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Wed, Mar 14, 2018 at 8:41 AM

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14-Mar-2018

Dear Ms. Nopianti,

Your manuscript, JTAS-1387-2018, entitled "PROTEASE INHIBITOR ACTIVITY OF CATFISH (Pangasius hypopthalmus) AND SWAMP EEL (Monopterus albus) BLOOD PLASMA" has been unsubmitted from the Journal of Tropical Agricultural Science. The reason of “unsubmission” is due to the following issue(s):

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01-Aug-2018

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Comment 1: to check on grammar and spelling.
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Some statements are sweeping statements while some statements are redundant.
Response 1: (Dear Author, please list down the Reviewer 2's comments that stated in Microsoft Word file and answer accordingly.)

[Reviewer 3]

Comment 1: This paper reports the potential of using plasma from two different fish species as inhibitor towards protease. The author appropriately addresses some of the comments made previously. However, some important comments made previously especially on the aspect of thoroughness of this manuscript are still missing.
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Response 1: (Dear Author, please respond accordingly and reply to the reviewer's comments in PDF file as well.)

[Reviewer 2]
Comment 1: Go through the Word file and please read the comments and track changes. Some aspects are not clear and please strengthen the discussion.
Some statements are sweeping statements while some statements are redundant.
Response 1: (Dear Author, please list down the Reviewer 2's comments that stated in Microsoft Word file and answer accordingly.)

[Reviewer 3]

Comment 1: This paper reports the potential of using plasma from two different fish species as inhibitor towards protease. The author appropriately addresses some of the comments made previously. However, some important comments made previously especially on the aspect of thoroughness of this manuscript are still missing.
Response 1: (Dear Author, please respond properly.)

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Wed, Sep 26, 2018 at 1:56 PM

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26-Sep-2018

Dear Ms. Nopianti:

It is a pleasure to accept your manuscript entitled "Protease Inhibitory Activity and Protein Analysis of Catfish (Pangasius hypopthalmus) and Swamp Eel (Monopterus albus) Blood Plasma" in its current form for publication in the Journal of Tropical Agricultural Science.

An official acceptance letter will be sent to you from my office in due course of time.

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Thank you for your fine contribution. On behalf of the Editors of the Journal of Tropical Agricultural Science, we look forward to your continued contributions to the Journal.

Sincerely,
Prof. Dato' Dr. Abu Bakar Salleh
Chief Executive Editor, Journal of Tropical Agricultural Science

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Re: URGENT (expiry 48 hours) : SCHEDULED FOR PUBLICATION IN JTAS VOL. 42 (1) FEB. 2019 [JTAS-1387-2018] 3

rodiana nopianti <nopi_81@yahoo.com>
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Dear Publication Officer,

According to email that we had received, the article didn't contain any comment (s) from pre-press officer. So, I agree to publish our article as it is without any correction from us.
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Best Regard

Rodiana Nopianti
(Corresponding author)

On Mon, 2/11/19, Pre Press Farrah <upmjournals.prepress@upm.edu.my> wrote:

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Thu, Jan 31, 2019 at 2:51 PM

Dear Author(s),

I am writing to you in reference to an article entitled, **"Protease Inhibitory Activity and Protein Analysis of Catfish (Pangasius hypopthalmus) and Swamp Eel (Monopterus albus) Blood Plasma"** with author(s): **Rodiana Nopianti, Herpandi, Ace Baehaki, Rinto, Sherly Ridhowati and Maggy Thenawidjaja Suhartono**, submitted to *Pertanika* on 21 March 2018 for intended publication in JTAS.

Your paper has been anonymously peer-reviewed by two to three referees competent in the specialized areas appropriate to your manuscript independently evaluating the scientific quality of the manuscript.

I am pleased to tell you that based on the clarity, technical approach and scientific validity presented; your paper **has been accepted** by the Editorial Board on 26 September 2018, and is ***tentatively*** scheduled for publication in **JTAS Vol. 42(1) Feb. 2019**.

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Protease Inhibitory Activity and Protein Analysis of Catfish (Pangasius hypopthalmus) and Swamp Eel (Monopterus albus) Blood Plasma

Journal:	*Journal of Tropical Agricultural Science*
Manuscript ID	JTAS-1387-2018.R2
Manuscript Type:	Regular Article
Scope of the Journal:	Fisheries sciences < Fisheries sciences < AGRICULTURAL SCIENCES, Food Sciences < Food and nutrition development < AGRICULTURAL SCIENCES, Biotechnology < BIOLOGICAL SCIENCES, Proteins < Biochemistry < BIOLOGICAL SCIENCES
Keywords:	Blood Plasma, Catfish, Protease Inhibitor, Swamp Eel
Abstract:	Protease inhibitor is a component that can prevent protein from degradation caused by protease activity. Blood plasma contains a variety of protease inhibitors. The objective of this study was to investigate the potential use of crude blood plasma from catfish (Pangasius hypopthalmus) and swamp eel (Monopterus albus) as protease inhibitor. The parameters observed were moisture content, protein content, ash content, inhibitory activity to trypsin and papain enzymes and protein profile of blood plasma. Generally, inhibitory activity increased as the volume of blood plasma increased (25µL, 50µL, 75 µL, 100 µL). The inhibitory activity of blood plasma from catfish was 7.66-50.73% to trypsin enzyme and 20.34-83.05% to papain enzyme. Meanwhile, the inhibitory activity of blood plasma from swamp eel was 9.49-46.35% to trypsin enzyme and 28.81-64.41% to papain enzyme. The highest inhibitory activity was demonstrated by swamp eel blood plasma with molecular weight between 19.84-174.14 kDa (13 proteins). Protein content of blood plasma from swamp eel (11.92%) was lower than catfish (16.63%) but it resulted higher enzyme inhibitory activity. For further research, we can carry out purification steps on blood plasma that are expected to get better inhibitory activity. ABSTRACT REVISED II.doc

Running Title:

Protease Inhibitory Activity of Blood Plasma

Author and Corresponding Author Information

Full Title:
Protease Inhibitory Activity and Protein Analysis of Catfish (*Pangasius hypopthalmus*) and Swamp Eel (*Monopterus albus*) Blood Plasma

A list of number of black and white figure and table:

1. Table 1. *The concentration of standard solution (Bovine Serum Albumin)*
2. Table 2. *Volume of catfish and swamp eel blood plasma sample for trypsin and papain inhibition assay*
3. Table 3. *The proximate analysis of catfish* (Pangasius hypopthalmus) *and swamp eel* (Monopterus albus) *blood plasma*
4. *Figure 1*. Inhibitory activity of catfish (*Pangasius hypopthalmus*) blood plasma to trypsin and papain enzymes
5. *Figure 2*. Inhibitory activity of swamp eel (*Monopterus albus*) blood plasma to trypsin and papain enzymes
6. *Figure 3*. Molecular weight of catfish (*Pangasius hypopthalmus*) (C1, C2, C3) and swamp eel (*Monopterus albus*) (S1, S2, S3) blood plasma (M= protein marker)

ABSTRACT

Protease inhibitor is a component that can prevent protein from degradation caused by protease activity. Blood plasma contains a variety of protease inhibitors. The objective of this study was to investigate the potential use of crude blood plasma from catfish *(Pangasius hypopthalmus*) and swamp eel (*Monopterus albus*) as protease inhibitor. The parameters observed were moisture content, protein content, ash content, inhibitory activity to trypsin and papain enzymes and protein profile of blood plasma. Generally, inhibitory activity increased as the volume of blood plasma increased (25µL, 50µL, 75 µL, 100 µL). The inhibitory activity of blood plasma from catfish was 7.66-50.73% to trypsin enzyme and 20.34-83.05% to papain enzyme. Meanwhile, the inhibitory activity of blood plasma from swamp eel was 9.49-46.35% to trypsin enzyme and 28.81-64.41% to papain enzyme. The highest inhibitory activity was demonstrated by swamp eel blood plasma with molecular weight between 19.84-174.14 kDa (13 proteins). Protein content of blood plasma from swamp eel (11.92%) was lower than catfish (16.63%) but it resulted higher enzyme inhibitory activity. For further research, we can carry out purification steps on blood plasma that are expected to get better inhibitory activity.

Keywords: Blood plasma, catfish, protease inhibitor, swamp eel.

INTRODUCTION

Fish is a commonly used food around the world but is associated with rapid decline in its quality (perishable food). One of the factors that affect the rapid change in fish quality is the high protein content. In general, protein content in fish is 15-20%, but protein content lower than 15% and higher than 28% is also found in some fish species (Murray & Burt, 2001). Protease enzymes act on the fish protein to bring about its deterioration in its quality.

The decline in quality after the post mortem phase is one of the most unfavourable changes in fish muscle. During the post mortem, degradation of muscle proteins contributes to the rapid softening of flesh. The protein proteolysis can be attributed to endogenous protease activity (Chéret et al., 2007). Two characterized proteolytics system are known to hydrolyze protein during post mortem storage of meat and fish muscle: calpains and cathepsins (Jiang, 2000; Ouali, 1992). Cathepsin L is one of the most important protease enzyme in the softening process in fish meat, and in surimi processing it is a problem because it can not be eliminated by washing. There is correlation between the active enzyme cathepsin with the rate of deterioration of fish quality. Cathepsin L enzyme activity is optimum at 60 ^{0}C, and this is the cause of gel texture softening in surimi-based end products during the slow thermal process due to the destruction of the protein, especially myofibril (An et al., 1994). Ho et al., (2000) also reported that cathepsin L and and L-like left in mackerel surimi had Myosin Heavy Chain (MHC)-degrading ability which consequently caused gel softening during setting at 40-45 ^{0}C. In addition, texture softening also occurs in grass carp fillets caused by Cathepsin B and L as the major endogenous enzymes that leads to proteolytic degradation (Ge et al., 2014). Ueki et al., (2016) reported that breaking strength white croacker gels prepared by adding the intestinal extracts was decreased. The authors

suggested that proteolitic enzymes (i.e. trypsin) in the intestinal extracts caused the weakening of the gel texture.

One way to inhibit proteolytic degradation by cathepsin enzymes is by using blood plasma. Blood plasma contains a variety of protease inhibitors, including α2-macroglobulin that is a protease inhibitor of some protease classes with its mechanism of action baits and traps (Barret, 198 Many studies have been conducted using plasma to inhibit the work of cathepsin enzymes, including cow blood plasma (Kang & Lanier, 1999; Marquez-Alvarez et al., 2015), pig blood plasma (Benjakul et al., 2001; Benjakul and Visessanguan, 2000), chicken blood plasma (Rawdkuen et al., 2007) and salmon blood plasma (Fowler & Park, 2015). However, the utilization of blood plasma from cattle and chickens is limited due to mad cow disease infection as well as avian influenza in poultry.

Blood plasma comprises 46-63% of total blood volume, with moisture content being 92% of total plasma (Martini, 2005). According to FAO (Nomura, 2007), fish catch and aquaculture globally amounted to 106 million tons in 2004. The percentage of blood is about 5% of body weight (Halliday, 197 d amounts to 5 million tons. Fish blood is generally not utilized but has the potential to be collected and processed to produce value-added foods based on its nutritional value and functional properties (Lynch et al., 2017). Fish blood can be extracted for application in the food, biomedical and pharmaceutical sectors (Lafarga et al., 2015, 2016; Mullen et al., 2015). The objective of this study was thus to investigate the ability of blood plasma from two freshwater tropical fish (catfish and swamp eel) to function as protease inhibitor towards the enzymes trypsin and papain.

MATERIAL AND METHODS

Materials

Live catfish *(Pangasius hypopthalmus)* (size 2-4/kg) and swamp eel (*Monopterus albus*) (size 10-15/kg) were obtained from Indralaya

traditional market, South Sumatera, Indonesia. Trypsin (from bovine pancreas), papain (from papaya latex), N_{α}-Benzoyl-$_{L}$-arginine-$_{DL}$ β-naphthylamide (BANA) and N_{α}-Benzoyl-$_{L}$-arginine 4-nitroanilide hydrochloride (BAPNA) were purchased from Sigma Aldrich (USA).

Collecting of Blood Plasma

The collecting of crude blood plasma was according to methods described in Fowler and Park (2015). Whole blood was collected from bleeding fish into bottles containing EDTA (Eethylenediaminetetraacetic acid), and then centrifuged for 15 min at 1500g at 4^0C. The supernatant was regarded as plasma and kept in freezer until it was used.

Proximate Analysis

Protein content (Bradford, 1976)

The protein analysis followed the method of Bradford (1976) and the Bovine Serum Albumin (BSA) was used as the standard.

Preparation of Bradford reagent solution

A 25 mL aliquot of ethanol 95% was mixed with 5 mg of Commassie Brilliant Blue G250. The solution was added with 50 mL H_3PO_3 85% and homogenized. The mixture was adjusted with distilled water until it reached a volume of 100 mL and kept at 4^0C before use.

Preparation of standard solution

The preparation of standard solutions for protein analysis is given in Table 1. 10 mg of BSA was added to 10 mL of distilled water and then stirred with a magnetic stirrer to give homogeneous solution. Specific volumes of BSA (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mL) were added to different volume of distilled water (0.9, 0.8, 0.6, 0.4, 0.2 and 0 mL) respectively, and then 5 mL of Bradford solution was added to each mixture. The mixtures were allowed to react for 30 minutes at room temperature. The absorbance was read at 595 nm.

Table 1.

The concentration of standard solution (Bovine Serum Albumin)

BSA (mL)	Distilled water (mL)	Concentration (mg/mL)
0.1	0.9	0.1
0.2	0.8	0.2
0.4	0.6	0.4
0.6	0.4	0.6
0.8	0.2	0.8
1	0	1

c. Preparation of blank

1 mL of distilled water was mixed with 5 mL of Bradford solution which was then homogenized and allowed to react for 30 minutes at room temperature. The absorbance was read at 595 nm.

d. Sample assay

A 1 mL of sample plasma was mixed with 9 mL of distilled water. A 0.5 mL of the mixture was then added to 4.5 mL of Bradford reagent. The solution was allowed to react at room temperature for 30 minutes. The absorbance was read at 595 nm. The concentration of protein was determined by the following equation:

y= ax+b

y = absorbance of sample

a = slope

b = intercept

x = protein concentration of sample

Moisture content

The moisture content analysis was done according to the AOAC (2005) procedure using the evaporation principle of water in the sample until it reaches a constant weight at 105 °C. The blood plasma sample used was 1 mL.

Ash content

The ash content analysis followed the AOAC (2005) procedure where the principle of analysis was combustion of organic compound to get an inorganic ash residue at 550 °C. The blood plasma sample used was 1 mL.

Trypsin Inhibition Assay

Trypsin inhibition was determined according to the methods of Fowler and Park (2015). Four different blood plasma volumes ((25, 50, 75, 100 µL) (Table 2) were diluted with distilled water and adjusted to 0.2 mL. 150 µL of the inhibitor solution was added to 300 µL of trypsin enzyme (20 µg/mL) and 150 µL of distilled water and pre-incubated at 37°C for 10 min. 750 µL of 0.4 mg/mL BAPNA in 50 mM tris-HCl buffer (pH 8.2) containing 20 mM $CaCl_2$ and pre-warmed to 37°C was than added, and the reaction mixture was incubated for 37^0C for 10 minutes. The reaction was stopped by adding 150 µL of 30% acetic acid (v/v). Absorbance was read at 410 nm and inhibitory activity was expressed as percent decrease in OD_{410} compared to the control.

Papain Inhibition Assay

Papain inhibition was determined according to the method of Fowler and Park (2015). Four different blood plasma volumes ((25, 50, 75, 100 µL) (Table 2) were diluted with distilled water and adjusted to 2 mL. 2 mL of 0.25 M sodium phosphate buffer (pH 6.0) containing 2.5 mM EDTA and 25 mM β-mercaptoethanol (βME) was added to 0.1 mL of papain solution (100 µg/mL) containing 25 mM sodium phosphate buffer (pH 7.0) and 2 mL of inhibitor solution. After preincubation at 37°C for 5min, 0.2mL of 2mM BANA was added to initiate the reaction. After 10 min of incubation, 1 mL of cold 2% HCl in ethanol was added to stop the reaction. 1 mL of 0.06% ρ-dimethylamino-cinnamaldehyde dye was then added to show the colour of the final solution. Absorbance was read at 540 nm and the inhibitory activity was expressed as the percent decrease in OD_{540} compared to the control.

Table 2.

Volume of catfish and swamp eel blood plasma sample for trypsin and papain inhibition assay

Sample	Papain Assay			Trypsin Assay		
	Plasma Volume (µL)	Protein Concentration (mg/mL)	Plasma Concentration (mg/mL)	Plasma Volume (µL)	Protein Concentration (mg/mL)	Plasma Concentration (mg/mL)
Catfish Plasma	25	0.21	1.25	25	2.08	12.5
	50	0.42	2.5	50	4.16	25
	75	0.62	3.75	75	6.24	37.5
	100	0.83	5	100	8.32	50
Swamp eel Plasma	25	0.15	1.25	25	1.49	12.5
	50	0.30	2.5	50	2.98	25
	75	0.45	3.75	75	4.47	37.5
	100	0.60	5	100	5.96	50

Molecular Weight of Inhibitor

The SDS-PAGE procedure was followed according to the method described by Laemmli (1970). A 12% resolving gel and a 3.5% stacking gel were used. The sample buffer consisted of 188mM M Tris-HCl pH 6.8, 15% β-mercaptoethanol, 3% SDS, 0.01% bromophenol blue, and 30% glycerol in deionized water. Sample preparation was conducted by mixing protein samples with loading buffer in a ratio 1:1 and heating the mixture to 95^0C for 10 min to denature the protein samples. An SDS-PAGE broad-range molecular weight standard (6.5 to 200 kDa) (Bio-Rad, Alfred Nobel Drive, Hercules, CA, U.S.A) was used. The running buffer consisted of 0.12 M Tris base, 0.95 M glycine and 0.5% SDS (w/v) in deionized water. Samples and protein standard were run into well. After finishing the process, gels were then immersed in deionized water for 5 min and stained with coomassie blue stain buffer for 20 minuntes and destained with a destaining buffer for 1 min. The gels were then immersed in 1% acetic acid and incubated for 1 h on a shaker. The acetic acid was replenished every hour until the gel was completely de-stained.

Statistical Analysis

Only descriptive statistics was utilized for the reporting of the data.

RESULTS AND DISCUSSIONS

Proximate Analysis of Blood Plasma

Table 3 shows the value of proximate analysis for each blood plasma. The moisture content of catfish blood plasma was 91.46%, while for the swamp eel was 93.05%.

Table 3.

The proximate analysis of catfish (Pangasius hypopthalmus) *and swamp eel* (Monopterus albus) *blood plasma*

Source of plasma	Proximate Analysis		
	Moisture content (%)	Protein (mg/mL)	Ash (%)
Cat Fish	91.46	16.63	0.75
Swamp eel	93.05	11.92	0.92

According to Kisia (2016) and Martini (2005), moisture content in plasma in plasma of vertebrates is 90-92%. The difference of moisture content in both of plasma is due to differences in other chemical composition, such as protein content and ash content, which affects moisture content. Protein is one of the important components in the body. Proteins have the function of maintaining water balance in the blood and tissues, regulating blood volume, helping the transport of fat, vitamins, and hormones and as an antibody defense.

The protein content of catfish plasma was 16.63 mg/mL and the swamp eel plasma was 11.92 mg/mL. Protein in plasma reflects the blood protein content, except for hemoglobin. Plasma proteins are divided into three main fractions i.e. albumin (42% w / v), globulin (56%) (α1-globulin, α2-globulin, β-globulin, and γ-globulin) and fibrinogen (1%). The largest proportions contained in solutes include proteins, which account for 6% -8% in blood plasma (Moure et al., 2003). Florkin and Scheer (1974) suggests

that solutes in fish's blood plasma vary between freshwater fish and marine fish and that the differences in plasma blood plasma protein is associated with the development and physiology of fish.

The ash content of catfish and swamp eel plasma were 0.75% and 0.92% respectively. The difference of ash content in both of plasma is related to endogenous (genetically controlled and associated with species-specific life cycles) and exogenous (such as environmental and dietary) factors (Perschbacher & Stickney, 2017). Ash content reflects the mineral content in blood plasma. Minerals in the blood plasma are part of a minor component of blood plasma which amounts to 1% (Moure et al., 2003; Martini, 2005). The minerals compose the normal extracellular fluid ion composition for vital cellular activity and contributes to the osmotic pressure of body fluids. The major plasma electrolytes are Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}, HCO^{3-}, HPO_4^{2-}, and SO_4^{2-} (Martini, 2005).

Inhibitory Activity of Blood Plasma

The inhibitory activity of blood plasma to protease enzyme increased as the concentration of blood plasma for both fishes increased. The protease inhibitory activity ranged from 7.66 to 50.73% for trypsin and 20.34 to 83.05% for papain for the catfish blood plasma (Figure 1). The protease inhibitory activity of the swamp eel blood plasma ranged from 9.49 to 46.35% for trypsin and 28.81 to 64.41% for papain (Figure 2).

The activity of blood plasma inhibitors to papain enzyme was higher than that of trypsin enzyme. This result was in contra[illegible]ith the recomendation of Yongswatdigul et al. (2014) and Sriket (2014), where the surimi made from tropical fish were susceptible to serine protease attacks that cause protein degradation. But this was in line with the result of Ge et al., (2014) that reported softening occurs in grass carp fillets caused by cathepsin B and L as the major endogenous enzymes that leading to proteolytic degradation.

Based on data, the highest value protease inhibitory activity on trypsin enzyme and papain was resulted by swamp eel blood plasma although its protein concentration was lower than catfish blood plasma (0.15-0.60 and 0.21-0.83 mg/mL, respectively). This was probably due to the higher efficacy of the protein plasma of the eel acting as a protease inhibitor as compared to that in the catfish. Fowler and Park (2015), showed that the protease inhibitory activity of salmon plasma ranged between 25% - 81% for papain and below 20% for trypsin. The higher inhibitory activity of the salmon blood plasma as compared to that of the catfish and swamp eel (this study) was probably related to it being free of impurities such as water, fat, ash, and other non-protein components.

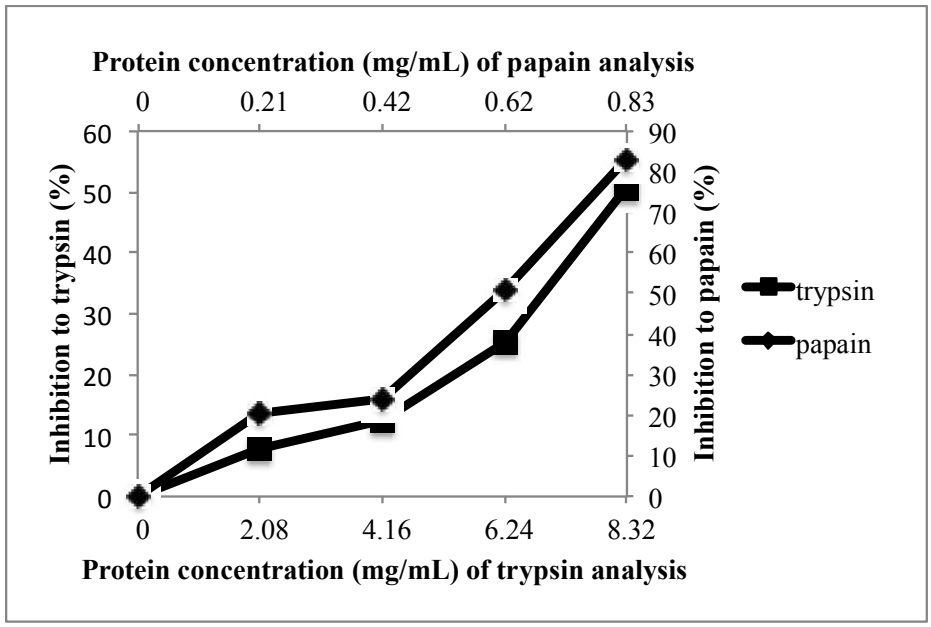

Figure 1. Inhibitory activity of catfish (*Pangasius hypopthalmus*) blood plasma to trypsin and papain enzymes

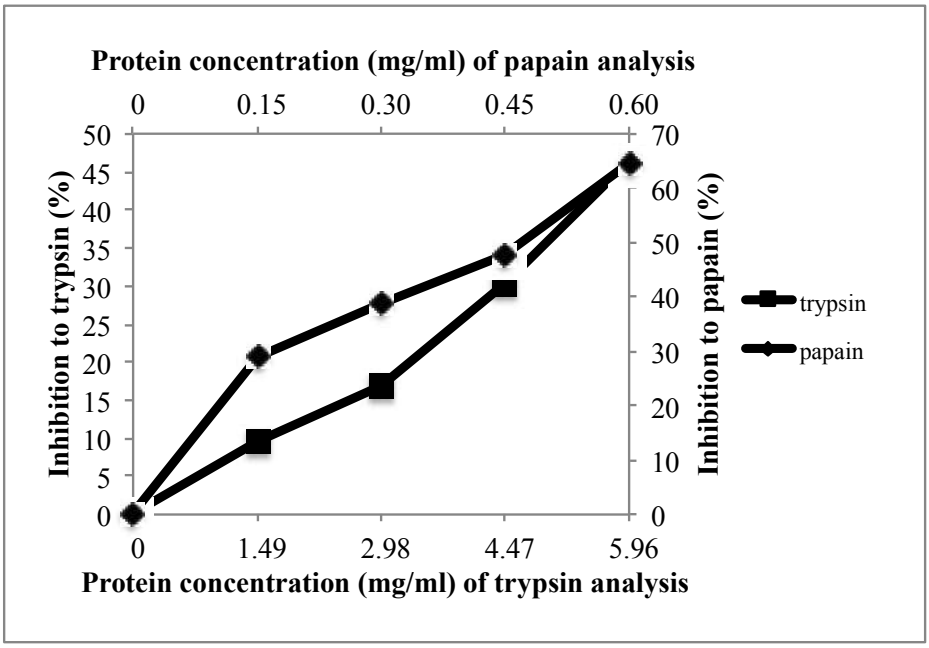

Figure 2. Inhibitory activity of swamp eel (*Monopterus albus*) blood plasma to trypsin and papain enzymes

Molecular Weight of Blood Plasma

The molecular weight of catfish (C1-C3) and swamp eel blood plasma (S1-S3) is the range of 9.64 to 124.36 kDa (10 proteins) and 19.84 to 174.14 kDa (13 proteins) respectively (Figure 3). According to Howell and Lawrie (198[illegible]e molecular weight seen in the protein bands detected in blood plasma of catfish and swamp eels showed similarities with molecular weight of pigs plasma, namely albumin (65-69 kDa), 12 bands of α-globulin protein (7-120 kDa), 8 bands of β-globulin protein bands (48-100 kDa), 3 bands of γ-globulin protein bands (33, 58, and 110 kDa), and fibrinogen at 40 kDa. According to Benjakul and Visessanguan (2000), the presence of serine protease inhibitor is in the range of 58-64 kDa and this is within the range of the proteins in catfish and eel plasma which was able to inhibit the enzymes chymotrypsin and trypsin. Li et al. (2008) reported, the presence of the cysteine protease inhibitor at 55 kDa. The variation of the

proteins from catfish and swamp eel as determined by the SDS-PAGE analysis determine their inhibitory activity. Therefore, swamp eel blood plasma activity was higher than catfish. The thickness of the protein band is associated with high inhibitory activity of plasma to papain.

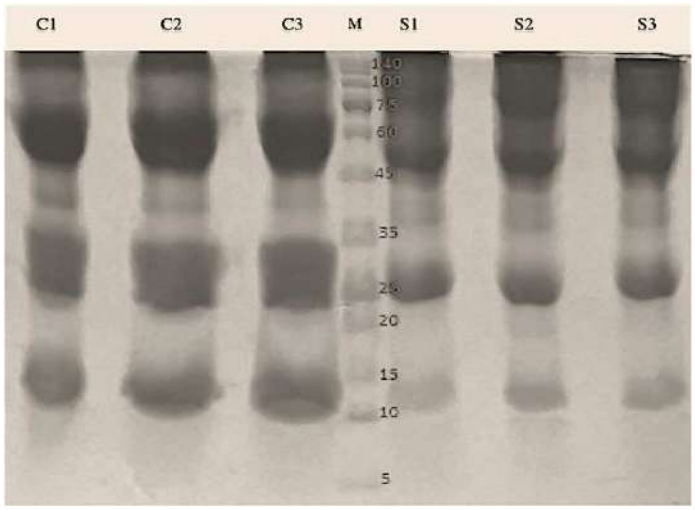

Figure 3. Molecular weight of catfish (*Pangasius hypopthalmus*) (C1, C2, C3) and swamp eel (*Monopterus albus*) (S1, S2, S3) blood plasma (M= protein marker)

CONCLUSIONS

Both the catfish and swamp eel blood plasma had inhibitory activity towards trypsin and papain. Papain is a cysteine protease and its inhibition was higher than trypsin by the protease inhibitor in both catfish and swamp eel plasma. The highest inhibitory activity to protease enzyme was from the swamp eel blood plasma.

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Protease Inhibitory Activity and Protein Analysis of Catfish (*Pangasius hypopthalmus*) and Swamp Eel (*Monopterus albus*) Blood Plasma

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ABSTRACT

Protease inhibitors can prevent protein from degradation caused by protease activity. Blood plasma contains a variety of protease inhibitors. The objective of this study was to investigate the potential use of crude blood plasma from catfish *(Pangasius hypopthalmus*) and swamp eel (*Monopterus albus*) as protease inhibitors. The parameters observed were moisture content, protein content, ash content, inhibitory activity to trypsin and papain enzymes and protein profile of blood plasma. The inhibitory activity increased as the volume of blood plasma increased (25 µL, 50 µL, 75 µL, 100 µL). The inhibitory activity of blood plasma from catfish was 7.66-50.73% to trypsin enzyme and 20.34-83.05% to papain enzyme while the inhibitory activity of blood plasma from swamp eel was 9.49-46.35% to trypsin enzyme and 28.81-64.41% to papain enzyme. The highest inhibitory activity was demonstrated by swamp eel blood plasma with molecular weight between 19.84-174.14 kDa (13 proteins). Protein content of blood plasma from swamp eel (11.92%) was lower than catfish (16.63%) but resulted higher enzyme inhibitory activity. Further research can be conducted to carry out purification steps on blood plasma that are expected to show better inhibitory activity.

Keywords: Blood plasma, catfish, protease inhibitor, swamp eel

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INTRODUCTION

Fish is a commonly used food around the world and is classified as perishable food. One of the factors that affect the rapid change in fish quality is the high protein content. In general, protein content in fish is 15-20%, but protein content lower than 15% and higher than 28% is also found in some fish species (Murray & Burt, 1983). Protease enzymes act on fish protein to bring about its deterioration in its quality.

The decline in quality after the post mortem phase is one of the most unfavourable changes in fish muscle. During post mortem, degradation of muscle proteins contributes to the rapid softening of flesh. The protein proteolysis can be attributed to endogenous protease activity (Chéret et al., 2007). Two characterized proteolytics are known to hydrolyze protein during post mortem storage of meat and fish muscle: calpains and cathepsins (Jiang, 2000; Ouali, 1992). Cathepsin L is one of the most important protease enzymes in the softening process in fish meat, and in surimi processing it is a problem because washing cannot eliminate it. The presence cathepsin enzyme in surimi or fish mince results in a decrease in gel strength with a brittle and non-elastic gel at temperature around 60^{o}C (Rawdkuen et al., 2007b). Ho et al. (2000) reported that cathepsin L and and L-like in mackerel surimi had Myosin Heavy Chain (MHC)-degrading ability which consequently caused gel softening during setting at 40-45^{o}C. In addition, texture softening also occurs in grass carp fillets caused by Cathepsin B and L as the major endogenous enzymes that leads to proteolytic degradation (Ge et al., 2014).

One way to inhibit proteolytic degradation by cathepsin enzymes is by using blood plasma. Blood plasma contains a variety of protease inhibitors, including α2-macroglobulin which is a protease inhibitor of some protease classes with its mechanism of action baits and traps (Barret, 1981). Many studies have been conducted using plasma to inhibit the work of cathepsin enzymes, including cow blood plasma (Kang & Lanier, 1999; Marquez-Alvarez et al., 2015), pig blood plasma (Benjakul et al., 2001; Benjakul & Visessanguan, 2000), chicken blood plasma (Rawdkuen et al., 2007a) and salmon blood plasma (Fowler & Park, 2015). However, the utilization of blood plasma from cattle and chickens is limited due to mad cow disease infection as well as avian influenza in poultry.

Blood plasma comprises 46-63% of total blood volume, with moisture content being 92% (Martini, 2005). According to the Food and Agriculture Organization (Nomura, 2007), the global fish catch and aquaculture amounted to 106 million tons in 2004, and since blood is about 7% of body weight (Philips & Williams, 2011), this would amount to 7 million tons. Fish blood is generally not utilized but has the potential to be collected and processed to produce value-added foods based on its nutritional value and functional properties (Lynch et al., 2017). Fish blood can be extracted for application in the food, biomedical and pharmaceutical sectors (Lafarga et al., 2015; Lafarga et al., 2016; Mullen et al., 2015).

The objective of this study was to investigate the ability of blood plasma from two freshwater tropical fish (catfish and swamp eel) to function as protease inhibitor towards the enzymes trypsin and papain.

MATERIAL AND METHODS

Materials

Live catfish *(Pangasius hypopthalmus*) (size 2-4/kg) and swamp eel (*Monopterus albus*) (size 10-15/kg) were obtained from Indralaya traditional market, South Sumatera, Indonesia. Trypsin (from bovine pancreas), papain (from papaya latex), N_{α}-Benzoyl-$_{L}$-arginine-$_{DL}$ β-naphthylamide (BANA) and N_{α}-Benzoyl-$_{L}$-arginine 4-nitroanilide hydrochloride (BAPNA) were purchased from Sigma Aldrich (USA).

Collecting of Blood Plasma

The collecting of crude blood plasma was according to methods described in Fowler and Park (2015). Whole blood was collected from bleeding fish into bottles containing EDTA (Eethylenediaminetetraacetic acid), and then centrifuged for 15 min at 1500 g at 4^{o}C. The supernatant was regarded as plasma and kept in freezer until it was used.

Proximate Analysis

Protein Content (Bradford, 1976). The protein analysis followed the method of Bradford (1976) and the Bovine Serum Albumin (BSA) was used as the standard.

***Preparation of Bradford Reagent Solution*.** A 25 mL aliquot of ethanol (95%) was mixed with 5 mg of Commassie Brilliant Blue G250. The solution was then added to 50 mL H_3PO_3 85% and homogenized. The mixture was adjusted with distilled water until it reached a volume of 100 mL and kept at 4^{o}C before use.

***Preparation of Standard Solution*.** The preparation of standard solutions for protein analysis is given in Table 1. Ten mg of BSA was added to 10 mL of distilled water and then stirred with a magnetic stirrer to give a homogeneous solution. Specific volumes of BSA (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mL) were added to different volume of distilled water (0.9, 0.8, 0.6, 0.4, 0.2 and 0 mL) respectively, and then 5 mL of Bradford solution was added to each mixture. The mixtures were allowed to react for 30 minutes at room temperature. The absorbance was read at 595 nm.

Table 1
The concentration of standard solution (Bovine Serum Albumin)

BSA (mL)	Distilled water (mL)	Concentration (mg/mL)
0.1	0.9	0.1
0.2	0.8	0.2
0.4	0.6	0.4
0.6	0.4	0.6
0.8	0.2	0.8
1	0	1

***Preparation of Blank*.** One mL of distilled water was mixed with 5 mL of Bradford solution which was then homogenized and allowed to react for 30 minutes at room temperature. The absorbance was read at 595 nm.

Sample Assay. A 1 mL of sample plasma was mixed with 9 mL of distilled water. A 0.5 mL of the mixture was then added to 4.5 mL of Bradford reagent. The solution was allowed to react at room temperature for 30 minutes. The absorbance was read at 595 nm. The concentration of protein was determined by the following equation:

y = ax+b
y = absorbance of sample
a = slope
b = intercept
x = protein concentration of sample

Moisture Content. The moisture content was determined using the evaporation principle until the sample reached a constant weight at 105°C (Association of Official Analytical Chemists [AOAC], 2005). The blood plasma sample used was 1 mL.

Ash Content. The ash content analysis was determined by the combustion of organic compounds at 550°C (AOAC, 2005). The blood plasma sample used was 1 mL.

Trypsin Inhibition Assay. Trypsin inhibition was determined according to the methods of Fowler and Park (2015). Four different blood plasma volumes ((25, 50, 75, 100 µL) (Table 2) were diluted with distilled water and adjusted to 0.2 mL. 150 µL of the inhibitor solution was added to 300 µL of trypsin enzyme (20 µg/mL) and 150 µL of distilled water and pre-incubated at 37°C for 10 min. 750 µL of 0.4 mg/mL BAPNA in 50 mM tris-HCl buffer (pH 8.2) containing 20 mM $CaCl_2$ and pre-warmed to 37°C was than added, and the reaction mixture was incubated for 37°C for 10 minutes. The reaction was stopped by adding 150 µL of 30% acetic acid (v/v). Absorbance was read at 410 nm and inhibitory activity was expressed as percent decrease in OD_{410} compared to the control.

Papain Inhibition Assay. Papain inhibition was determined according to the method of Fowler and Park (2015). Four different blood plasma volumes ((25, 50, 75, 100 µL) (Table 2) were diluted with distilled water and

Table 2
Volume of catfish and swamp eel blood plasma sample for trypsin and papain inhibition assay

Sample	Papain Assay			Trypsin Assay		
	Plasma Volume (µL)	Protein Concentration (mg/mL)	Plasma Concentration (mg/mL)	Plasma Volume (µL)	Protein Concentration (mg/mL)	Plasma Concentration (mg/mL)
Catfish Plasma	25	0.21	1.25	25	2.08	12.5
	50	0.42	2.5	50	4.16	25
	75	0.62	3.75	75	6.24	37.5
	100	0.83	5	100	8.32	50
Swamp eel Plasma	25	0.15	1.25	25	1.49	12.5
	50	0.30	2.5	50	2.98	25
	75	0.45	3.75	75	4.47	37.5
	100	0.60	5	100	5.96	50

adjusted to 2 mL. 2 mL of 0.25 M sodium phosphate buffer (pH 6.0) containing 2.5 mM EDTA and 25 mM β-mercaptoethanol (βME) was added to 0.1 mL of papain solution (100 μg/mL) containing 25 mM sodium phosphate buffer (pH 7.0) and 2 mL of inhibitor solution. After preincubation at 37°C for 5min, 0.2mL of 2mM BANA was added to initiate the reaction. After 10 min of incubation, 1 mL of cold 2% HCl in ethanol was added to stop the reaction. 1 mL of 0.06% ρ- dimethylamino-cinnamaldehyde dye was then added to show the colour of the final solution. Absorbance was read at 540 nm and the inhibitory activity was expressed as the percent decrease in OD_{540} compared to the control.

Molecular Weight of Inhibitor

The SDS-PAGE procedure was followed according to the method described by Laemmli (1970). A 12% resolving gel and a 3.5% stacking gel were used. The sample buffer consisted of 188mM M Tris-HCl pH 6.8, 15% β-mercaptoethanol, 3% SDS, 0.01% bromophenol blue, and 30% glycerol in deionized water. Sample preparation was conducted by mixing protein samples with loading buffer in a ratio 1:1 and heating the mixture to 95^0C for 10 min to denature the protein samples. An SDS-PAGE broad-range molecular weight standard (6.5 to 200 kDa) (Bio-Rad, Alfred Nobel Drive, Hercules, CA, U.S.A) was used. The running buffer consisted of 0.12 M Tris base, 0.95 M glycine and 0.5% SDS (w/v) in deionized water. Samples and protein standard were run into well. After finishing the process, gels were then immersed in deionized water for 5 min and stained with coomassie blue stain buffer for 20 minuntes and destained with a destaining buffer for 1 min. The gels were then immersed in 1% acetic acid and incubated for 1 h on a shaker. The acetic acid was replenished every hour until the gel was completely de-stained.

Statistical Analysis

Only descriptive statistics was utilized for the reporting of the data.

RESULTS AND DISCUSSION

Proximate Analysis of Blood Plasma

Table 3 shows the proximate analysis for each blood plasma. The moisture content of catfish blood plasma was 91.46% and 93.05% for the swamp eel.

Table 3
The proximate analysis of catfish (Pangasius hypopthalmus) *and swamp eel* (Monopterus albus) *blood plasma*

Source of plasma	Proximate Analysis		
	Moisture content (%)	Protein (mg/mL)	Ash (%)
Cat Fish	91.46	16.63	0.75
Swamp eel	93.05	11.92	0.92

According to Kisia (2016) and Martini (2005), moisture content in plasma of vertebrates is 90-92%. The difference of moisture content in plasma is due to differences in the chemical composition, such as protein content and ash content, which affects moisture content. Proteins are important components and functions in maintaining water balance in the blood and

tissues, regulating blood volume, helping the transport of fat, vitamins, and hormones and as antibodies.

The protein content of catfish plasma was 16.63 mg/mL and the swamp eel plasma was 11.92 mg/mL. Protein in plasma reflects the blood protein content, except for hemoglobin. Plasma proteins are divided into three main components i.e. albumin (42% w / v), globulin (56%) (α1-globulin, α2-globulin, β-globulin, and γ-globulin) and fibrinogen (1%). The largest proportions contained in solutes include proteins, which account for 6% -8% in blood plasma (Moure et al., 2003). Kisia (2016) suggested that solutes in fish blood plasma varied between freshwater fish and marine fish. The protein content of fish plasma is realtively low when compared to other vertebrates. The blood proteins are important in the maintenance of the right osmotic pressure and viscosity of blood.

The ash content of catfish and swamp eel plasma were 0.75% and 0.92% respectively. The difference of the ash content in plasma is related to endogenous (genetically controlled and associated with species-specific life cycles) and exogenous (such as environmental and dietary) factors (Perschbacher & Stickney, 2017). Ash content reflects the mineral content in blood plasma. Minerals in the blood plasma are part of a minor component of blood plasma which amounts to 1% (Martini, 2005; Moure et al., 2003). Minerals make up the normal extracellular fluid ion composition for vital cellular activity and contributes to the osmotic pressure of body fluids. The major plasma electrolytes are Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO^{3-}, HPO_4^{2-}, and SO_4^{2-} (Martini, 2005).

Inhibitory Activity of Blood Plasma

The inhibitory activity of blood plasma to protease enzyme increased as the concentration of blood plasma for both fishes increased. The protease inhibitory activity ranged from 7.66 to 50.73% for trypsin and 20.34 to 83.05% for papain for the catfish blood plasma (Figure 1), while the protease inhibitory activity of the swamp eel blood plasma ranged from 9.49 to 46.35% for trypsin and 28.81 to 64.41% for papain (Figure 2).

The blood plasma protease inhibitory activity to papain was higher than that of trypsin. This result was in contrast with the recommendation of Yongswatdigul et al. (2014) and Sriket (2014), where the surimi made from tropical fish were susceptible to serine protease attacks that caused protein degradation. But this was in line with the result of Ge et al., (2014) that reported softening occured in grass carp fillets caused by cathepsin B and L as the major endogenous enzymes leading to proteolytic degradation.

The highest protease inhibitory activity on trypsin and papain was by the swamp eel blood plasma even though its protein concentration was lower than that of the catfish blood plasma (0.15-0.60 mg/mL and 0.21-0.83 mg/mL, respectively). This was probably due to the higher efficacy of the protein plasma of the eel acting as a protease inhibitor as compared to that in

the catfish. Fowler and Park (2015) showed that protease inhibitory activity of salmon plasma ranged between 25% - 81% for papain and below 20% for trypsin. The higher inhibitory activity of the salmon blood plasma as compared to that of the catfish and swamp eel (this study) was probably related to it being free of impurities such as water, fat, ash, and other non-protein components.

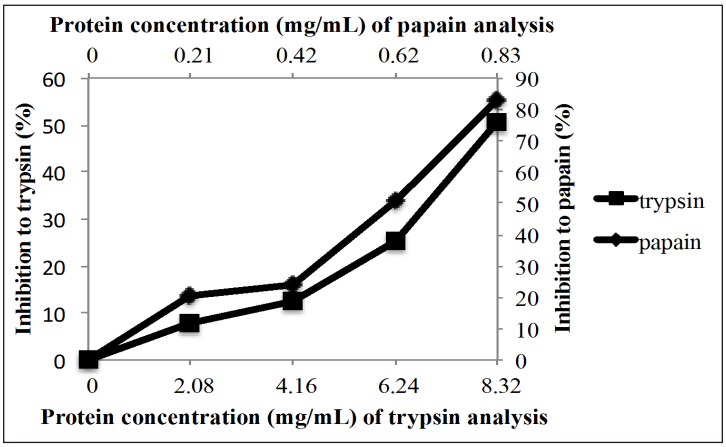

Figure 1. Inhibitory activity of catfish (*Pangasius hypopthalmus*) blood plasma to trypsin and papain enzymes

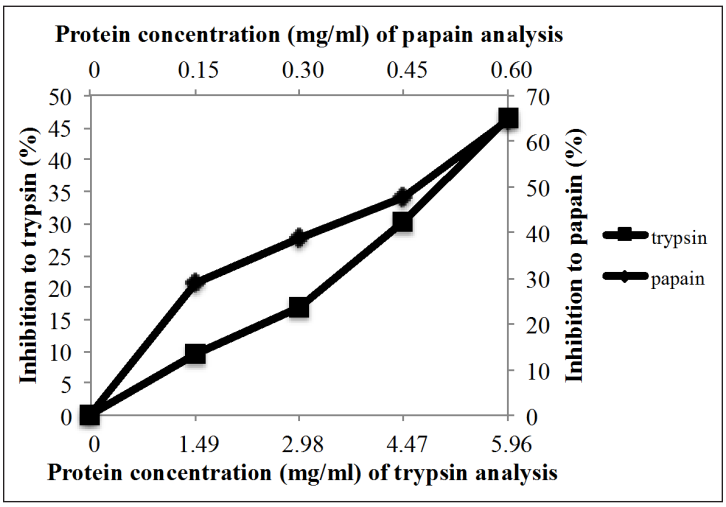

Figure 2. Inhibitory activity of swamp eel (*Monopterus albus*) blood plasma to trypsin and papain enzymes

Molecular Weight of Blood Plasma

The molecular weight of catfish (C1-C3) and swamp eel blood plasma (S1-S3) is in the range of 9.64 to 124.36 kDa (10 proteins) and 19.84 to 174.14 kDa (13 proteins) respectively (Figure 3). The molecular weight of the protein bands detected in the blood plasma of the catfish and swamp eel (this study) showed similarities with

molecular weight of pig plasma. The band is presumed to be α-globulin protein to have molecular weight ranging from 105 kDa - 150 kDa, while albumins had molecular weight of 65 kDa - 69 kDa. α_2-globulin is a protease inhibitor found indigenously in blood plasma and shows inhibitory activity againts serine, cysteine, carboxyl and metallo-proteinases (Benjakul et al., 2001; Lee e al., 2000). According to Benjakul and Visessanguan (2000), the serine protease inhibitor is in the range of 58-64 kDa and this is within the range of the proteins in catfish and eel plasma which is able to inhibit the enzymes chymotrypsin and trypsin. Li et al. (2008) reported the presence of the cysteine protease inhibitor at 55 kDa. The variation of the proteins from the catfish and swamp eel determined by the SDS-PAGE analysis reflected their inhibitory activity. The higher variation of the proteins in the swamp eel blood plasma may explain its higher inhibitory activity than that of the catfish.

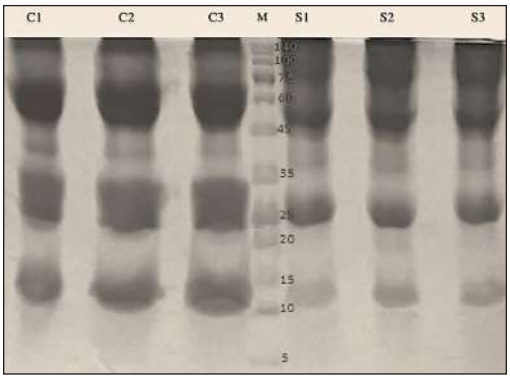

Figure 3. Molecular weight of catfish (*Pangasius hypopthalmus*) (C1, C2, C3) and swamp eel (*Monopterus albus*) (S1, S2, S3) blood plasma (M= protein marker)

CONCLUSION

Both the catfish and swamp eel blood plasma showed inhibitory activity towards trypsin and papain. Papain is a cysteine protease and its inhibition was higher than that of trypsin. The highest inhibitory activity to protease enzyme was from the swamp eel blood plasma.

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