (5%). Whereas, protein with unknown function (43%) was the major protein detected by UniProt/TrEMBL database. This followed by structural proteins (23%), ribosomals (13%), enzymes (6%), contractiles (6%), regulatory (4%), antioxidants (3%), and calcium-binding protein (2%). From this study, a wide range of proteins with various functions were detected within the mucus of wild *Channa striatus,* which needs further studies and exploration.

Introduction

Seven species of fish from genus *Channa*, including *C. bankanensis, C. gachua, C. lucius, C. marulioides, C. melasoma, C. micropeltes* and *C. striatus* can be encountered in Peninsular Malaysia [1]. The common snakehead fish or *C. striatus* is ubiquitos and living in various type of waterway such as ponds, lakes, streams, drains [2-3], small ditches, rice fields and rivers [4]. This medium to large-sized of fish can attained a total length up to 75 cm. However, the common size of this fish is between 30 and 40 cm [3]. It is native to Bangladesh, Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Pakistan, Sri Langka, Thailand and Vietnam [3]. Morphologically, the body of *C. striatus* is cylindrical, having elongated dorsal, anal fins supported by rays, plate like scales on head and a round caudal fin. The coloration of upper surface is greenish brown, to almost black with an oblique stripe [5-6], and has a white belly [2]. This obligate air-breathing fish preferring shallow and slow-moving water, temperature between 20-30ºC, good level of dissolved oxygen and a low turbidity [7].

In Malaysia, several fish species, such as *C. micropeltes, C. striatus, C. gachua, Periophtalmus sp.* (Mudskipper) and *Monopterus albus* (Swampy eel) are known to be used in Malay traditional medicine [7-8]. Because of the highly resources of medicinal properties, *C. striatus* have attracted numerous scientists to study on it. Works by Mat Jais *et al.* [9-10], documented the amino acid profile of *C. striatus* fillets, which riches in glycine residue, and also recorded antinociceptive activity in *C. striatus*. Other studies on *C. striatus*, included Baie and Sheikh [11-12] on wound healing properties, Mat Jais *et al.* [13] on antifungal activities, Dhanaraj *et al.* [14] on antibacterial activity and Dahlan-Daud *et al.* [15] on amino and fatty acids composition. The medicinal property of this fish is suitable for post-surgical, convalescence and arthritis patients [16-17]. It also had a potential to be used as wound healer, pain reliever, energy booster, ACE-inhibitor, anti-depressant and neuroregenerative agent [7].

Recently, Kwan *et al.* [18] has identified 137 and 194 proteins from spray and freeze-dried water extract samples of *C. striatus*. Several protein molecules such as actin, myosin, tropomyosin, calcium ion-related protein and collagen were detected, which suspected to be involved in the wound healing process. Kwan *et al.* [19] also performed a comprehensive proteomic profiling of *C. striatus* meat, by using high sensitivity liquid chromatography, and detected 75 proteins. These included several potential proteins for wound healing process, such as structural proteins, enzymes, calcium related proteins and collagen. These findings revealed the rich of bioactive compounds in *C. striatus*, which lead to various pharmacological effects, including the wound healing process. In this study, the epidermal mucus of wild *C. striatus* were extracted, and its protein compounds and functions were determined.

Materials and methods

Collection of fishes and epidermal mucus

Several medium-sized of adults *Channa striatus* were collected from paddy field and irrigation channel, at Kampung Selinsing, Perak, Peninsular Malaysia (4º55'N, 100º33'E; elevation 22 m a.s.l.). After captured the fishes were placed in the water tank and brought back to the laboratory. In the laboratory the fishes were transfer to another water tank and maintained for 24 hours before the extraction. Prior extraction the fishes were rinsed with distilled water and placed into the plastic containers. Their epidermal mucus was collected by gently massages throughout its body with hand. To avoid contamination, the cloacal areas of the fishes were not massage. The collected epidermal mucus was stored into several 50 mL Falcon tubes, and kept in the freezer (-35ºC), prior to further analysis. The weight and body length of the fishes were measured by using electronic balance and digital calliper.

Freeze drying

After overnight, the frozen mucus was transferred into freeze-dry machine (Labconco) and ran for 24 hours. The optimal temperature and vacuum condition were -47ºC and 0.025 mBar, respectively. Fifteen milligrams of freeze-dried mucus were added into 1 mL of 40 mM Tris-HCl (pH 8.8) extraction buffer and leaved for 20 minutes in room temperature with occasionally vortex. Afterwards, the mixture samples were centrifuged at 12,000 x g for 30 minutes, collected its supernatants, and placed at -35ºC.

Bradford assay

The total protein concentration of the mucus was quantified by using Bradford assay [20]. Five microliters of supernatant were mixed with 250 µL Bradford reagent in a 96-well plate and incubated at ambient temperature for 15 minutes. A standard curve ranging from 0-2.0 mg/mL was constructed with absorbance at 595 nm. The total protein concentration of each sample was determined and averaged by comparing the absorbance value against the standard curve.

Protein fractionation

This process was conducted by using Gelfree 8100 fractionation system (Expedeon, CA, USA) according to the protocol provided by Witkowski and Harkins [21]. Two hundred micrograms of sample were loaded to 10% Tris-Acetate cartridge. Twelve fractions were collected and subsequently concentrated by using a vacuum concentrator.

Protein digestion

Procedure for protein digestion was carried out following the methodology developed by Kinter and Sherman [22]. The samples were re-suspended in 100 µL of 6 M urea and 100 mM Tris buffer at 10 mg/mL. 200 mM DTT was added to each sample and kept in room temperature for an hour. Later, 200 mM of iodoacetamide was added and incubated at room temperature for an hour, followed by the excess of DTT to consume unreacted iodoacetamide. The concentration of urea in the sample was reduced by adding 775 µL of water. For digestion purpose, 20 µg trypsin solution (Promega, WI, USA) was added to each sample and incubated overnight at 37ºC. The digestion process was ended on the next day by adjusting the pH of the buffer to pH < 6.

LC-MS/MS analysis

Each sample was mixed with 100 µL of 0.1% formic acid in deionized water and filtered using 0.45 µm regenerated cellulose (RC) membrane syringe filter (Sartorius AG, Goettingen, Germany). The analysis was carried out by using LTQ-Orbitrap Velos Pro mass spectrometer, coupled with Easy-nLC II nano liquid chromatography system. Easy column C18 (10 cm, 0.75 mm i.d., 3 µm; Thermo Scientific, San Jose, CA, USA) was used as the analytical column, whereas Easy column C18 (2 cm, 0.1 mm i.d., 5 µm; Thermo Scientific, San Jose, CA, USA) was used as the pre-column. Analytical column was equilibrated at the flow rate of 0.3 µL/min for 4 µL, while the pre-column at 3 µL/min for 15 µL. Later, 5 µL of samples were injected and chromatographically separated at the flow rate of 0.3 µL/min. Two running buffers were used: (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile. Samples were eluted using the gradient 5% to 100% of buffer B in 80 minutes. The eluent was sprayed into the mass spectrometer at 2.1 kV (voltage source), and the capillary temperature was set up at 220ºC. Protein and peptides were detected by full scan mass analysis from m/z 300-2,000 at resolving power of 60,000 (at m/z 400, FWHM; 1-s acquisition). Data-dependent MS/MS analyses (ITMS) triggered by the

8 most abundant ions from the parent mass list of predicted peptides, with rejection or unassigned charge state. Collision-induced dissociation (CID) was applied as the fragmentation technique with a collision energy of 35. For each sample it was analysed twice.

Protein and peptide identification (De Novo Sequencing)

Software PEAKS Studio Version 7 (Bioinformatics Solution, Waterloo Canada) was used to perform *de novo* sequencing and database matching. National Centre for Biotechnology Information (NCBI) fish database from October 2014 was used as database matching. Parent mass and precursor mass tolerance were set at 0.1 Da. False detection rate (FDR) < 0.1% and significant score (-10lgP) for protein > 30 were used for protein acceptance. Minimum unique peptide was set at 1, while maximum variable post-translational modification was set at 4.

Results

Several adults of *Channa striatus* ranging between 24-36 cm in length and 130-210 g weight were collected from its natural habitats, the paddy fields at Kampung Selinsing, Perak. Beside the paddy fields, the fishes were also collected from the ditches and irrigation canals nearby. Based on the seven-point calibration curve, ranging from 0-2 mg/mL ($y=0.2959x + 0.0478$; $R^2 = 0.9579$), the total protein concentration of *C. striatus* epidermal mucus was determined as 2.45 mg/mL (Figure 1).

The epidermal mucus protein of *C. striatus* has been identified by using Uniprot/Swisprot and Uniprot/TrEMBL databases, which detected 192 and 157 proteins, respectively. Out of 192 proteins detected by UniProt/SwissProt database, 40 were keratins (21%), followed by 31 histones (16%), 25 14-3-3 proteins (13%), 21 calmodulins (11%), 19 ras-related proteins (10%), 15 creatine kinases (8%), 12 actins (6%), 7 peroxiredoxins (4%), 7 40S ribosomal proteins (4%), 6 60S ribosomal proteins (3%) and 4 gradient proteins (2%). Other proteins, including trypsin, serum albumin, glycolysis related protein, fatty acid binding protein and ATP-dependent RNA helicase contributed 2% (Figure 2). From 157 proteins detected by UniProt/TrEMBL database, 68 were uncharacterized proteins (43%), followed by 33 histones (21%), 13 60S ribosomal proteins (8%), 9 actins (6%), 8 40S ribosomal proteins (5%), 8 glycolysis related proteins (5%), 5 natural killer cell enhancing factors (3%), 5 ras-related protein (3%) and 4 calcium ion related proteins (2%). Other proteins, including keratin, annexin, trypsinogen and creatine contributed 4% (Figure 3).

Based on types of protein, 73 structural proteins (38%), followed by 49 regulatory (26%), 27 catalytic/enzymatic (14%), 22 calcium-binding/transport (11%), 12 ribosomals (6%) and 9 contractile proteins (5%) were perceived from UniProt/SwissProt database (Figure 4). On the other hand, 68 unknown proteins (43%), followed by 36 structural proteins (23%), 20 ribosomals (13%), 10 enzymes (6%), 9 contractiles (6%), 7 regulatory (4%), 4 antioxidants (3%), and 3 calcium-binding (2%) were detected from UniProt/TrEMBL database (Figure 5).

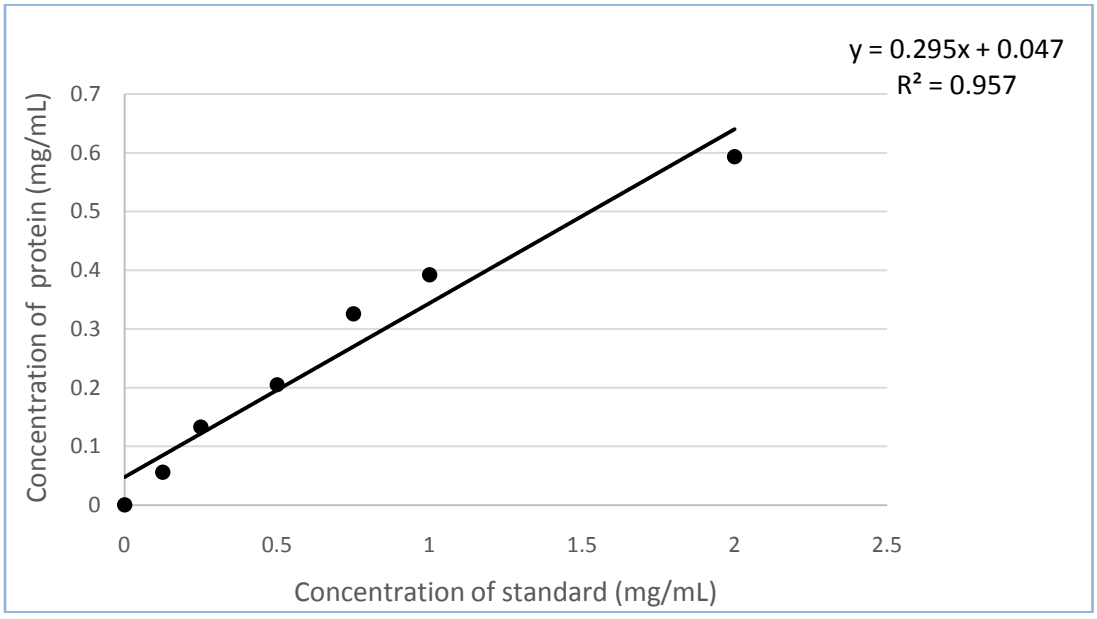

Figure 1. The seven-point calibration curve (protein concentration of *C. striatus* = 2.45 mg/mL).

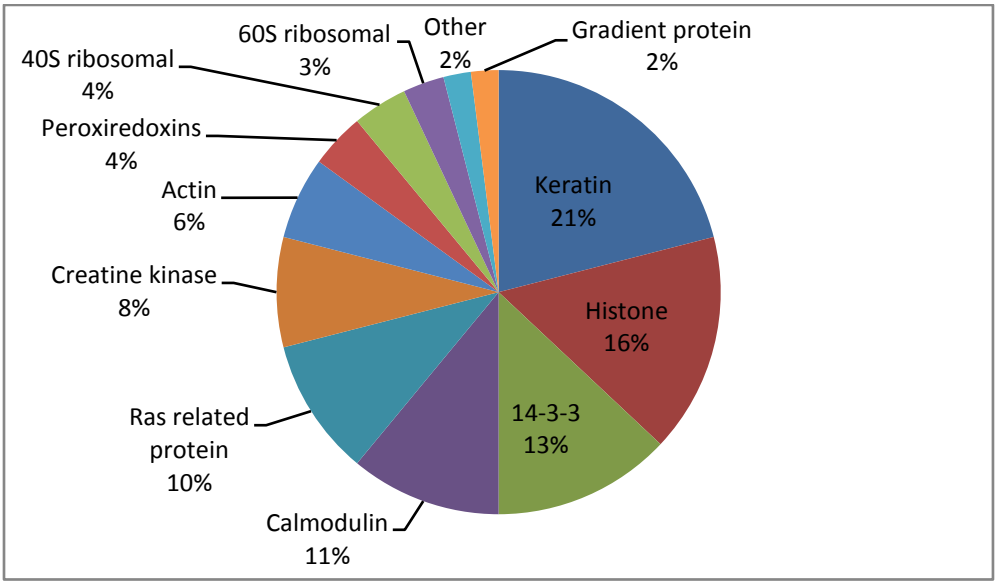

Figure 2. Proteins obtained from Swissprot database.

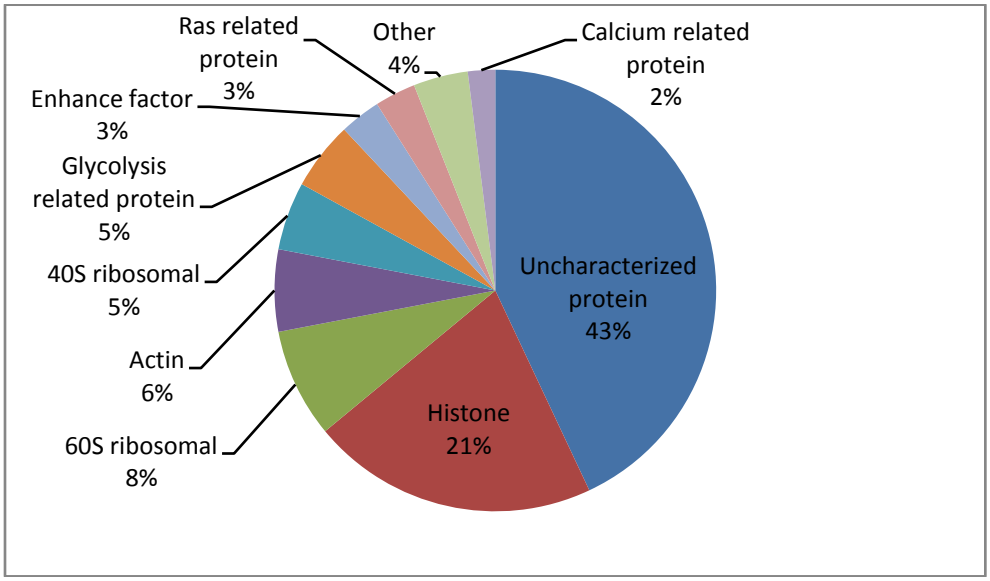

Figure 3. Proteins obtained from trEMBL database.

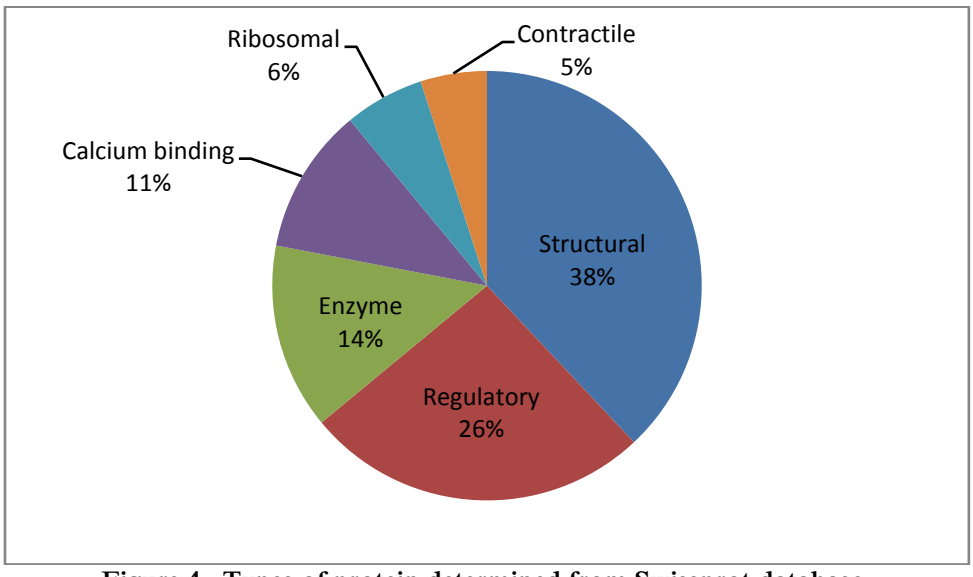

Figure 4. Types of protein determined from Swissprot database.

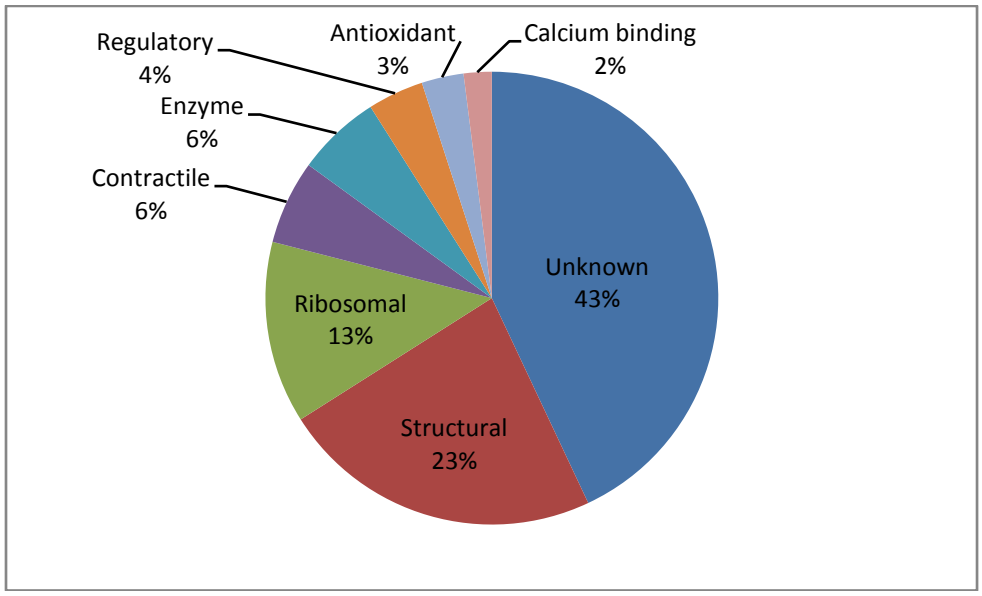

Figure 5. Types of protein determined from trEMBL database.

Discussion

Channa striatus or locally known as Haruan living in various type of water bodies, including streams, lakes, swampy areas, ditches, irrigation channels and paddy fields, thus exposing its body to numerous types of pathogenic microorganisms. For a protection purposes, its epidermal surface produced a slippery and slimy fluid called mucus. Beside a protection, this mucus exerted a variety of functions, including respiration, ionic and osmotic regulation, reproduction, excretion, disease resistance, communication, feeding and nest building [23].

Various regulatory proteins, such as 14-3-3 proteins, ras-related protein, transforming proteins, rho-related GTP binding, anterior gradient, ras-liked GTP binding and serum albumin were detected within the epidermal mucus of *C. striatus*. Among these, 14-3-3 (13%) was the most abundance regulatory protein detected. This type of protein can be found in all eukaryotic organisms and often formed in multiple isoforms (alpha, beta, gamma and zeta). It involved in many process, including signal transduction, cell cycle regulation, apoptosis, stress responses and malignant transformation [24]. Researched by Mori *et al.* [25] found that 14-3-3 protein consist of antimicrobial properties and involved in secretion of antibacterial proteins. Study by Wei *et al.* [26] showed the acidic mucus extracted of *C. striatus* can inhibited the growth of three human pathogens, including *Bacillus subtilis, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Whereas, the aqueous mucus extracted can only inhibited the growth of fish pathogen, *Aeromonas hydrophila*. Hence the abundance of 14-3-3 proteins present in the mucus of *C. striatus* may be the key components of antimicrobial properties, which protected the fishes from pathogenic microorganisms.

Structural proteins were the most abundance protein encountered from the epidermal mucus of *C. striatus*. These included keratins, histones, filaments and intermediate filaments. Keratin (21%) was the most abundance, produced by keratinocytes and formed the basic cell structure of epidermis. Its play roles in mechanical stability and regulatory functions and involved in intracellular signalling pathways such as wound healing and apoptosis process [27]. In human, keratin is critical for regulating wound healing, by assist in re-epithelialisation process. Keratin-based products had been shown to enhance the activation of keratinocytes, increasing migration and cell proliferation rates [28]. Thus, the presence of keratin in the mucus of *C. striatus* may contribute to the wound healing process.

Calmodulin (11%) is a calcium ions-binding protein, which also found within the mucus of *C. striatus*. The function of this protein is to modulate the concentration of cytosolic calcium ions. In human, the concentration of cytosolic calcium ions will affect the biological processes, including muscle contraction, cell proliferations and apoptosis [29]. During disruption of plasma membrance, calcium ions transported by calmodulin will enter the site, and trigger the local fusion of internal membrances to repair the wound [30]. Similar with keratin, the finding of calmodulin molecules in the mucus of *C. striatus* may assist in wound healing process.

Actin (6%) was the foremost contractile protein detected within the mucus of *C. striatus*. The actin is important in many cellular functions, including cell motility, maintenance the cell shape and regulation of transcription [31]. According to Cowin [32], actin is an essential network of filaments, found in all cells, and contributed in migration, adhesion and proliferation process. These processes are fundamental in wound healing progression. Cowin [32] also identified actin as a pivotal, for foetal

scar-free wound healing, by a process of regeneration. The presence of actin may help in improving wound healing process and reduce scar formation.

The discovery of many uncharacterized proteins with unknown function, may indicated the existing of many novel proteins within the mucus of *C. striatus*. These types of protein need further studies to determine its roles and functions. Perhaps one or more uncharacterized proteins, which detected in this study may have antimicrobial properties or involved in wound healing process.

Conclusion

Channa striatus is a common freshwater fish found in the water bodies of Peninsular Malaysia. The epidermal mucus of this fish consists many valuable protein molecules. From LC-MS/MS analysis, 192 and 157 protein molecules were detected by Uniprot/Swisprot and Uniprot/TrEMBL database, respectively. These including keratin, calmodulin and actin, which involved in wound healing process and 14-3-3 protein which have antimicrobial properties.

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