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Protease inhibitory activity profile of Indonesia wild swamp eel (*Monopterus albus*)

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Abstract

A protease inhibitor is a compound that potentially could inhibit the activity of a protease or some protease enzymes. It has a significant role in fish processing to prevent quality deterioration. The study aimed to investigate the inhibitory activity of wild swamp eel (*Monopterus albus*) plasma fractionated with ethanol to papain enzyme. The parameters analyzed were protein content, inhibitory activity to papain enzyme, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis to demonstrate the protein profile. The result showed that the amount of protein in the plasma fractionated with ethanol was decreased. The effectiveness in inhibiting papain enzyme was increased when compared to that of the crude plasma, from 65 to 128.56% per mg protein. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that the protein complex alpha-2-macroglobulin in wild swamp eel plasma consists of two different subunits of 120 kDa and 110 kDa. This study suggested that plasma fractionated with ethanol showed inhibitory activity to papain enzyme greater than that of the crude plasma.

1. Introduction

Fish is well known for its high nutritious protein content, a significant amount of minerals, rich in some vitamins, and unique fatty acid with relatively low lipid content. They have a wide variety of nutritional compositions, not only among species but also within the same species from one individual to the other. The variation was affected by several factors, such as maturity, sex, environment, and season. The protein in fish amounts to 16-21%, and fish contains all essential amino acids (Petricorena, 2014). Fish harbours internal protease enzymes. Protease can be divided into endopeptidase and exopeptidase according to the cleavage site. Concerning the active site, protease is classified into four groups: serine, cysteine, aspartic, and metalloprotease as proposed by the Enzyme Commission of the International Union of Biochemists (Sriket, 2014).

Being highly perishable contributes to the limited shelf life of fish and fish products due to their high protein content. The internal protease enzymes were one of the causes of this condition, and they are excreted from the fish's digestive organ and muscle. Singh and Benjakul (2018) reported that the predominant type of protease in fish's digestive system and skin was serine, whereas in muscle was cysteine protease. Self-digestion by the internal protease occurred during post-mortem handling and storage of fish, known as autolysis, and the enzymes worked with no control of their host. Felberg *et al.* (2010) reported that the phenomenon of autolysis both in herring (*Clupea harengus*) and sardine (*Sardina pilcharus*) was due to protease enzymes, such as elastase, trypsin, and aspartyl aminopeptidase based on the result of partial sequences the peptide derived from the proteases. In addition, Cathepsin and Calpain, a group member of cysteine protease, also induced the autolysis and softening of muscle fish. Tang *et al.* (2019) confirmed Cathepsin L as a cause of gel weakening or modori in Sturgeon (*Acipenseridae*) surimi gel. The gel strength of surimi decreased as the result of hydrolysis of Myosin Heavy Chain (MHC) by Cathepsin L, known as a lysosomal cysteine protease.

Modori caused by Cathepsin L generally can be evaluated at the temperature range of 50 to 70°C (Tang *et al.,* 2019). Park *et al*. (2013) reported the optimum

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temperature for Cathepsin L to induce gel weakening during slow heating was 55°C. Softening gel became a problem in the surimi processing industry. To solve the problem, the surimi industry uses protease inhibitors, such as dried egg white, potato powder, bovine plasma, and chicken plasma. However, bovine and chicken plasma were no longer used due to cow disease and avian influenza, whereas dried egg white and potato powder were more effective against serine protease. Blood plasma contains alpha-2-macroglobulin that acts as a protease inhibitor for all types of proteases. Alpha-2-macroglobulin is a nonspecific protease inhibitor and is widely available in fish plasma (Li *et al.*, 2008b). The wild swamp eel is a kind of freshwater fish. They are classified as nocturnal animals that spend their daytime in the holes and breath assisted by additional respiratory organs (Hilles *et al*., 2018). Swamp eel provided a lot of blood when gutted or headed compared with the other fish. Studies on the use of fish blood plasma as a protease inhibitor had been reported, and the result showed their ability as an inhibitor, such as grass carp (*Ctenipharyngodon idellus*) plasma (Li and Lu, 2006); rainbow trout (*Oncorhynchus mykiss)* plasma (Li *et al.*, 2008b); chum salmon (*Oncorhynchus keta*) plasma (Li *et al.,* 2008a); chinook salmon blood plasma (Fowler and Park, 2015a). Nopianti *et al*. (2019) reported catfish (*Pangasius sutchi*) and wild swamp eel (*Monopterus albus*) plasma could inhibit the activity of trypsin and papain. The highest inhibition was resulted by wild swamp eel plasma to papain enzyme. Following the result, this study aimed to further investigate the inhibition by fractionated wild swamp eel plasma and analyze its inhibitory activity.

2. Materials and methods

2.1 Material

Life swamp eel (Figure 1) from natural catch with the size 10-15/kg derived from Karawang, West Java, Indonesia transported by bus at night to avoid sun exposure. Papain (from papaya latex), trypsin (from bovine pancreas), N_{α}-benzoyl-$_{DL}$-arginine-β-naphthylamide (BANA), Na-Benzoyl-$_{L}$-arginine 4-nitroanilide hydrochloride (BAPNA), 2-mercaptoethanol, ρ-dimethylamino-cinnamaldehyde were purchased from Sigma Aldrich (USA). A broad range protein standard (10-260kDa) was purchased from Thermo Fisher Scientific (USA).

Figure 1. Swamp eel (*Monopterus albus*)

2.2 Collecting of blood plasma and fractionation sample preparation

Whole blood was collected immediately from the bleeding wild swamp eel with the addition of 10% EDTA (Ethylenediaminetetraacetic acid) to avoid coagulation at the ratio of 100:1, then centrifuged (Eppendorf™5804 R, Fisher Scientific, Sweden) at 3000 rpm for 25 mins. The collecting of plasma was followed by centrifugation of the supernatant at 3000 rpm for 25 mins. The plasma was kept at -20°C before being used.

Fractionation of plasma was conducted using the principal differences in the solubility of plasma protein in ethanol. Preparation of the fractionation sample was done by thawing the plasma. Fractionation followed the method of Cohn *et al*. (1946). A 40% ethanol with a pH of 5.5, made from 96% ethanol, was used to collect the samples rich in alpha-2-macroglobulin. Plasma was diluted into distillation water with a ratio of 1:1 to reach the protein concentration of 30-35 mg/mL. Then the sample was mixed with cold ethanol at a ratio of 2:1 (Denizli, 2011). The precipitation of the samples lasts for 1.5 hrs. After precipitation, the samples were centrifugated at 3000 rpm for 10 mins. The pellets were taken and dried with nitrogen gas for 30 mins or until their weight was constant. Dry pellets solubilized with 200 µL of 20 mM Tris-HCl pH 8.0. Then, the fractionation samples were kept at -20°C until used. All the precipitation process was under cold conditions (4°C).

2.3 Protein content

The protein content analysis was carried out by following the method of Bradford (1976), and Bovine Serum Albumin was used as the standard (1000, 800, 600, 400, 200, and 100 ppm). The samples were measured by using the instrument iMark™ Microplate Absorbance Reader (Bio-Rad, USA). A 10 µL of the sample was mixed with 250 µL of Bradford reagent and allowed to react at room temperature. The absorbance was read at 595 nm.

2.4 Papain inhibition assay

The measurement of papain inhibition was by following the method Abe *et al.* (1994). Four different volumes of plasma (25, 50, 75, and 100 µL) were adjusted to 1.1 mL. An amount of 0.2 mL of 0.25 M sodium phosphate buffer (pH 6.0) that contained 2.5 mM EDTA, 25 mM 2-mercaptoethanol, was added to 0.1 mL

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of papain (5 µg/mL) in sodium phosphate buffer (pH 7.0), and then followed by addition of 0.2 mL inhibitor solution. The mixture was homogenized with vortex and incubated at 37°C for 5 mins. After the first incubation, for starting the reaction, 0.2 mL of 1 mM BANA was added into the solution and then continued to the second step of incubation at 37°C for 10 mins. The reaction was stopped by using 1 mL of 2% HCl in ethanol. As much as 1 mL of 0.06% ρ-dimethylamino-cinnamaldehyde was added to form colour. The absorbance read at 540 nm and the inhibitory activity was shown as the per cent decrease in OD_{540} compared to the control.

2.5 Trypsin inhibition assay

The measurement of trypsin inhibition was by following the method of Fowler and Park (2015a). Four different volumes of plasma (25, 50, 75, and 100 µL) were adjusted to 250 µL. An amount of 150 µL of inhibitor solution was mixed with 300 µL of trypsin enzyme (20 µg/mL) and 150 µL of distilled water. Then, the mixture was homogenized and incubated at 37°C for 10 mins. As much as 750 µL of 50 mM BAPNA in Tris HCl buffer (pH 8.2) containing 20 mM $CaCl_2$ and pre-warmed to 37°C was added to the mixture solution. The reaction mixture was incubated at 37°C for 10 mins and then stopped by adding 150 µL of 30% acetic acid. The absorbance read at 410 nm. The inhibitory activity was reflected as the per cent decrease of OD_{410} from the control.

2.6 Molecular weight of plasma ethanol fraction as an inhibitor

Molecular weight was determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to Laemmli (1970). The analysis used a 12% separating gel and 4% stacking gel. The sample buffer stock consisted of 0.5 M Tris HCl (pH 6.8), 26.3% glycerol, 1% bromophenol blue, 10% SDS, and then adjusted to 10 mL with deionized water. Freshly prepare 5% of 2-mercaptoethanol mixed with the sample buffer based on the total volume, and then used at the ratio of 1:1 to the sample. The samples were heated at 90°C for 5 mins to denature the protein samples. A broad range protein standard (6.5-205 kDa) was used as a marker. The running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.1% SDS. The concentration protein sample load was 20 µg for each well. After the running samples process finished, gels were immersed in deionized water for 5 mins, and then stained with coomassie blue stain for 30 mins. The destaining step was by allowing the gels to be soaked in the destaining solution overnight at room temperature. The molecular weight of the protein was determined by comparing it to the protein standard.

2.6 Statistical analysis

The only statistical analysis used descriptive statistics to report the data. All measurements were conducted in triplicates.

3. Results and discussion

3.1 Protein contents of plasma and plasma ethanol fraction

Protein is one of the major compositions in fish. Both marine and freshwater fish offer protein sources that contain all essential amino acids (Petricorena, 2014). Table 1 shows the protein content types of plasma. The protein contents of crude plasma and plasma fractionated with ethanol were 63.05 mg/mL and 6.36 mg/mL, respectively. The wild swamp eel crude plasma contained lower protein content than rainbow trout plasma (612.3 mg/mL) (Li *et al.*, 2008b). Generally, the protein content of the plasma is 60-80 mg/mL (Phillips and Williams, 2011). It indicated that the protein content of plasma in the wild swamp eel was within the normal range. The difference in plasma protein levels resulted is due to differences in the separation or fractionation process (Li *et al.,* 2008b). Crude plasma had higher protein content compared to that plasma fractionated with ethanol. It was probably due to the fractionation process that used 40% ethanol with pH 5.5 which targeted the fraction rich in α-2-macroglobulin. Rawdkuen *et al.* (2007) reported that the proteinase inhibitor from chicken plasma after fractionation by ammonium sulfate decreased compared to that of the crude chicken plasma protein. It means, that regardless of the chemicals used for fractionation, the protein contained in the resulting fraction will decrease.

Table 1. The protein contents of wild swamp eel (*Monopterus albus*) crude plasma and plasma fractionated with ethanol

Types of plasma	Protein content (mg/mL)
Crude plasma	63.05±8.10
Plasma fractionated with ethanol	6.36±0.22

3.2 Inhibition assays of protease

The wild swamp eel crude plasma had inhibitory activity on both trypsins (Figure 2) and papain enzyme (Figure 3). The percentage of inhibitory activity increased as the amount of protein increased. The plasma contained a protease inhibitor, which was active on both serine protease (trypsin) and cysteine/thiol (papain). Alpha-2-macroglobulin is one of the inhibitors of proteases in plasma, which can inhibit almost all protease classes (serine, carboxyl, thiol, metalloprotease) (Rehman *et al*., 2013). Fowler and Park (2015b) reported chinook salmon plasma that inhibits the activity of both trypsin and papain. Lin *et al.* (2008) also revealed

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rainbow trout (*Oncorhynchus mykiss*) plasma which can work against the activities of papain and trypsin as the plasma concentration used was increased.

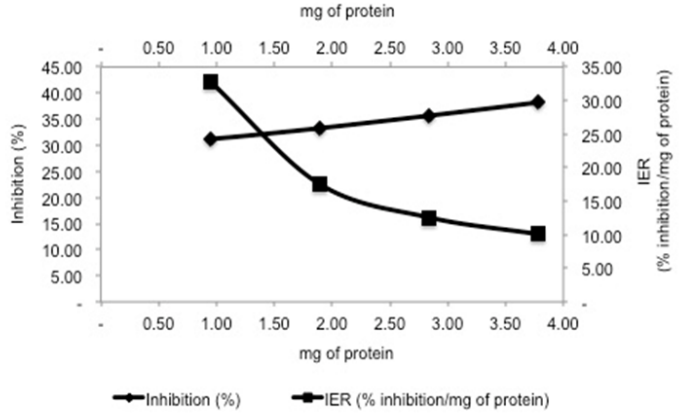

Figure 2. Inhibitory activity crude plasma of wild swamp eel to trypsin enzyme.

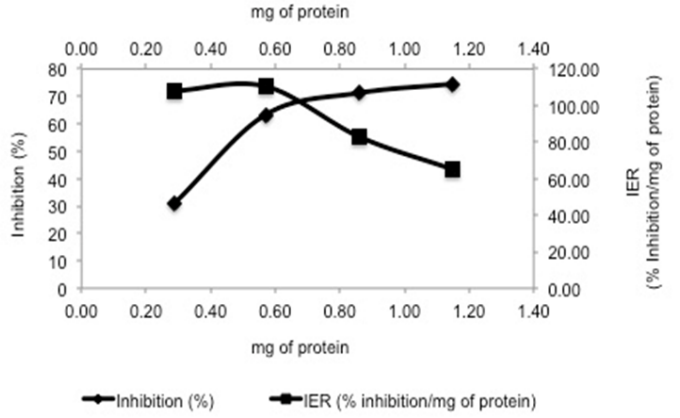

Figure 3. Inhibitory activity crude plasma of wild swamp eel to papain enzyme

The highest inhibition of wild swamp eel plasma was to papain enzyme, with the working concentration of protein lower than that of the trypsin. It means that wild swamp eel plasma is more effective to inhibit papain, a cysteine protease, than trypsin. These imply a new outlook to apply one of the wild swamp eel plasma functions as the inhibitor agent of cysteine protease. The objective is to prevent heated gel degradation or modori caused by Cathepsin L. The inhibition activity of the swamp eel plasma (30.98-74.52%) showed as if lower than that of salmon blood plasma (20-87%) (Fowler and Park, 2015a). It might be related to the different amounts of protein tested. The previous study reported modori phenomenon in sturgeon (*Acipenseridae*) surimi gels as a result of Cathepsin L activity at 40°C (Tang *et al.,* 2019). Hu *et al.* (2012) reported that the gel strength of carp (*Cyprinus carpio*) surimi was reduced significantly with the addition of purified cathepsin L. Yamada *et al.* (2020) reported the modori reaction in surimi blue grenadier and Alaska Pollock involved cysteine protease at 50°C and 60°C.

On the contrary, the % inhibitory/mg of protein or Inhibition Efficiency Ratio (IER) was decreased as the amount of protein (mg) increased (Figure 2 and Figure 3), both in trypsin and papain. IER expressed inhibition efficiency ratio (Chen *et al.,* 2007) of plasma (inhibitor) to papain and trypsin. The decrease in IER value was due to the number of substrates and enzymes that were fixed in this analysis, hence saturation reaction occurred between inhibitors and enzymes. As the impact, the speed of the reaction rate of inhibitor decreased as the inhibitors increased because fewer enzymes were available to bind for inhibiting their activities. Alpha-2-macroglobulin is a non-competitive inhibitor. The action mechanisms of the non-competitive inhibitor are in two ways, namely direct binding to the sites other than the enzyme active site or binding to the enzyme-substrate complex to the sites other than the enzyme active sites. These actions alter the enzyme conformation such that the substrate is inhibited to bind to the enzyme. The enzyme also cannot conduct catalytic activity in the presence of an inhibitor (Satyanarayana and Chakrapani, 2020).

A different trend resulted from plasma fractionated with ethanol (Table 2) compared to that of the crude plasma, where the highest amount of protein (0.44 mg) gave the highest inhibition and also IER values. It may be related to the purity level of the plasma sample. After the fractionation process using 40% ethanol, the end product became richer in alpha-2-macroglobulin. The evidence also can be seen from SDS-PAGE. There were two thick bands in the protein profile of the plasma fractionated with ethanol. It indicated that the sample was rich in alpha-2-macroglobulin (concentrated). Therefore, the inhibitory and IER value of the plasma fractionated with ethanol were higher than the crude plasma. Lin *et al*. (2018) reported the IER trend of ACE-inhibitory peptide from *Chlorella sorokiniana* protein hydrolysate increased after purification through Reversed-Phase HPLC compared to size exclusion chromatography.

3.3 Protein profile of plasma ethanol fraction

In determining the molecular weight of the protein in the fraction, the samples were run under reducing conditions with heated and no heated treatment. Two bands of estimated molecular weight of 146 kDa and 105

Table 2. Inhibitory activity of plasma fractionated with ethanol.

Sample	Volume of fraction (µL)	mg of protein	% Inhibition	IER (%Inhibition/mg of protein)
Plasma fractionated with ethanol	25	0.11	10.43	93.59±17.22
	100	0.44	56.99	128.56±0.02

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kDa were detected in crude plasma (Figure 4). Li *et al.* (2008b) reported protein bands with molecular weights around 60, 80, 110, and 180 appeared on the SDS-PAGE protein profile of rainbow trout plasma. On the other hand, two bands with molecular weights of 120 kDa and 110 kDa were detected in plasma fractionated with ethanol (Figure 4). Li and Lu (2006) reported the alpha-2 -macroglobulin from grass carp (*Ctenopharyngodon idellus*), which consisted of two different subunits of 95 kDa and 80 kDa, after purification by ion-exchange chromatography. The groupers (*Epinephelus coioides*) also resulted in two bands with molecular weight 97 and 80 kDa after the purification step by hydrophobic interaction chromatography (Chuang *et al*., 2013). The purification process made the protein components separate. The presence of mercaptoethanol in the sample buffer and the heating process of the sample after mixing with the sample buffer before running into SDS-PAGE gels led to the complete denaturation of the protein samples. Mercaptoethanol is a reducing agent, which can denature the protein samples by breaking disulfide bonds, which then separate the polypeptides that constitute the protein multisubunit such that they can be analyzed (Albert *et al*., 2008). Another compound in the sample buffer that caused protein denaturation is sodium dodecyl sulfate (SDS) which is an anionic surfactant with a long hydrophobic tail and a negatively charged head (Eidhammer *et al.*, 2007).

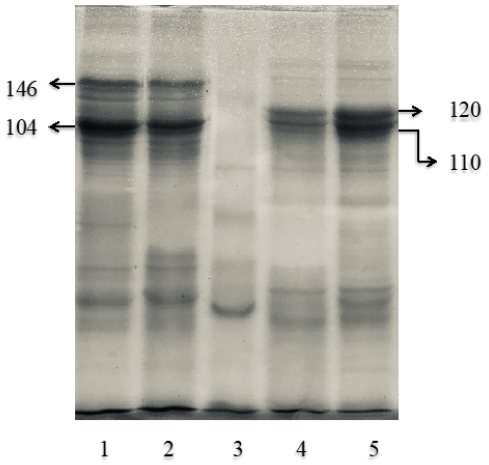

Figure 4. Protein profile of plasma ethanol fraction. Lane 1: crude plasma+mercaptoethanol, heated; Lane 2: crude plasma+mercaptoethanol, no heated; Lane 3: marker; Lane 4: plasma ethanol fraction+mercaptoethanol, no heated; Lane 5: plasma ethanol fraction, heated).

It appears that after the fractionation process, the protein complex alpha-2-macroglobulin in the wild swamp eel plasma consists of two different subunits of 120 kDa and 110 kDa. The protein result still requires purification steps to confirm the molecule characteristics.

4. Conclusion

Plasma fractionated with ethanol showed inhibitory activity to papain enzyme greater that of the crude plasma. The value of IER (% inhibition/mg protein) plasma fractionated with ethanol of 128.56 was higher than that of crude plasma of 65. It indicates the effectiveness of fractions as an inhibitor. Two bands of estimated molecular weight of 146 kDa and 105 kDa were detected in crude plasma. The protein complex of alpha-2-macroglobulin from wild swamp eel plasma consists of two different subunits of 120 kDa and 110 kDa. As the recommendation, further purification step is still needed to further characterize the molecular features and activities of the inhibitor.

Conflict of interest

The authors declare no conflicts of interest.

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