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Characterization of trypsin enzyme extracted from intestines of yellowfin tuna (*Thunnus albacares*) and bigeye tuna (*Thunnus obesus*) from Indonesian seawater

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Abstract

Recently, there has been a major concern about fisheries waste produced by tuna processing factories. There are many ways to handle the waste from tuna processing factories, specifically the tuna viscera which contains the stomach, pyloric caeca, kidneys, and intestines. The tuna viscera have been known as a source of digestive enzymes, one of them is a protease, including trypsin. The objective of this research was to analyze the enzymatic characteristic of trypsin extracted from two different tuna species. In this study, the trypsin extracted from the intestines of yellowfin and bigeye tuna was characterized by analyzing its enzyme activity as well as optimum temperature and pH, protein concentration, kinetic studies, and molecular weight. The results reveal that the enzyme activity and protein concentration observed in bigeye tuna (*Thunnus obesus*) were significantly higher than in yellowfin tuna (*Thunnus albacares*). In addition, trypsin bigeye and yellowfin tuna had the optimum temperature and pH from both crude extracts within the range of 50-60˚C and 7-8, respectively. Moreover, the molecular weights of trypsin enzymes extracted from yellowfin (*T. albacares*) and bigeye tuna (*T. obesus*) were 29 kDa.

1. Introduction

It is widely recognized that Indonesia has a large marine ecosystem from which fisheries commodities have been harvested. One of the most valuable commodities is tuna fisheries with the fishing grounds situated in Eastern Indonesia. As the leading country in tuna fisheries, this species has been widely exported to various countries, including Southeast Asian countries, Japan, the Middle East, France, and the USA (AP2HI, 2021). However, there has been a major concern faced by the fisheries industries. Globally, it is estimated that the fish processing industries produce and discard waste in a large volume annually, accounting for up to 60% of the harvested biomass (Siddik *et al.,* 2020). This condition has been leading to economic loss and environmental problems. Therefore, the development of several recovery processes to produce biomolecules from fish by-products (including head, skin, fins, viscera, and roe) is required (Zamora-Sillero *et al.,* 2018). These biomolecules can be used as the source of natural enzymes for various types of industries.

The demand for enzymes has continuously increased every year, especially for the leather, pulp and paper, textile, feed, and detergent industries (BPPT, 2019). However, the supply mostly comes from imported products. The amount of enzyme needed for industrial needs was estimated to be 2,500 tons with import values of IDR 200 billion in 2017 (BRIN, 2017). Regarding that, it is important to explore potential sources to produce a high amount of enzyme, thus, the national demands could be fulfilled. Another crucial factor is that there has been an increasing trend in halal product consumption, including cosmetics and pharmaceuticals. According to the report of State of the Global Islamic Economy 2018/2019 by the Centre of Dubai Islamic Economy Development (CDIED) (2018), Indonesia has not yet reached the top ten leading countries in terms of the halal industry for cosmetics and pharmaceuticals. Hence, the use of fish as an alternative to raw material in enzyme production would be very beneficial for those aspects.

In the last few decades, several studies about

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producing the natural enzyme from tuna viscera have been conducted. The tuna viscera which contains the stomach, pyloric caeca, kidneys, and intestines have been known as the source of digestive enzymes, one of which is a protease. Proteases are the group of enzymes that hydrolyze the peptide bonds in proteins and polypeptides, which are classified into several groups based on their properties, such as substrate specificity, catalytic mechanism, as well as optimum pH and temperature (Zhu *et al.,* 2019). One of the proteases studied in this article is trypsin which is known as serine proteases secreted from the pancreas (Bond, 2019).

The isolation of trypsin from various fish species has been performed, for instance in oil sardine (*Sardinella longiceps*), albacore tuna (*Thunnus alalunga*), Atlantic cod (*Gadus morhua*), and Kawakawa tuna (*Euthynnus affinis*). Although it was isolated from various digestive organs of fish species, such as fish a whole of fish viscera, spleen, and intestines, the trypsin enzymes revealed similar biochemical properties. Those fish trypsin indicated the optimum catalytic activity within the specific range of temperature and pH, accounting for 40-60˚C and 6-11, respectively (Khandagale *et al.,* 2017; Poonsin *et al.,* 2019; Sandholt *et al.,* 2019; Nurhayati *et al.,* 2020; Khoa *et al.,* 2021).

Moreover, fish trypsin can be beneficial for further application. Fish protein hydrolysate containing antioxidant activity could be produced from fish trypsin purified from unicorn leatherjacket (*Aluterus monoceros*) (Zamani and Benjakul, 2016). The fish trypsin immobilized onto magnetic-chitosan composite can be used to detect the antinutritional factors in aquafeeds that influence animal growth during husbandry (Azevedo *et al.,* 2018). Another potential application in specific biomedicines production as to its effectiveness against pathogens, including bacteria and viruses (Jesús-de la Cruz *et al.,* 2018). However, the information about trypsin isolated from the digestive tract of tuna as tropical fish, especially from its intestines, is very limited. Meanwhile, it is estimated that the trypsin extracted from fish intestines, including tuna intestines, would produce more benefits as to the presence of abundant digestive enzymes. Therefore, the objective of this research is to determine the properties of trypsin extracted from the intestines of yellowfin (*T. albacares*) and bigeye tuna (*T. obesus*) species.

2. Materials and methods

2.1 Materials

A total of two species of tuna viscera used in this study were yellowfin tuna (*T. albacares*) (purchased from local fishermen in Gorontalo) and bigeye tuna (*T. obesus*) (purchased from PT Pahala Bahari Nusantara). Besides, there were several chemicals used, such as tris base (SIGMA), distilled water, acetic acid (Merck KGaA), N-α-benzoyl-DL-arginine-p-nitroaniline (BAPNA) (Sigma), $CaCl_2.2H_2O$ (Merck), *bovine serum albumin* (BSA) (SIGMA), DMSO, and the chemicals for SDS-PAGE analysis, including buffer (0,125 M tris-HCl pH 6,8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol (βME), 4% separating and 15% stacking gels, *Coomassie Brilliant Blue* (AppliChem), acetic acid, and methanol (Merck). All chemicals are for analysis.

2.2 Sample preparation and morphometric measurement

The intestines were separated from other parts of the tuna viscera. Fish intestines were then stored in a freezer at -20°C to minimize protease autolysis. When it will be used, the intestine is thawing and stored at a low temperature (4°C). After that, the length, width, and weight of intestines were measured (the number of samples for each species was 10 fishes). Then, the intestines were washed, cut into small pieces, and ground for proximate analysis, enzyme extraction, and further enzymatic assays.

2.3 Proximate analysis of tuna intestines

Chemical compositions of tuna intestines were analyzed in duplicate consisting of four parameters, namely water, ash, fat, and protein following the methods of AOAC (2005).

2.4 Extraction of trypsin from the tuna intestines

The extraction of trypsin from the tuna intestines was performed following the method by Barkia *et al.* (2010). The intestines are cut into 3-5 cm, then immersed in liquid nitrogen until frozen. Furthermore, the intestine sample was homogenized (homogenizer) with buffer Tris -HCl (0.01M; pH 8) with a 1:4 ratio (b/v), and then it was centrifuged for 30 mins (11,000 rpm) at 4˚C, producing supernatant as the crude extract of trypsin. The properties of this crude extract then analyzed, including enzyme activity, optimum temperature and pH, protein concentration, and enzyme kinetics were analyzed according to the following procedures.

2.5 Analysis of enzyme activity, optimum temperature and pH

The method by *Barkia et al.* (2010) was used to analyze the enzyme activity, optimum temperature and pH of the activity. The crude extract of trypsin was evaluated by using BAPNA as the substrate. The solution of BAPNA was made from 0.0435 g of BAPNA and 1 mL of DMSO, then mixed with buffer Tris-HCl 0.05M containing $CaCl_2.2H_2O$ 0.02M until the volume

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reached 100 mL. Then, as many 0.05 mL of crude extract mixed with BAPNA as many as 2.5 mL, it was incubated for 10 mins at 37˚C. Next, as many as 1 mL of acetic acid (30%) was added to the mixture and then incubated for 10 mins at 7˚C. The sample absorbance was measured using a spectrophotometer with a wavelength of 410 nm, after which the enzyme activity was calculated based on the formulation below.

$$\text{Enzyme activity (U/mL)} = \frac{(A - A0) \times Vt \times 1\,000}{8{,}800 \times T \times V1}$$

Where A: Sample absorbance at λ= 410 nm, A0: Blank absorbance at λ= 410 nm, T: Incubation time (mins), Vt: Total volume (mL), V1: Sample volume (mL) and 8,800: coefficient of p-nitroaniline

The same procedure previously mentioned was also applied, but the incubation temperature was set as follows: 20˚C, 30˚C, 40˚C, 50˚C, 60˚C, and 70˚C, while the pH of BAPNA substrate were set at 5, 6, 7, 8, 9, and 10.

2.6 Analysis of protein concentration

According to the Bradford (1976) method, as many as 0.1 mL of sample was mixed with 5 mL Bradford reagent. The mixture was then incubated for 5 mins at 35˚C and measured using a spectrophotometer with wavelength 595 nm. Then, the standard solutions (2 mg/mL of BSA) were prepared and measured using the same wavelength. After that, the standard curve was made to determine the absorbance values of samples, which are equal to the concentration of protein. This number was used for calculating the specific enzyme activity by using the equation below.

$$\text{Specific enzyme activity (U/mg)} = \frac{\text{enzyme activity (U/mL)}}{\text{protein concentration (mg/mL)}}$$

2.7 Kinetic studies

To determine the kinetics of the enzyme reaction following Lehninger (1977), firstly, trypsin enzyme activity was measured using BAPNA as a substrate at concentrations of 0, 1, 1.5, 2, 2.5, 3, and 3.5 mM. Furthermore, the calculation of the value of K_m and V_{max} using the Lineweaver-Burk equation can be seen below.

$$\frac{1}{V_0} = \frac{1}{Vmax} + \frac{Km}{Vmax}\frac{1}{[S]}$$

Where V_0: Initial rate of reaction, [S]: Substrate, V_{max}: Maximum rate of reaction and K_m: Michaelis-Menten constant value

2.8 Determination of molecular weight using SDS-PAGE

The molecular weight of trypsin was determined by using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) with the molecular weight marker (Precision Plus Protein™ Standards, Size 10-250 kDa, Bio Rad) as well as the 4% concentration of gel and 15% concentration of separation gel. The electrophoresis was running under the system at 13 mA/gel at 100 V for 4 hrs. After that, the staining using 0.1% *Coomassie Brilliant Blue* R-250 dissolved in 50% methanol and 7% acetic acid, following which rinsing using the 7% acetic acid until the bands were visible.

2.9 Data analysis

Data collected from this study were processed by using Microsoft Excel 2010 to calculate the mean and deviation standard, and then the data were presented in a table and graph and described descriptively. Especially for morphometric, proximate, and enzyme activity data, a t-test was carried out to compare the characteristics of yellowfin and bigeye tuna. Mathematical model t-test test according to Mattjik and Sumertajaya (2006) is:

$$t\ count = \frac{X_1 - X_2}{Sg\sqrt{\left(\frac{1}{n_1}\right) + \left(\frac{1}{n_2}\right)}}$$

$$Sg = \sqrt{\frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1+n_2-2}}$$

Where t = value t count, X_1 = Average value of the first group, X_2 = Average value of the second group, n_1 = Number of subjects in first group, n_2 = Number of subjects in second group, $S^2{}_1$ = First group variant, $S^2{}_2$ = Second group variant.

3. Results and discussion

3.1 Morphometric and proximate analysis of fish intestines

The morphometric parameters measured in both tuna species were length, width, and weight (Table 1). The result indicates that the intestine length of both yellowfin and bigeye tuna was in the range of 24-32 cm, yet the bigeye tuna possesses slightly longer and heavier intestines than the yellowfin tuna. The results of the t-test showed that the length, width, and weight of the intestines of bigeye tuna were significantly longer and heavier than those of yellowfin tuna but the width of the intestines of bigeye tuna was smaller than that (*p-value*<0.05). In comparison with kawakawa tuna (*Euthynnus affinis*), the intestine length and weight of yellowfin and bigeye tuna remain higher. Arbajayanti *et al*. (2021) reported that the intestines of yellowfin tuna were 51.43 cm long, 7.33 cm wide, and weighed 2.07 g. This might be due to the difference in fish sizes, especially body length and weight, which could affect the morphology of the digestive tract, including the intestines. Regarding the body length, the most

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representative biological parameter in fish is fork length. Darondo *et al.* (2020) reported that the average fork length of yellowfin tuna captured by handline and longline fishing gears in the eastern Indonesian sea was around 120-127 cm. Meanwhile, the average fork of kawakawa tuna (*E. affinis*) was only 34.1 cm.

Table 1. Comparison of the morphometric parameters of yellowfin and bigeye intestines with other tuna species.

Parameters	Yellowfin tuna (*T. albacares*)	Bigeye tuna (*T. obesus*)	Kawakawa tuna (*E. affinis*)[1]
Length (cm)	24.75±0.86[a]	32.33±6.90[b]	25.27±1.17
Width (cm)	1.77±0.20[b]	0.89±0.35[a]	N/A
Weight (g)	1.95±0.27[a]	6.75±3.41[b]	4.43±0.86

[1]Nurhayati *et al*. (2020)
Values with different superscripts are statistically significantly different based on t-test on yellowfin and bigeye tuna. Comparisons were made per test parameter. N/A: Not analyzed.

For the proximate analysis, Table 2 summarizes that the most dominant component in both yellowfin and bigeye tuna intestines was water (ranging from 75.02±3.37 to 78.05±0.04% w/w), which was also found in the kawakawa tuna (75.09 %w/w). It can also be seen that the protein and fat contents were also revealed as the major component in yellowfin and bigeye tuna, respectively. This might be influenced by the prey composition in the stomach. The result of the t-test showed that moisture, protein, and ash content of both bigeye and yellowfin tuna were relatively the same (*p-value*>0.05), but the fat content of yellowfin tuna was significantly higher than bigeye tuna (*p-value*<0.05).

Table 2. Comparison of the proximate analysis of yellowfin and bigeye intestines with other tuna species.

Parameters	Yellowfin tuna (*T. albacares*)	Bigeye tuna (*T. obesus*)	Kawakawa tuna (*E. affinis*)[1]
Water content (%w/w)	75.02±3.37[a]	78.05±0.04[a]	75.09
Ash content (%w/w)	1.01±0.75[a]	4.55±0.06[a]	N/A
Fat content (%w/w)	2.56±0.10[b]	0.71±0.01[a]	0.87
Protein content (%w/w)	19.56±1.25[a]	16.32±0.13[a]	16.72

[1]Nurhayati *et al*. (2020)
Values with different superscripts are statistically significantly different based on t-test on yellowfin and bigeye tuna. Comparisons were made per test parameter. N/A: Not analyzed.

Arbajayanti *et al*. (2021) reported that the intestine of yellowfin tuna, which has a larger morphometric size than the intestine of yellowfin tuna used in the study, had a lower water content (71.41%), higher protein (23.84%), ash 1.9% and 1.03% fat. The high protein content has an impact on the activity of the trypsin enzyme which is higher as well. A study conducted by Setyadji *et al.* (2012) regarding the stomach content of yellowfin and bigeye tuna showed that the main diet was fish (56-82%), followed by cephalopods (squids) as the complementary diet (0-8%) and crustaceans (shrimps) as the additional diet (2-4%). Interestingly, the ash content in bigeye tuna was higher than in yellowfin tuna, which could be caused by the presence of a component containing calcium carbonate called otolith in the stomach content. Lin *et al.* (2020) discovered that the stomach of bigeye tuna collected in the western Indian Ocean contains not only 642 fish but also 1,021 otoliths.

3.2 Enzyme activity and protein concentration from the crude extract of trypsin

The crude extract of trypsin produced from the intestine of yellowfin dan bigeye tuna was analyzed further by determining the enzyme activity and the protein concentration using the same substrate (BAPNA) (Table 3). The result of the t-test showed that the specific activity of trypsin was slightly lower in yellowfin tuna than in bigeye tuna (0.049 U/mg and 0.158 U/mg, respectively) (*p-value*<0.05). These values are still lower compared to the specific activity of the trypsin enzyme in kawakawa tuna (*E. affinis*) (Nurhayati *et al.,* 2020), and even much lower compared to other fish species. This could have resulted from different species used during enzyme assay and sources of organs for enzyme extraction. In comparison with the use of fish viscera for enzyme extraction, the use of a more specific internal organ is foreseen to decrease the amount of enzyme extracted. Fish viscera is the internal organ body composed of the heart, liver, and intestine (FishBase, 2021), resulting in abundant digestive enzymes compared to a single internal organ only.

Differences in the content and types of enzymes found in fish are thought to be correlated with the number of amino acids or peptide sequences in the fish body because each enzyme has a specific marker peptide. Trypsin has a catalytic ability that characterizes all serine proteases, namely, it can catalyze a series of amino acid residues histamine, aspartic acid, and serine (de Albuquerque *et al*., 2001). However, this enzyme has characteristics that distinguish it from other serine proteases, namely its specificity on the peptide bond formed on the carboxyl side of arginine or lysine (Burnett, 2001; de Albuquerque *et al*., 2001) and its ability to activate other pancreatic zymogens (de Albuquerque *et al*., 2001).

3.3 Characterization of crude extract of trypsin

The crude extracts from the intestines of both tuna species were assessed by using BAPNA as a substrate at

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Table 3. Specific activity of crude extract trypsin on yellowfin tuna and bigeye tuna in comparison with other species.

Species	Common name	Organ	Enzyme activity (U/mL)	Protein Concentration (mg/mL)	Specific Activity (U/mg)	Substrate
T. albacares	Yellowfin tuna	Intestine	0.023	0.47	0.049[a]	BAPNA
T. obesus	Bigeye tuna	Intestine	0.121	0.768	0.158[b]	BAPNA
E. affinis[1]	Kawakawa tuna	Intestine	0.205	0.7	0.29	BAPNA
Sardinella longiceps[2]	Oil sardine	Fish viscera	8778	3850	2.28	BAPNA
Luphiosilurus alexandri[3]	Catfish	Pyloric caeca	481.81	229.9	2.09	BAPNA

[1]Nurhayati *et al*. (2020), [2]Khandagale *et al*. (2017), [3]dos Santos *et al.* (2016).
Values with different superscripts are statistically significantly different based on t-test on yellowfin and bigeye tuna.

different temperatures and pH values (Table 4). It is observed that the optimum temperature and pH from both crude extracts were within the range of 50-60˚C and 7-8, respectively. This result indicates that the trypsin extracted from both tuna species is estimated to have similarity with the biochemical characterization of other fish trypsin with the optimum temperature of 40-65˚C and pH of 6-11 (dos Santos *et al.,* 2016; Jesús-de la Cruz *et al.,* 2018). Likewise, the purified trypsin from silver carp (*Hypophthalmichthys molitrix*) sample collected during the summer and the winter seasons in China demonstrated the optimum temperature of 65˚C (Abe *et al.,* 2020).

Additionally, the SDS-PAGE analysis demonstrated that the molecular weight of trypsin enzymes extracted from yellowfin and bigeye tuna were identical, accounting for 29.192 and 29.346 kDa, respectively (Figure 1). Those molecular weights are higher in comparison with purified trypsin from pyloric caeca of unicorn leatherjacket (*Aluterus monoceros*), which was 23.5 kDa. Nevertheless, the molecular weights of trypsin extracted from tuna intestines were still in the range of molecular weight examined in marine animals and mammalian trypsin (22-30 kDa) (Bougatef, 2013). In contrast, dos Santos *et al.* (2016) reported that the range of molecular weights for fish trypsin is 23-28 kDa, indicating that the values obtained from this current research are slightly above the range. This might be due to the existence of several different parameters, for instance, the type of fish species used and the source of trypsin. Poonsin *et al.* (2019) also found that genetic variations may be the cause of different molecular weights in fish trypsin.

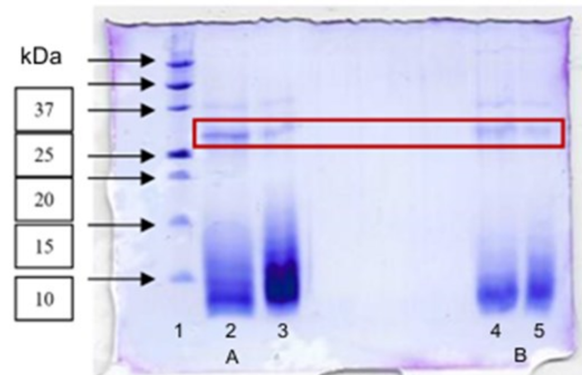

Figure 1. Crude extract of trypsin enzyme from the intestine of (A) yellowfin tuna and (B) bigeye tuna on an SDS-PAGE stained with Coomassie Brilliant Blue R-250. Lane 1: Marker (Precision Plus Protein™ Standards, Size 10-250 kDa), Lane 2 and 3: Crude extract of yellowfin tuna intestine – replication 1 and 2, Lane 4 and 5: Crude extract of bigeye tuna intestine – replication 1 and 2.

3.4 Kinetic studies of trypsin

A study about enzyme kinetics is used to determine enzymatic reactions in hydrolyzing the substrate. Some factors influence the enzymatic reaction, one of which is substrate concentration. The presence of a maximum level of substrate concentration would lead to the maximum enzymatic reaction from which the Michaelis-Menten constant value can be derived. The Michaelis-Menten constant value (K_m) represents the value of substrate concentration required to obtain half of the maximum rate of reaction (V_{max}).

In this current study, the influence of substrate concentration on trypsin activity was analyzed and the K_m values were calculated (Figure 2). It can be seen in Table 5 that the value of maximum enzymatic reaction (V_{max}) of trypsin enzyme extracted from yellowfin tuna

Table 4. Trypsin characteristics of yellowfin and bigeye tuna.

Species	Common name	Organ	Types of extract	Optimum temperature (˚C)	pH optimum
T. albacares	Yellowfin tuna	Intestine	Crude	60	8
T. obesus	Bigeye tuna	Intestine	Crude	50	7
E. affinis[1]	Kawakawa tuna	Intestine	Crude	60	9
Sardinella longiceps[2]	Oil sardine	Fish viscera	Crude	60	8
Luphiosilurus alexandri[3]	Catfish	Pyloric caeca	Crude	50	9

[1]Nurhayati *et al*. (2020), [2]Khandagale *et al*. (2017), [3]dos Santos *et al.* (2016).

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intestines was ten times higher than bigeye tuna Similarly, the value of Michaelis-Menten Constant (K_m) was eight times higher in yellowfin tuna than in bigeye tuna. However, these V_{max} and K_m values obtained from this study were lower than in the kawakawa tuna observed in the research of Nurhayati *et al.* (2020). Hence, it can be suggested that the species difference affects the enzymatic activity of trypsin. The K_m values also have been reported in previous research, for instance in *Paralichthys olivaceus* 0.017 mM (Kim and Jeong, 2013) and *Luphiosilurus alexandri* 0.517 mM (dos Santos *et al.,* 2016).

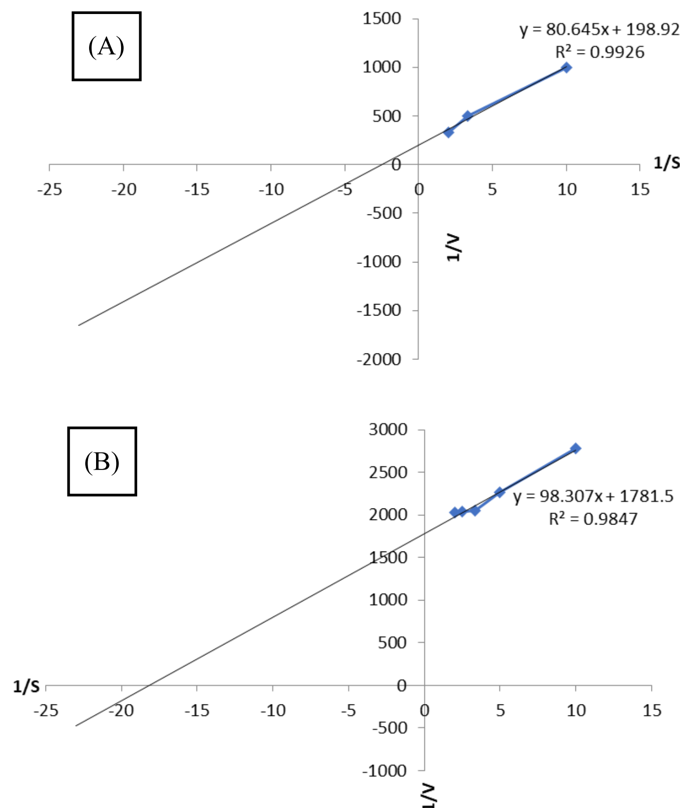

Figure 2. Enzymatic velocity rate of trypsin extracted from intestines of (a) yellowfin and (b) bigeye tuna.

Table 5. Trypsin reaction kinetics of yellowfin and bigeye tuna.

Species	Organ	Types of extract	V_{max} (mmol/s)	K_m
T. albacares	Intestine	Crude	0.00503	0.40541
T. obesus	Intestine	Crude	0.00056	0.05520
E. affinis[1]	Intestine	Crude	0.42	1.12

[1]Nurhayati *et al*. (2020)

4. Conclusion

The crude trypsin extracted from the intestine of both yellowfin and bigeye tuna reveals several different characteristics. The enzyme activity and protein concentration observed in bigeye tuna were significantly higher than in yellowfin tuna. In addition, trypsin bigeye and yellowfin tuna had the optimum temperature and pH from both crude extracts within the range of 50-60°C and 7-8, respectively. Moreover, both molecular weights of trypsin enzymes extracted from yellowfin and bigeye tuna were 29 kDa.

Conflict of interest

The authors declare no conflict of interest.

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