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Short Communication: Protein profiles of Giant Mudskipper and its potential use as biomarker candidate for heavy metal contamination in Barito Estuary, Indonesia

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Abstract. *Hidayaturrahmah, Mabrur, Santoso HB, Sasmita R, Rahmy USA, Badruzsaufari. 2019. Short Communication: Protein profiles of Giant Mudskipper and its potential use as biomarker candidate for heavy metal contamination in Barito Estuary, Indonesia. Biodiversitas 20: 745-753.* Giant mudskipper (*Periphthalmodon schlosseri*, Pallas 1770) is one of important biological diversity that potential as bio-indicator in environment assessment which is heavy metal contamination. These fish are susceptible to ambient environment, that effect to their physiological mechanism including protein synthesis and expression. This research aims to identify protein profiles of giant mudskipper muscle and review its potential application as a biomarker for heavy metal contamination in Barito Estuary of South Kalimantan. Total proteins were isolated from fish muscle using Tris EDTA buffer and then precipitated using Ammonium sulfate salt. The protein concentration was measured using Bradford assay and then separated based on molecular weight using the SDS-PAGE method. The result showed muscle proteins of giant mudskipper could be precipitated on optimum condition at Ammonium sulfate saturation 60-70% which protein concentration 5.106 $mg.mL^{-1}$. The protein separated into 20 bands for raw and precipitated proteins which molecular weight ranging from 33 into 184 kDa. Further study is needed for protein identification based on amino acids sequence of protein bands for biomarker discovery and validation.

Keywords: Biomarker, Bradford, *Periophthalmodon schlosseri*, protein, SDS-PAGE

INTRODUCTION

Periopthalmodon schlosseri or popular as giant mudskipper (Gobiidae: Oxudercinae) is one of amphibious fish that live in a coastal area of Indonesia. This fish is commonly found on muddy shores of intertidal habitat, estuaries or mangrove ecosystem. Previously, this fish is reported to occur in Australia, Straits of Malacca (Singapore and Peninsular Malaysia), Sarawak, Thailand, Indonesia (Murdy 1989), India and New Guinea (Weber and De Beaufort 1953). In Indonesia, giant mudskipper reported in Bangka, Sumatra, Java, Jakarta, Madura, Sulawesi, Ambon, Waigeu (West Papua) and Kalimantan islands (Weber and De Beaufort 1953). Hidayaturrahmah and Muhamat (2013) reported the giant mudskipper commonly found in the coastal area around Barito Estuary of South Kalimantan.

Periophthalmodon schlosseri are uniquely adapted to a completely amphibious lifestyle (Graham 1997; Bob-Manuel 2011). This fish is a euryhaline, which able to adapt to a wide range of salinity due to tides (Lauriano et al. 2018). They are obligate air-breathing, which stores air in a vascularized orobranchial cavity containing reduced gills. The gills are only ventilated with water when the fish expires the air from its orobranchial cavity (Taylor et al. 2010). They are live and preserved individuals, quiet and active when out of the water, feeding, interacting with others and defending their territories (Jaafar et al. 2006; Bob-Manuel 2011). The fish also spend over 90% time out of the water, in the mudflats (Lauriano et al. 2018). This carnivorous fish can grow up to 27 cm in length (Ip et al. 2004a; Ip et al. 2004b). Based on their standard length, "giant mudskipper" refer to *Pn. schlosseri* as the largest species among Oxudercinae. Zulkifli et al. (2012) reported that the giant mudskipper food are fiddler crabs (*Uca* sp.), medaka fish (*Oryzias* sp.) and other fish.

Economic development in the coastal area has led to the heavy metal contamination on the aquatic environments and its accumulation in sediments or living organisms into a toxic level (Abdel-Baki et al. 2013). The response of heavy metal exposure was varied among the fish or even in a different organism. In fish, heavy metal exposure resulted in significant mortality, morphological anomalies, developmental retardation, and pathological changes in brain, heart, cranial or caudal kidney. The activity of antioxidant enzymes always changes as the effect of heavy metal exposure (Sehonova et al. 2018). Moreover, heavy metal exposure affected the histopathological changes in fish, including gills, liver, hemolymph organ and digestive gland (Pagano et al. 2017). Various tissue damages were reported, such as hyperplasia, erythrocyte infiltration, hypertrophy, lamellar fusion, epithelial lifting, tissue lesions and membrane destabilization (Vajargah et al. 2018 and Aliko et al. 2015). In addition, heavy metal

accumulation in fish tissue triggers oxidative stress through reactive oxygen species (ROS) overproduction leading to structural damage of proteins and DNA, which can be associated with mutations, chromosomal, and carcinogenesis (Burgos-Aceves et al. 2018).

Heavy metals are naturally found in soil and originated from the soil. Human activities including urbanization, industrialization, agriculture, and mining are contributed to heavy metal accumulation in aquatic ecosystems including in Barito Estuary of South Kalimantan (Zhuo and Guo 2015). Dwiyitno et al. (2008) reported in April and September 2005, some heavy metals including Hg, Pb, Cd, and Cu has been found in water, sediment and fish living in Barito Estuary, but still under maximum concentration. In 2009, Hg and Pb content on water has been exceeding the maximum concentration, but even under maximum level on sediment (Sofarini et al. 2010). Several researchers demonstrated that the giant mudskipper fish can accumulate and regulate heavy metals (Cu, Zn, Pb, Cd, and Ni) in its tissues: scale, bone, operculum, intestine, liver, and cartilage (Buhari and Ismail 2016). In addition, the fish can accumulate heavy metals (Cu, Zn, Pb, Cd, and Ni) on their muscle, but lower than liver, intestine, and cartilage (Buhari and Ismail 2017).

Ansari et al. (2014) suggested that the giant mudskipper has been important as bio-indicator for environmental monitoring and assessment, including heavy metal concentration. These fish are susceptible to ambient environment, that affects their physiological response especially protein synthesis and expression. Previous research reported some proteins from fish tissue that important for heavy metal regulation and detoxification. Therefore, a proteomic study is needed for heavy metal biomarker development. This study aimed to identify protein profiles of giant mudskipper muscle and review its potential application as a biomarker candidate for heavy metal contamination in Barito Estuary of South Kalimantan.

MATERIALS AND METHODS

Sample collection

The fish sample was collected from the tidal area of Kuala Lupak, District of Barito Kuala, South Kalimantan, Indonesia (Fig. 1 and Fig. 2). The study site was located not far from the Barito Estuary, which affected by several human activities including coal transportation, Trisakti port, and other activities. The fish were fished with fresh shrimp as bait and then transported into Laboratory of Biology, Universitas Lambung Mangkurat in Banjarbaru. The body weight and length of giant mudskipper were measured. The muscle was separated from the bone, digestive tract, gills, and other organs, and stored at -20 ^{0}C for protein profiles analysis.

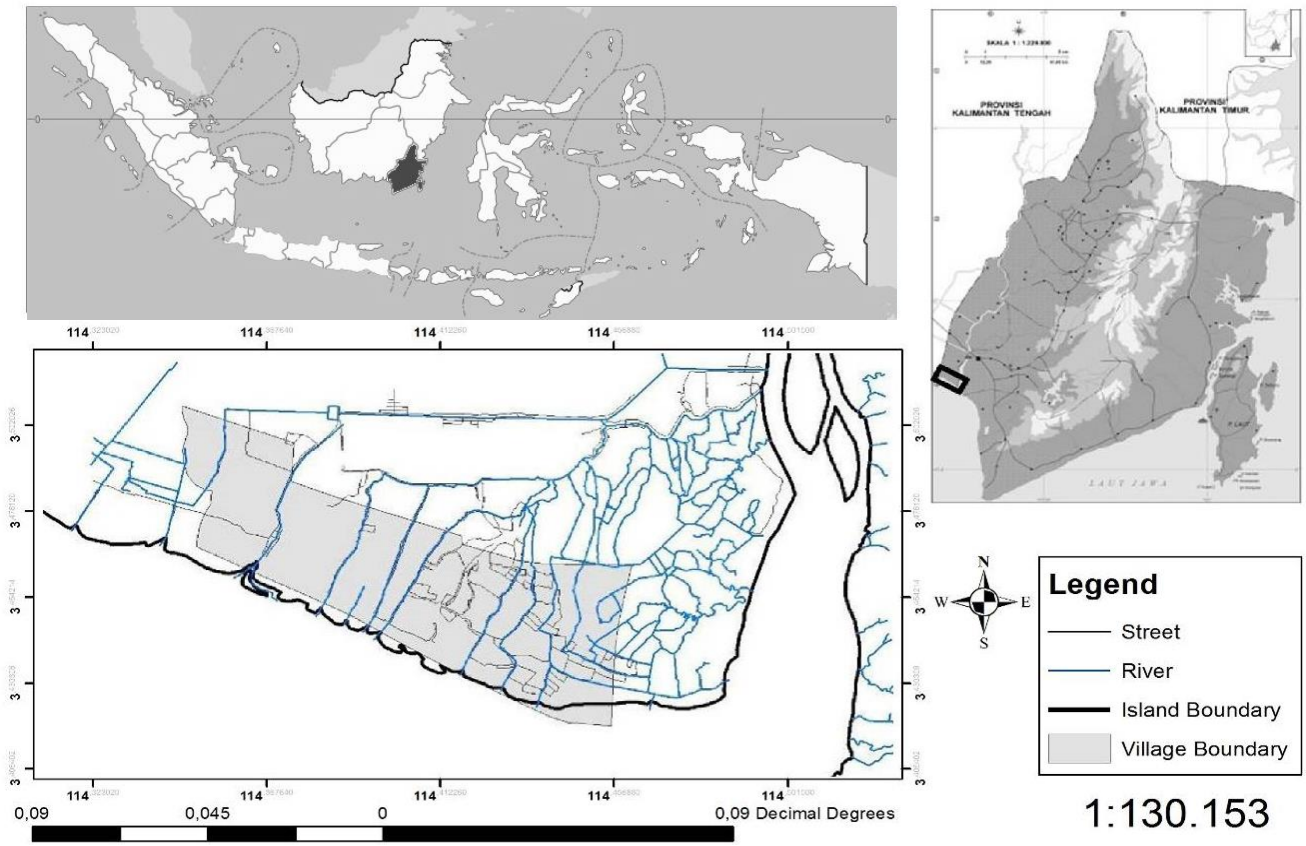

Figure 1. Location of a sample collection of *Periophthalmodon schlosseri* in Kuala Lupak, Barito Estuary, South Kalimantan Province, Indonesia

Figure 2. Location of sample collection A. intertidal ecosystem of Kuala Lupak B. nest of the giant mudskipper (←) *Peripopthalmodon schlosseri*

Protein extraction

The fish muscle was homogenized with Tris EDTA buffer (0.5 M Tris on pH 8.3 and 1 mM EDTA) with tissue: buffer ratio was 1: 3 (w/v) and then centrifuged on 10.000 g for 10 minutes at 4ºC. The supernatant was collected and stored in the freezer at -20ºC (Dekic et al. 2016).

Protein precipitation using ammonium sulfate

Protein supernatant was precipitated according to Burgess (2009) using different Ammonium Sulfate (AS) saturation (from 20% to 80%) at the temperature 0ºC. The AS was added on initial saturation (20, 30, 40, 50, 60 and 70%), and then incubated for 30 minutes at 0ºC. The protein-AS solution than centrifuged on 15.000 g for 15 minutes at 4ºC. After the centrifugation, the AS was added to raise the saturation into 30, 40, 50, 60, 70, and 80% from initial saturation. The protein-AS solution was incubated for 30 minutes at 0ºC and centrifuged on 15.000 g for 15 minutes at 4ºC. After the centrifugation, supernatants were discarded and pellet collected. The pellet was suspended with Tris HCl 10 mM pH 8,0 for further analysis.

Bradford assay

Protein content was analyzed based on Bradford Assay (1976) using Bradford reagent (Amresco, USA). The assay was carried out according to the manual, which used microplate. The absorbance of protein was measured at 595 nm using Spectrostar Nano Spechtofotometer (BMG Labtech). The concentration of protein was determined based on a linear regression fit of Bovine Serum Albumin (HiMedia) as the standard protein.

Electrophoresis and staining

SDS-PAGE was performed according to Laemmli (1970). Protein bands were separated on 1 mm thick slab containing 10% polyacrylamide in separating gel and 5% in stacking gel using Maxi Vertical Gel Electrophoresis Apparatus – MV 20 DSYS (Major Science).

Separating gel contained a mixture of 14.8 mL of distilled water, 11.75 mL of 30% acrylamide solution (29.2 gr acrylamide and 0.2 gr N.N'-methylene-bis-acrylamide in 100 mL distilled water), 8.8 mL of 4X Resolving Tris Solution (1.5 M Tris HCl on pH 8.8 and 0.4% SDS), 350 µL of 10% Ammonium Persulfate (APS) (0.1 gr APS in 1 mL distilled water) and 70 µL of N,N,N',N'-tetra-methyl-ethylene-diamine (TEMED). The solution was poured into a 1 mm thick slab up to ¾ of plate height, and isopropanol was added to avoid bubble from the gel, and sit for 15 minutes until acrylamide gel was formed. Isopropanol is removed from separating gel and stacking gel solution was added.

Stacking gel contained a mixture of 3.05 mL of distilled water, 0.65 mL of 30% acrylamide solution, 1.25 mL of 4X Stacking Solution (0.5 M Tris HCl on pH 6.8 and 0.4% SDS), 30 µL of 10% APS and 10 µL of TEMED. The solution was poured into the slab and then put the comb to make wells for sample loading, and sit for 30 minutes until acrylamide gel was formed.

Running. Protein sample was mixed with 4X Laemmli sample buffer with protein: buffer ratio 1: 4 (v/v). The solution was heated on a water bath for 5 minutes at 70ºC. After heating the solution loaded into gel and electrophoresis was running at 120 voltage, 40 mA for 3 hours. Chromatein Prestained Protein Ladder (Vivantis) were used as protein ladder to determine the molecular weight of giant mudskipper protein.

Staining. Gel was stained in staining solution (1.25 gr of Coomassie Brilliant Blue R-250 in 225 mL methanol, 50 mL glacial acetic acid and 225 mL distilled water) for 30 minutes on an orbital shaker. After the staining process, the gel was destained to rinse dye excess with destaining solution (225 mL methanol, 50 mL glacial acetic acid and 225 mL distilled water) for 30 minutes and replicate overnight.

Data analysis

The molecular weight of separated bands was determined based on linear regression fit between Relative mobility value (Rf) and log molecular weight of the protein ladder. Rf was measured based on the equation (Haniffa et al. 2017):

$$Rf = \frac{\text{protein migration distance (cm)}}{\text{tracking dye distance (cm)}}$$

RESULTS AND DISCUSSION

Sample collection and identification

Periophthalmodon schlosseri that collected on this research has been identified and confirmed based on their morphological character (Fig. 3) and identification key according to Murdy (1989). The species has a black longitudinal strike on the upper body, nostril position on the posterior body, color pattern and shape of the first fin and lip morphology. According to Jaafar et al. (2006), the color pattern of *Pn. schlosseri* dorsal fin is not different from *Periophthalmus walailakae* with dark brown or black and white distal margin. However, caudal fin of *Ps. walailakae* is marked with black spots. At the ventral view, the upper lip of *Pn. schlosseri* was continuous, differ to *Ps. walailakae* which is constricted at the two points. *Periophthalmodon* and *Periophthalmus* significantly different in having a row of teeth in the upper jaw (Murdy 1989).

The coloration of this fish uniformly yellow or buff and blue spot are found on the flanks. Black longitudinal stripe runs uninterrupted from posterior margin of the eye, along with the upper part of each side of the body to the caudal base. A similar strip is found on *Ps. walailakae*, but the stripes are always interrupted, discontinuous and originate from behind the head (Jaafar et al. 2006). Dorsal fins blackish or dusky brown with pale margins and caudal fin blackish or dusky without black spots mark (Murdy 1989; Jaafar et al. 2006). Based on their morphological, *Pn. schlosseri* is sympatric with *Pn. septemradiatus* and allopatric with *Ps. walailakae* (Murdy 1989; Darumas and Tantichodok 2002).

Local people of Kuala Lupak know these fish as "Glodok" or "Timpakul", along with *Apocryptes, Pseudapocryptes, Parapocryptes, Zappa, Oxuderces, Scartelaos, Apocyptodon, Boleophthalmus* and *Periophthalmus*, *Periophthalmodon* has been classified as a subfamily of Oxudercinae (Gobiidae) (Murdy 1989). However, only seven genera (*Apocryptes, Pseudapocryptes, Zappa, Scartelaos, Boleophthalmus, Periophthalmus* and *Periophthalmodon*) belong to mudskipper, which has amphibious character and use their pelvic fin to walk on mudflats or land. Four genera of mudskipper including *Periophthalmodon, Periophthalmus, Boleophthalmus* and *Scartelaos* consisted of 25 air-breathing species, which can exchange gases directly with the aerial environment (Ansari et al. 2014). *Periophthalmodon* is sister taxa with *Periophthalmus* and consisted of three species which is *Pn. schlosseri, Pn. septemradiatus,* and *Pn. freycineti* (Murdy 1989; Polgar et al. 2010).

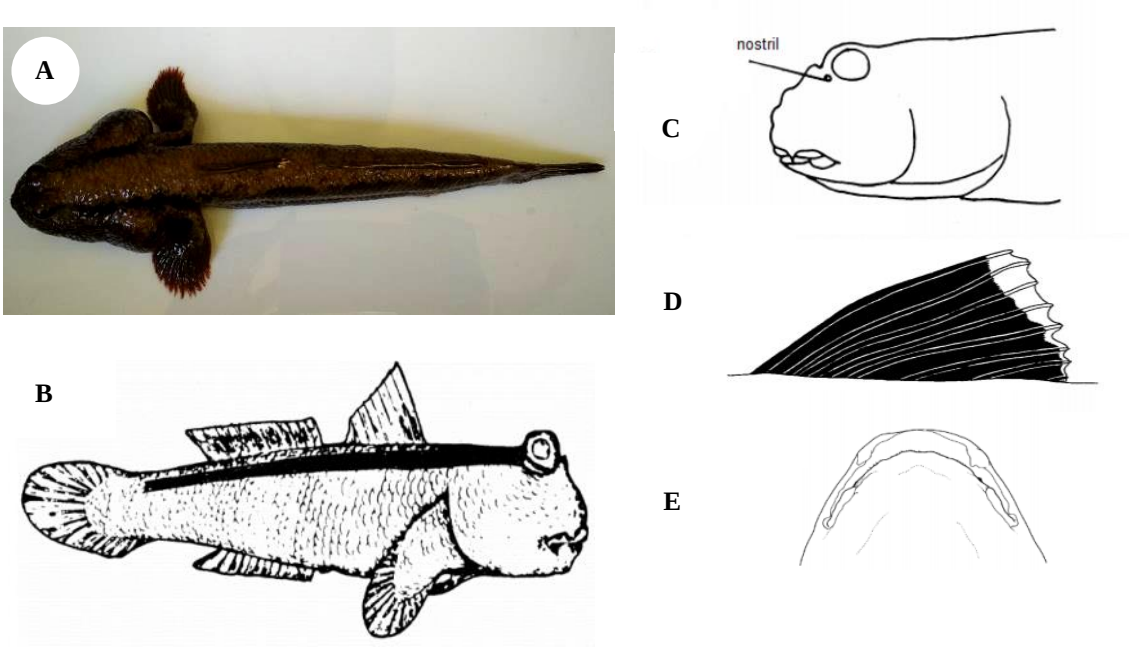

Figure 3. Giant mudskipper fish and its morphological character (A). Giant Mudskipper (*Pn. schlosseri*) collected from Kuala Lupak (B). Black longitudinal strike on the upper giant mudskipper body (Murdy 1989) (C). Cephalic sensory and nasal pores of the *Periophthalmodon* genera (D). Color pattern and shape of the first dorsal-fin and (E). Lip morphology (ventral view of head) of *Pn. schlosseri* (Jaafar et al. 2006). Scale bar = 5 cm

Table 1. Protein concentration which precipitated in different Ammonium sulfate saturation (%)

Fish sample	Protein concentration (mg.mL^{-1})						
	0	20-30	30-40	40-50	50-60	60-70	70-80
P. schlosseri	4.249 ± 0.139^{d}	3.097 ± 0.081^{a}	3.414 ± 0.147^{b}	3.764 ± 0.179^{c}	4.615 ± 0.177^{e}	5.106 ± 0.084^{f}	5.030 ± 0.210^{f}

Note: Values represent the mean of three replicate ± standard deviation. The same letter indicates that there was no significant difference between a column in the row by Duncan test at the 5% level

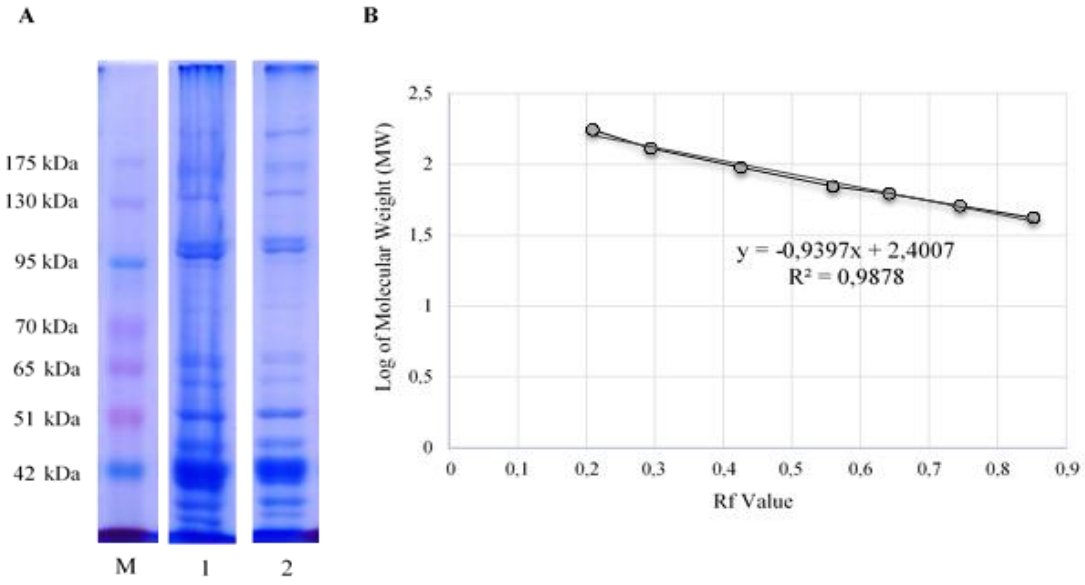

Figure 4. Protein pattern of giant mudskipper (A). Protein profiles of giant mudskipper (B). Linear regression fit between the log of molecular weight and Rf value of protein ladder. Notes: M. Protein Ladder (Cromatein Prestained Protein Ladder, Vivatis); 1. Not precipitated protein profile, 2. Precipitated protein profiles of giant mudskipper

Effect of precipitation on the concentration of muscle protein extract

Protein quantification was performed according to Bradford (1976) using Bradford reagent containing Coomassie Brilliant Blue G-250 (CBB) as a dye. The CBB can bind with proteins and form a protein-dye complex which has a blue color and absorbing at 595 nm. Dye binding process approximately formed in 2 minutes and stable for one hour (Bradford, 1976). The dye actively binds to Lysine (Lys), Histidine (His) and Arginine (Arg) residues on protein (Congdon et al. 1993). The assay was very sensitive and can detect low protein concentration up to 1 µl.mg^{-1} (Maldonado and Young, 2018). The concentration of giant mudskipper muscle protein was determined based on equation that obtained from standard curve of linear regression between protein concentration and absorbance of Bovine Serum Albumin (BSA) as standard protein. Linear regression analysis has expressed high accuracy with R^2 value of 0.991.

Table 1. were demonstrated the concentration of protein extract, which extracted from the muscle of giant mudskipper. The result showed that the difference ammonium sulfate saturation affected the concentration of protein extract. Giant mudskipper protein can be precipitated on optimum condition at 60-70% of ammonium sulfate saturation, with protein concentration 5.106 mg.mL^{-1}. However, there is no significant difference to 70-80% of ammonium sulfate saturation with the protein concentration is 5.030 mg.mL^{-1}. The result showed that the addition of higher ammonium sulfate concentration could affect a protein concentration of extract. Globular proteins solubility increases by the addition of salt (< 0.15 M), an effect of salting-in. At higher salt concentration, protein solubility usually decreases, an effect of salting-out. When ammonium sulfate added to the solution, hydrophobic interaction between protein and water were increased. As a response to salt addition, the protein decreasing its surface to minimize contact with the solvent. Protein was folded (the folded is more compact than the unfolded) and then self-association leading to precipitation (Wingfield, 2001).

The optimum ammonium sulfate saturation for protein precipitation are different between the species or even in same species collected from different geographical region. Our previous research, demonstrated the optimum ammonium sulfate saturation for muscle protein precipitation of *Pangasius hypophthalmus* and *Hemibagrus nemurus* is 60-70% and 70-80%, respectively (Sasmita et al. 2018). In addition, geographical origin of the sample may be contributing to the optimum ammonium sulfate saturation. In the snakehead fish (*Channa striata*), optimum ammonium sulfate saturation was different between fish which is collected from Nagara and Gambut (South Kalimantan). Protein of snakehead which collected from Nagara can be precipitated on the optimum condition with 70-80% ammonium sulfate saturation and 60-70% for snakehead fish which collected from Gambut (Mabrur et al. 2018). According to Chiew et al. (1995), it became a challenge to understand and determine an optimum condition for protein precipitation. Many factors are contributed to protein solubility in salt solution, including protein hydrophobicity, surface charge density, the net charge, hydration, size, polarizability, and valency of salt ions (Zhang 2012).

Table 2. The data of protein profiles extracted from the muscle of giant mudskipper

No. of Band	Migration distance (cm)	Rf	y = -0,939x + 2,400	Molecular Weight (kDa) [antilog 10 y]
Protein profiles of not precipitated extract				
1	0.97	0.1431	2.2657	184.36
2	1.41	0.2080	2.2047	160.22
3	1.52	0.2242	2.1895	154.70
4	1.83	0.2699	2.1466	140.14
5	2.18	0.3215	2.0981	125.34
6	2.50	0.3687	2.0538	113.18
7	2.65	0.3909	2.0330	107.89
8	2.82	0.4159	2.0094	102.20
9	2.92	0.4307	1.9956	98.99
10	3.22	0.4749	1.9540	89.96
11	3.45	0.5088	1.9222	83.60
12	4.11	0.6062	1.8308	67.73
13	4.21	0.6209	1.8169	65.60
14	4.45	0.6563	1.7837	60.77
15	4.92	0.7257	1.7186	52.31
16	5.31	0.7832	1.6646	46.19
17	5.67	0.8363	1.6147	41.18
18	5.81	0.8569	1.5953	39.39
19	6.12	0.9027	1.5524	35.68
20	6.30	0.9292	1.5275	33.69
Protein profiles of AS-precipitated extract (15 µg protein/wells)				
1	0.94	0.1414	2.2673	185.04
2	1.44	0.2165	2.1967	157.28
3	1.53	0.2301	2.1840	152.74
4	1.85	0.2782	2.1388	137.65
5	2.20	0.3308	2.0894	122.84
6	2.55	0.3835	2.0399	109.63
7	2.67	0.4015	2.0230	105.44
8	2.81	0.4226	2.0032	100.74
9	2.93	0.4406	1.9863	96.89
10	3.21	0.4827	1.9467	88.46
11	3.45	0.5188	1.9128	81.82
12	4.12	0.6195	1.8182	65.80
13	4.22	0.6346	1.8041	63.70
14	4.47	0.6722	1.7688	58.72
15	4.91	0.7383	1.7067	50.90
16	5.31	0.7985	1.6502	44.69
17	5.66	0.8511	1.6008	39.88
18	5.82	0.8752	1.5782	37.86
19	6.10	0.9173	1.5387	34.57
20	6.32	0.9504	1.5076	32.18

As previously mentioned by Wingfield (2001) and Zhang (2012), protein hydrophobicity will be a major factor that affects optimum ammonium sulfate saturation for protein precipitation. The optimum ammonium sulfate saturation depends on the hydrophobic amino acid residue in fish muscle. This residue was differ between species, organism or even in different protein type. Protein with high hydrophobic amino acid residues will be folded when ammonium sulfate added to the solution. Other protein with high hydrophilic amino acid residues has not folded and precipitated. This protein will be eliminated from the solution when centrifuged.

Protein profiles of giant mudskipper

Protein profiles of giant mudskipper obtained by two different protein extract from non-precipitated and precipitated with ammonium sulfate (Fig. 4) were performed in 10% resolving gel. The result of linear regression analysis between the Rf and log molecular weight of protein ladder obtained standard curve graph with the equation y = -0.939x + 2.40 shown in Figure 4B. The value of $R^2 = 0.9878$ indicated that the standard curve of protein ladder expressed has high accuracy (Hidayati et al. 2016). The molecular weight of protein profiles was measured based on the equation from the standard curve of protein ladder. The result showed that the molecular weight of giant mudskipper muscle protein ranging from 32 to185 kDa, shown in Table 2.

Twenty of protein bands were separated from not-precipitated and precipitated muscle protein extract of giant mudskipper fish on 10% resolving gels. Our previous study successful separated snakehead protein with molecular weight ranging 24-191 kDa, 24 - 146 kDa from *Pangasius hypophthalmus* protein and 25 to 135 kDa from *Hemibagrus nemurus* protein on 10% resolving gels (Sasmita et al. 2018). According to Rath et al. (2013), 10% resolving gels are the best concentration for protein separation with molecular weight ranging 14 to 200 kDa. The results of SDS-PAGE analysis of non-precipitated and precipitated protein has demonstrated that there is not different in number of bands or molecular weight. In our study, 15 µg protein loaded into wells gives great screening result with the maximum number of band.

According to Asfar et al. (2014) and Romadhoni et al. (2016) fish muscle also containing Albumin protein, which found on snakehead fish (*Channa striata*). Molecular weight of fish albumin protein were varied among the organism and its sources, which is 70 kDa on catfish (Hasnain et al. 2004) and Atlantic Salmon (Maillou et Nimmo 1993), 71-145 kDa on *Cyprinus carpio* (Yanagisawa et al. 1977) and 160-180 kDa on *Seriola quinqueradiata* (Nakagawa 1978). Albumin is an important protein, which has functional properties and used for increased levels of albumin in hypoalbuminemia and accelerates wound healing on post-operative patients (Mustafa et al. 2012). Albumin is one of the important protein as biomarker for heavy metal contamination in aquatic ecosystem. According to Javed and Usmani (2015), albumin concentration has been increased in the serum, liver, and muscle of *Channa punctatus* that living in heavy metal contaminated river by thermal power plant waste.

Giant mudskipper muscle hypothesized containing Ovalbumin protein which has molecular weight 44.69 kDa. Some research reported that the ovalbumin has molecular weight 43 kDa (Fish et al. 1969), 45 kDa (Chun et al. 2011) or ranging from 43 to 45 kDa (Urisu 2001). This protein has an immunogenic property and used by scientist to induced allergic asthma (Alenius et al. 2017) and atopic dermatitis (AD) on an animal model (Kim et al. 2017). Ovalbumin protein is containing 385 amino acid residues, which is on 323-339 amino acid residue are have IgE-binding activity and promote histamine release (Johnsen and Elsayed 1990).

Discussion

Protein was the major component in muscle (Zilhadia et al. 2014). Fish muscle consists of 18-23% protein or sometimes more depending on the species and harvesting time. Fish protein consisted of 20-30% sarcoplasmic protein, 66-77% myofibrillar protein and 2-3% insoluble protein of connective tissue (Kristinsson and Rasco 2000). Based on their molecular weight, muscle protein of giant mudskipper are hypothesized as tropomyosin, actin and C-protein. According to Sasmita et al. (2018) fish muscle containing myosin light chain (15-25 kDa), troponin (30 kDa), tropomyosin (35 kDa), actin (42 kDa), gelsolin (90 kDa) and C-protein (140 kDa) which is important for muscle contraction. Other protein reported on muscle which is myofibril, actinin, M-protein, filament protein, protease and collagen (Price and Schweigert 1987). Muscle protein has an important role in fish metabolism, especially as structural protein, enzymes, ribosomal protein, transport protein, DNA-RNA binding protein, transcription factor, translation factor, calcium-ion binding and signal transduction (Gam et al., 2006).

Water pollution, including heavy metal contamination, promote some genes expression, which was affected to the protein level in aquatic organism. Total protein fluctuation has been reported in *Channa striata* which exposed by 2,4-D pesticide (Anusuya and Hemalatha 2014). However, total protein has been increasing at *Channa punctatus* that living in heavy metal contaminated river by thermal power plant waste. When total protein separated on SDS-PAGE, the result showed different banding pattern between heavy metal exposure and normal condition, with disappearance or formation extra band on heavy metal exposed fish (Hamdy et al. 2016).

Some protein bands that resulted from SDS-PAGE analysis are hypothesized as Albumin (71-145 kDa or 160-180 kDa), Ovalbumin (43-45 kDa), Transferrin (80 kDa), Superoxide dismutase (32.5 kDa), Glutathione Peroxidase (88 kDa), Heat Shock Protein (HSP) 70 (66-78 kDa) and HSP90 (83-110 kDa) based on their molecular weight. Other protein like metallothioneins (6-10 kDa) and catalase (230 kDa) are not separated from this study, cause this study performed 10% polyacrylamide of separating gel. 10% polyacrylamide gels are recovered for separation of a protein band with a molecular weight ranging from 30 into 180 kDa. Separation of low or high molecular weight protein is needed higher or lower polyacrylamide concentration, respectively.

Albumin, Transferrin, Superoxide Dismutase, Glutathione Peroxidase, HSP70, and HSP90 is an important protein as biomarker for heavy metal contamination and assessment. Previous study reported this protein can regulate and detoxify heavy metal ions in fish tissue. The response and mechanism of the protein usually different between fish species or depend on type of heavy metal ions. In addition, other protein like Superoxide dismutase and Glutathione peroxidase is an important antioxidant enzyme as defense mechanism against reactive oxygen species (ROS) production affected by heavy metal accumulation in fish tissue. ROS production may be leading to oxidative stress, DNA damage, protein oxidation, nitric oxide formation and peroxidation of cell constituents (Javed et al. 2017). Thus, this protein very important for fish adaptation in heavy metal contaminated water.

Information of the protein profiles obtained on this study is very important for biomarker development of heavy metal contamination in Barito Estuary. However, several studies are needed to verify the protein bands for biomarker discovery and validation before application in the field. According to Sabullah et al. (2015), the protein band resulted from SDS-PAGE would be identified using mass spectrometry (MS), such as liquid chromatography/ MS (LC/MS) and matrix-assisted laser desorption/ ionization-time of flight analysis/ MS (MALDITOFF/MS). The MS analysis resulting in an amino acid sequence of the protein, and then the data compared with the database search engine. Information about proteins functions and characteristic can obtained from the Swiss Prot (www.expasy.ch/sprot/sprot-top.html) or PubMed (www.ncbi.nlm.nih.gov/entrez).

According to Qoronfleh (2017), the development of protein biomarker is divided into two stage which is discovery and validation. Biomarker discovery stage were including three steps: (i) identification of candidate protein biomarker (identification based on mass spectrometry, proteomic study and transcriptomic), (ii) relative assay development (develop relative quantification assay for protein that identified previously) and (iii) biomarker testing (apply relative quantification assay to a representative samples of normal and exposed with heavy metal group). Validation stage were including two steps; (i) absolute quantitation assay development (refinement assay using previous data from biomarker testing of biomarker discovery stage elevation to absolute quantification based on easy, reliable and reproducible methods) and (ii) biomarker testing (relative or absolute quantification of target protein across of hundreds of samples). After biomarker validated, it can be used in the field for assessment and monitoring heavy metal contamination. However, protein biomarker also can be developed based on the protein that previously reported in the literature. This method automatically saves time and cost for biomarker development, especially for protein identification using mass spectrometry.

In conclusion, the protein of giant mudskipper has great potential as a biomarker for heavy metal contamination in Barito Estuary cause this fish can accumulate and regulate heavy metal in their tissue. A preliminary study for development protein biomarker to monitoring heavy metal contamination has been done, resulted in protein profiles data of giant mudskipper muscle protein and successfully separated 20 protein bands with a molecular weight ranging from 32 into 185 kDa. Furthermore, several studies are needed for biomarker discovery and validation before application in the field.

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