Characteristics of Trypsin Isolated From Intestines Different of Fish and Correlation Toward Trypsin Activity

Tati Nurhayati[1*], Asadatun Abdullah[1], Septiya Rahmawati[1], and Riki Kurniawan[1]

OPEN ACCESS

[1] Department of Aquatic Product Technology, Faculty of Fisheries and Marine Science, IPB University 16680, Indonesia

[*]Corresponding Author:
nurhayati7870@yahoo.com

Received: 16 January 2024

Accepted: 6 July 2024

Published: 31 August 2024

Academic Editor: Ima Wijayanti, Ph.D.

 Accreditation Number:148/M/KPT/2020. ISSN: 2089-5690, e-ISSN: 2406-9272. https://doi.org/10.15578/squalen.904

Abstract

Trypsin is a protease that breaks protein peptide bonds. Fish intestines can be used as an alternative raw material for trypsin. Trypsin enzymes from the intestines of different fish species have different characteristics. This study aimed to determine the characteristics and stability of trypsin enzyme in NaCl extracted from fish intestines based on differences in fish species. Trypsin activity was optimal at 50 °C and pH 8, with specific activity values of 0.5993 U/mg in rabbitfish, 0.3880 U/mg in sharks, and 0.6964 U/mg in flatfish. The maximum reaction speed (V_{max}) was the highest for trypsin from the intestine of rabbitfish (0.2585 mmol/s), followed by flatfish (0.1042 mmol/s), and shark (0.0599 mmol/s). The lowest K_m value was obtained for trypsin from sharks (0.4084 mM), followed by flatfish (1.0253 mM), and rabbitfish (4.5952 mM). Trypsin from the intestines of rabbitfish and flatfish was stable in NaCl solution (concentration 5-30%), as it can maintain a relative activity of more than 50%. In contrast, trypsin extracted from the intestines of milk fish had a relative activity below 21%. The average molecular weights of the three trypsin enzymes were 26.8, 27.2; and 21.9 kDa, respectively. Differences in the type of fish affected trypsin enzyme activity. Flatfish are omnivorous, and rabbitfish, as herbivores, have better enzyme activity values than sharks, as carnivores.

Keywords: flatfish, shark, NaCl stability, rabbitfish, trypsin enzyme characteristics

Introduction

In the fishing industry, most of them only use the meat part, while the rest are waste, for example, heads, bones, fins, and innards, which are less than optimally utilized. The fish-processing industry generates and discards a significant volume of waste each year, accounting for as much as 60% of the harvested biomass (Siddik et al., 2021). Waste is a low-quality raw material that, if not utilized, can cause problems. This has resulted in economic losses and environmental issues. Fish processing waste and by-products can be found in the intestines of fish (Nurhayati et al., 2023). Fish intestines are known to be an abundant source of digestive enzymes, especially as a good source of digestive proteases. Intestinal waste from fish has the potential to serve as an alternative raw material for trypsin enzymes. Trypsin enzyme (EC 3.4.21.4) is an enzyme that acts as a protein peptide bond breaker. Trypsin is an enzyme produced by the pancreas by a zymogen called the trypsinogen. According to Elgendy and Abdelrasool (2016), this enzyme is found in the digestive systems of many mammals and vertebrates. Muallifah (2017) reported that pig and cow pancreases are widely used as raw materials for the manufacture of this enzyme. However, the use of pig pancreas as an enzyme source has become controversial owing to concerns regarding its halal certification. Cattle are also feared to cause transmission of Bovine Spongiform Encephalopathy (BSE) or mad cow disease (Trismillah et al., 2014). Halal and health issues encourage the substitution of other raw materials.

Trypsin is an important bioactive compound in plants (Pamungkas et al., 2022). A digestive enzyme belonging to the serine protease group, trypsin hydrolyzes proteins on the carboxyl side of the amino acids arginine and lysine (Kulsum & Nugrahapraja, 2023). Different types of fish can affect enzyme characteristics owing to their internal and external factors. The types and living environments of fish can influence the characteristics of trypsin derived from them. Environmental factors included food, temperature, pH, and water salinity. Different fish food habits produce different enzymatic activities. Fish were divided into three categories based on their eating habits:

herbivores, omnivores, and carnivores. Research conducted by Gioda et al. (2017) showed that trypsin protease activity varies among carnivorous, herbivorous, and omnivorous freshwater fish. Trypsin from fish living in cold waters typically exhibits optimal temperature and stability at lower temperatures than fish inhabiting tropical habitats or mammals (Arbajayanti et al., 2021).

The purified enzyme showed an alkaline pH of 8.5-11, stability at temperatures of 40-50 °C, and a molecular weight of 23-28 kDa. Trypsin enzymes extracted from the intestines of freshwater or seawater fish exhibit a high level of reactivity under extreme conditions (dos Santos et al., 2016). The ability of trypsin enzymes to function under extreme conditions has led to their widespread application in various fields. These extreme conditions include temperature, pH, and substrate concentration. The stability of trypsin from sardine viscera at high pH and low temperatures suggests that this enzyme has promising applications in both the detergent and food industries (Manni et al., 2024). The NaCl concentration can also affect the activity of trypsin enzymes (Villalba-Villalba et al., 2017). The activity of the trypsin enzyme, which is stable towards NaCl concentration, can be used in industry, one of which is in the food sector. Trypsin can improve the quality of fish sauce (Rianingsih et al., 2016). Adding 0.3% of the enzyme to fish sauce products with a NaCl concentration of 20% can increase the degree of hydrolysis and lower TVB, TMA, and pH values. This causes spoilage microorganisms to produce alkaline substances that can evaporate easily. Ketnawa et al. (2017) noted that trypsin enzymes can also be used to produce protein hydrolysates and surimi products. The surimi industry and insulin precursors are fields that use trypsin (Mao et al. 2018; (Zhang et al., 2020). Trypsin is used as an ingredient in insulin production, which converts insulin precursors into insulin esters and can accelerate the healing of damaged tissues and aid in wound recovery (Harini et al., 2021).

The applications of the trypsin enzyme play an important role in humans, and the need for enzymes is increasing every year. Based on data from GVR (2020), the global enzyme market is worth USD 11.47 billion in 2021, and is projected to continue to rise. The increase in the demand for enzymes is due to the development of a Compound Annual Growth Rate (CAGR) of 6.5%. The demand for enzymes continues to increase owing to the availability of alternative raw materials, such as fish intestinal waste. The abundant potential of fish intestinal waste can meet the increasing need for enzymes. We hope that the availability of these raw materials can help overcome halal, health, and environmental problems. Research on trypsin enzymes is very important because it can determine the characteristics of each type of fish used, making it appropriate for industrial applications. There is a need for research to analyze the relationship between the characteristics and stability of the intestinal trypsin enzyme in different types of fish, namely rabbitfish, shark, and flatfish. This study aimed to determine the characteristics and stability of trypsin enzyme in NaCl extracted from fish intestines based on differences in fish species.

Material and Methods

Materials and Tools

The intestines of rabbitfish (*Siganus canaliculatus*), shark (*Rhizoprionodon acutus*), and flatfish (Pleuronectiformes) were the main materials used in this study. Rabbitfish and sharks were obtained from Muncar Waters, Banyuwangi, East Java, whereas flatfish were obtained from Palabuhan Ratu, Sukabumi, West Java. The other materials used for analysis were N-á-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Sigma-Aldrich, Missouri, United States), Bovine Serum Albumin (BSA) (AppliChem, Darmstadt, Germany), distilled water, DMSO (Sigma-Aldrich, Missouri, United States), HCl (Merck, Darmstadt, Germany), $CaCl_2$ $2H_2O$ (Merck, Darmstadt, Germany), NaCl (Merck, Darmstadt, Germany), SDS (Merck, Darmstadt, Germany), glycerol (Merck, Darmstadt, Germany), â-mercaptoethanol (Sigma-Aldrich, Missouri, United States), and Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc. United States). Tools used for trypsin enzyme characterization included an analytical balance (OHAUS AX224, New Jersey, US), spectrophotometer UV-Vis (Rayleigh, Beijing, China), pH meter (Hanna Instruments, Rhode Island, US), vortex mixer (DLAB MX-S, US), centrifuge (OHAUS FC5718R, New Jersey, US), incubator, and micropipette (Thermo Fisher Scientific).

Methods

Trypsin Enzyme Extraction

Trypsin was extracted from the intestines of the rabbitfish, sharks, and flatfish. The intestines were separated from the other fish innards and cleaned to remove adhering dirt. Extraction was performed according to the method described by Bougatef et al. (2008) with modifications. Intestinal samples were cut into pieces (± 1-1.5 cm), and liquid nitrogen was added and ground using a mortar and pestle. The sample was then suspended in buffer (50 mM Tris–HCl, pH 8.0) at a ratio of 1:4 (w/v) between the sample and buffer. The mixture was homogenized and centrifuged at 9,

500 × g for 30 min at 4°C. The supernatant (crude extract of trypsin) was separated for further analysis.

Trypsin Enzyme Preparation

The trypsin enzyme crude extract was frozen and thawed. The thawing process was performed at room temperature (±25°C) using ice cubes to maintain a cold temperature (±16°C) until the enzyme melted, approximately ±15-20 minutes. The thawed enzyme was characterized.

Characteristics of Trypsin Enzyme

The initial stage of this test involved the preparation of the BAPNA solution. BAPNA solution was prepared by dissolving 0.0435 g BAPNA in 1 mL DMSO, then dissolved with Tris HCl 0.05 M containing $CaCl_2$ $2H_2O$ 0.02 M to 100 mL. BAPNA was then added to the enzyme samples and incubated for 10 min. A 30% acetic acid solution was added to a volume of 1 mL and the mixture was incubated at the same temperature and time. Absorbance was measured using a spectrophotometer at 410 nm. A blank was prepared similarly by replacing the sample with distilled water. Testing the characteristics of trypsin enzyme in the intestines of rabbitfish, sharks, and flatfish includes several tests. The tests performed were as follows:

Determination of Optimum Temperature

The optimum temperature for the trypsin enzyme based on its activity of the trypsin enzyme was determined by mixing the enzyme with the substrate. The procedures described by Nurhayati et al. (2020) BAPNA substrate (2.5 mL of BAPNA substrate was mixed with trypsin (0.05 mL) at pH 8. The enzyme-substrate solution was incubated using different temperature treatments, namely 30, 40, 50, 60, and 70ºC for 10 minutes using a water bath. Subsequently, 1 mL of acetic acid solution was added, and the mixture was incubated for 10 min. The absorbance of the solution was measured using a UV-Vis spectrophotometer at 410 nm.

Determination of Optimum pH

The optimum pH was determined based on enzyme activity, and trypsin was used in the same way as the optimum temperature determination method. The procedures described by Nurhayati et al. (2020). The quantities used to determine the stable pH of the trypsin enzyme were pH 6, 7, 8, 9, and 10. The buffer used was a 0.05 M Tris buffer. The analysis was performed by mixing 2.5 mL of the BAPNA substrate (pH 6-10) with 0.05 enzyme solution. The enzyme-substrate solution was incubated at the optimal temperature for 10 min, and then 1 mL of acetic acid was added. The absorbance of the solution was measured using a UV-Vis spectrophotometer at 410 nm.

Trypsin Enzyme Kinetics

Trypsin enzyme kinetics procedure was described by Lehninger (1997). Enzyme kinetic analysis was carried out by preparing BAPNA substrates at different concentrations. This analysis was performed to determine the relationship between the concentration of the substrate used and its enzyme activity at concentrations of 1, 1.5, 2.5, 3 and 3.5 Mm. The analysis results were then calculated using the Michaelis-Menten equation, modified into a linear form called the Lineweaver-Burk equation. The equation used is as follows:

$$\frac{1}{V0} = \frac{1}{Vmax} + \frac{Km}{Vmax\ [S]}$$

Information:

V_0= Initial velocity at substrate concentration

[S]= Substrate concentration

V_{max}= Maximum speed

K_m= Michaelis-Menten constant for a particular substrate

V_{max} showed the fastest reaction rate at the highest substrate concentration. K_m can be determined from the reaction speed profile at different substrate concentrations. The result of the equation was entered into the conversion table of the relationship between concentrations of substrate and enzyme activity, and a graph of the relationship between them was made.

The Effect of NaCl on Enzyme Activity

The effect of NaCl on enzyme activity was tested by preparing NaCl solutions of various concentrations, namely, 5, 10, 15, 20, 25, and 30% (w/v). The procedures described by Nurhayati et al. (2020). The enzyme samples were mixed with various concentrations of NaCl at a ratio of 1:1 (v/v) at room temperature for 30 min. Trypsin activity was tested using the BAPNA substrate (optimum pH) and then incubated for 10 min at the optimum temperature. Next, 1 mL 30% acetic acid was added to the solution and incubated for 10 min. The absorbance of the filtrate was measured using a spectrophotometer at 410 nm. The absorbance data were analyzed using UV-Vis spectrophotometry to determine trypsin enzyme activity after adding NaCl solution.

Trypsin Enzyme Molecular Weight Measurement (SDS-PAGE)

The molecular weight of the trypsin crude extract was measured using SDS-PAGE (Laemmli, 1970). This test had several stages, starting with the pretreatment process. Pretreatment was carried out by mixing 10 µL of the sample obtained from the protein concentration of trypsin crude extract with 10 µL of sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, and 10% βmercaptoethanol (βME)). The solution was vortexed, centrifuged for 1 min, and stored at 0 °C. In the second stage, stocking gels with 3% and 15% concentrations were used. Samples were mixed with buffer, placed into the wells (20 µL), and run. Running samples were carried out using a current of 24 mA/gel and 70V voltage for 15 min, then continued at 170V for 1 h or until the band on the marker reached the gel lower limit. The next stage is coloring. Gel staining was carried out using 0.1% Coomassie Brilliant Blue (CBB) R-250 in 50% methanol and 7% acetic acid to be left alone for one hour, then rinsed using 7% acetic acid until the band was visible.

Analysis Procedure

Trypsin Enzyme Activity Analysis

Enzyme activity was determined using the method described by Arbajayanti (2022), with modifications. The initial stage of the analysis was the BAPNA solution. BAPNA solution was prepared by dissolving 0.0435 g BAPNA in 1 mL DMSO, then dissolved with Tris HCl 0.05 M containing $CaCl_2$ $2H_2O$ 0.02 M to 100 mL. Enzyme samples (0.05 mL) were added to 2.5 mL, then incubated at 60 °C for 10 min. A 30% acetic acid solution was added to a volume of 1 mL and the mixture was incubated at the same temperature and time. Absorbance was measured using a UV-Vis spectrophotometer at 410 nm. Blanks were prepared by replacing the samples with distilled water.

Analysis of Trypsin Enzyme Protein Concentration

Protein concentration testing was carried out according to Bradford's method (1976) by adding 0.1 mL of enzyme solution to a test tube containing 5 mL of Bradford reagent. After treatment with Blanko, the enzyme solution was replaced with distilled water. The solution was then vortexed and incubated for 5 min at 35 °C. The absorbance of the solution was measured at a wavelength of 595 nm using a spectrophotometer. The protein standard used was Bovine Serum Albumin (BSA) Fraction. The protein standards were prepared by dissolving 20 mg of BSA 9 in 20 mL of distilled water to obtain a 1 mg/mL BSA stock solution. The next step was to make a dilution series of 0.1 to 1 mg/mL. The series, 0.1 mL of solution was diluted, 5 mL of Bradford solution was added, left for 30 min, and the absorbance was measured at a wavelength of 595 nm.

Statistical Analysis

Quantitative data from the research results were processed using Microsoft Excel 2013, and analysis of variance (ANOVA) was performed using the Statistical Product and Service Solutions (SPSS) application. Research data can be categorized as normally distributed if they have a significance value greater than á (á = 0.05). Data values that were normally distributed were then analyzed by ANOVA analysis of variance at the 95% confidence level (α = 0.05) in SPSS. The results of the data obtained show that the data are significantly different (significance value <0.05) and not significantly different (significance value>0.05). Data that were significantly different (significance value <0.05) were subjected to Duncan's test. The relationship between trypsin activity and fish species was analyzed using Principal Component Analysis (PCA). The observed variables included fish-eating habits and enzyme activity. The variable of fish-eating habits was used to determine its relationship with trypsin enzyme activity. Data were processed using the XLSTAT 2022 software in Microsoft Excel 2013.

Results and Discussion

Trypsin Enzyme Activity

The results of the analysis of variance indicated that there were no significant differences among fish types (significance value < α), warranting further Duncan tests. Subsequent Duncan tests demonstrated that while fish type did not affect the specific activity of the trypsin enzyme, it did influence the overall enzyme activity. This suggests that different types of fish affect the resulting enzyme activity. The highest specific activity was found for trypsin in flatfish (0.6964 ± 0.1024 U/mg). Rabbitfish had the second highest enzyme activity, amounting to 0.5993 ± 0.0295 U/mg, whereas the lowest order was shark trypsin enzyme activity (0.3880 ± 0.0712 U/mg). This is a crude enzyme. Commercially available pure enzymes, one of which is produced by pharmaceutical companies, have a specific activity of 250-2500 U/mg. These results were obtained after a long process of processing and refining. Table 1 compares the specific activity of several types of fish.

Table 1. The specific activity of enzymes from different fish species

No.	Different of fish	Specific activity (U/mg)	Reference
1	Flatfish	0.6964 ± 0.1024	research result
2	Rabbitfish	0.5993 ± 0.0295	research result
3	Shark	0.3880 ± 0.0712	research result
4	Yellowfin tuna	0.049	Nurhayati et al., 2023
5	Bigeye tuna	0.158	Nurhayati et al., 2023
6	Kawakawa tuna	0.29	Nurhayati et al., 2020
7	Sardine	2.28	Khandagale et al., 2017
8	Catfish	2.09	dos Santos et al., 2016

These values are higher compared to the specific activity of the trypsin enzyme in yellowfin tuna (0.049 U/mg), bigeye tuna (0.158 U/mg) (Nurhayati et al., 2023), and Kawakawa tuna (0.29 U/mg) (Nurhayati et al., 2020). However, it is lower when compared with the specific activity of the trypsin enzyme from viscera oil sardines 2.28 U/mg (Khandagale et al., 2017) and in catfish pyloric caeca, 2.09 U/mg (dos Santos et al., 2016). Factors that influence trypsin activity are classified into external and internal factors. Fahisyah et al. (2019) stated that several external factors, including temperature, pH, volume, and concentrations of enzymes, substrates, cofactors, and enzyme inhibitors, can influence the enzymatic activity of a reaction. Other external factors were also mentioned by Hani et al. (2018), namely temperature, fish environment, environmental seasons, and food, and internal factors such as fish size, age, and origin. The factors influencing the action of trypsin can cause an increase or decrease in trypsin enzyme activity.

Characteristics of Trypsin Enzyme

Optimum Temperature

Temperature testing of the trypsin enzyme aims to determine at what temperature the trypsin enzyme can work optimally. Optimal temperature testing was performed using five test points in a temperature range of 30-70ºC at 10 °C intervals. The results of testing trypsin enzyme activity at different temperatures are shown in Figure 1.

The analysis of variance results revealed that differences in fish types did not significantly affect trypsin enzyme activity at different temperatures (significance value < α). Therefore, further Duncan's test is warranted. Duncan's test results demonstrated that the optimal temperature for trypsin enzyme activity across all three fish species was 50 °C, with the highest activity observed in flatfish. The optimum temperature was similar to that used in the previous studies.

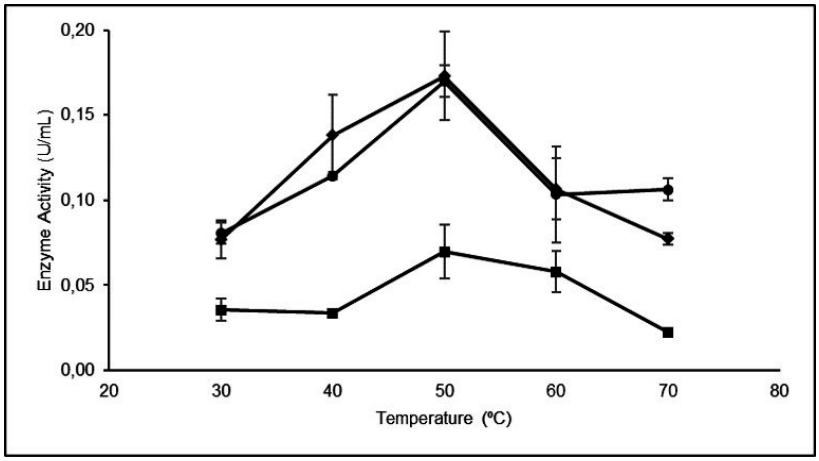

Figure 1. Trypsin activity at pH 8 with different incubation temperatures in the intestines of (•) rabbitfish, (▪) shark, and (♦) flatfish

Temperature affects whether enzymes are active or inactive (Deeth, 2021). A temperature increase that exceeds the optimum limit can cause damage or denaturation of enzymes, whereas a temperature decrease below the optimum limit results in enzyme inactivation. According to Larassagita et al. (2018), using eel fish samples, the enzyme sample was optimal at 50 °C. The findings of studies on bigeye tuna by Nurhayati et al. (2023) and catfish by dos Santos et al. (2016) were consistent in that trypsin preferred a temperature of 50. This optimum temperature was lower than that of trypsin from *Sardinella longiceps* (Khandagale et al., 2017) and tuna (Nurhayati et al., 2020), with an optimum temperature of 60ºC.

The difference in activity was due to the specific optimum temperature for each fish species. Fish have different habitats at different ambient temperatures, resulting in differences in the optimum incubation temperature of the enzyme (Rungruangsak, 2014). Temperature affects fish digestion by influencing the gut evacuation rate and by determining digestive enzyme activity (Solovyev et al., 2021). The optimum temperature for each fish enzyme was different, which can be attributed to different fish habitats. The high optimal temperature may be due to the fish habitat, which usually lives in warm waters. According to Namjou et al. (2019), there is a direct correlation between habitat temperature and thermal stability of fish trypsin. These variations might stem from the temperature of the fish's natural habitat or the specific experimental conditions employed during the evaluations (Zamani et al., 2023). Another factor is the difference in the substrate. Trypsin belongs to the alkaline proteinase group and cannot properly bind to substrates under acidic conditions (Dos Santos et al., 2016). The trypsin enzyme can still function even though it is not at its optimum temperature. The movement of enzyme molecules slows, and the rate of enzyme-substrate complexes decreases if the temperature is lowered below the optimum temperature. The enzyme can still function, but is very slow. Using temperatures above the optimum causes some enzyme molecules to undergo changes that prevent them from forming compounds with the substrate; thus, the reaction rate is slow. An increase in temperature also causes most enzyme molecules to become inactive, and the enzyme molecules undergo permanent changes.

pH Temperature

Trypsin enzyme can work optimally if it gets a favorable environment. A pH test was performed to determine the effect of pH on the trypsin enzyme activity. This test was conducted using pH values ranging from 6 to 10. The test results are shown in Figure 2.

Analysis of variance suggested that differences in fish types did not have a significant effect (significance value < α) on trypsin enzyme activity at different pH levels, thus necessitating further Duncan tests. Duncan's test results revealed that the optimal pH for trypsin enzyme activity across all three fish species was 8, with the highest enzyme activity observed in flatfish. The higher the pH value, the higher the enzyme activity; however, after pH 8, the enzyme activity decreased again. Trismillah et al. (2014) mentioned that changes in the degree of ionization of the acid and base groups of the enzyme will affect the speed of enzyme reactions. Enzymes have a specific pH range that can affect their optimal and stable activities. In this study, trypsin enzyme activity was generally stable over a wide pH range; from pH 6 to 10, the enzyme

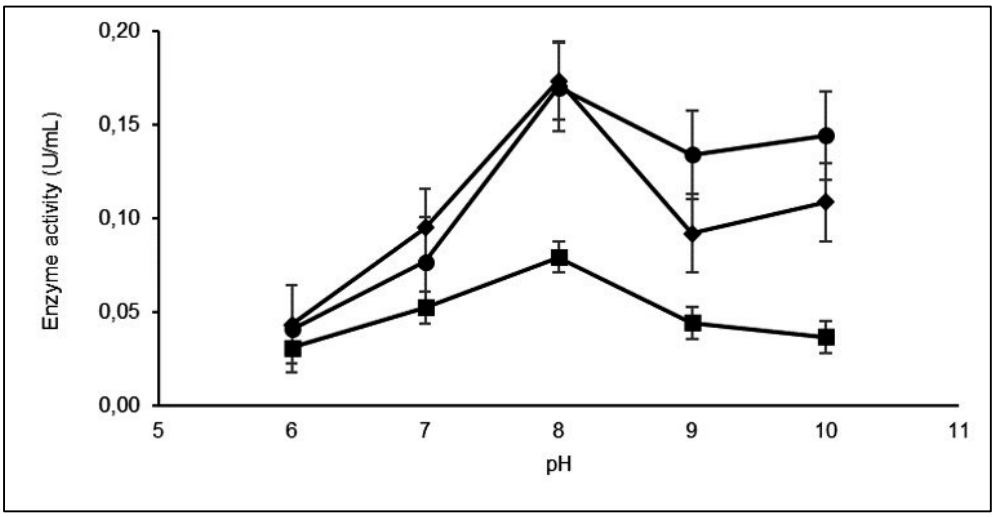

Figure 2. Trypsin activity at 50 ºC with different pH in the intestines of (•) rabbitfish, (▪) shark, and (♦) flatfish

could still function, and at pH 8, the highest enzyme activity was observed. Trypsin exhibits high stability within a range of alkaline pH values (Manni et al., 2024).

This result is the same as that reported by Nurhayati et al. (2023) using yellowfin tuna, unicorn leatherjacket (Zamani & Benjakul, 2016), thornback ray (Lassoued et al., 2015), common kilk (Zamani et al., 2014), catshark (Blanco et al., 2014), and *Sardinella longiceps* (Khandagale et al., 2017), which had an optimum pH of 8. This pH value is different from those of several other fish trypsin enzymes. The trypsin enzyme from *S. basilica* research results in Charu & Ragini (2020) was active between pH 4.0 and 1.0, with an optimum around pH 8.5. Klomklao et al. (2014)used the trypsin enzyme from Pacific saury to obtain an optimum pH of 8.5. With Atlantic cod samples, Stefansson et al. (2017) reported a stable pH range of 8.5 11. Assays to define the optimal pH revealed more significant trypsin enzyme activity in golden grey mullets at pH 10 (Bkhairia et al., 2016). The type of fish and its genetic characteristics, location, habitat, and anatomy determine the optimal pH for the trypsin enzyme (Vannabun et al., 2014). The difference in optimum pH can occur because of irreversible protein denaturation, which causes a decrease in BAPNA hydrolysis by pure trypsin at low and high pH. It has been proposed that pH stability correlates with the charge acquired by the enzyme at various pH levels (Jesús-de la Cruz et al., 2018). Enzymes have a specific chemical structure and a certain pH range for reactions; therefore, enzymes are sensitive to changes in pH. Changes in pH that are more acidic or basic cause a denaturation process in the three-dimensional structure of the enzyme, resulting in the cessation of enzyme activity. Most enzymes undergo irreversible denaturation in acidic or alkaline solutions, resulting in loss of activity. Patil et al. (2023) explained the denaturation of trypsin under a very alkaline pH, in which the repulsive force of the negative charge of the trypsin molecule was dominant. This may have caused an altered conformation, thus losing its activity. Nurkhotimah et al. (2017) reported that enzymes function efficiently in a relatively small pH range in their environment; this is because the enzyme structure begins to change, and the substrate cannot bind back to the active side of the enzyme.

Trypsin Enzyme Kinetics

This test uses a substrate, BAPNA, with concentrations of 1; 1.5; 2; 2.5; 3; and 3.5. The effect of the substrate concentration on trypsin enzyme activity is shown in Figure 3. The results of the enzymatic reaction speed tests are shown in Figure 4.

The substrate concentration can influence the activity of trypsin. The results of the measurements taken at various substrate concentrations are presented in Figure 4. Analysis of variance suggested that differences in fish types did not have a significant effect (significance value < α) on trypsin enzyme activity at different substrate concentrations, thus necessitating a further Duncan test. Duncan's test results revealed that the optimum substrate concentration for the trypsin enzyme across all three types of fish was 3.5 mM, with the highest activity observed in the rabbitfish intestine. The activity value of rabbitfish intestinal trypsin enzyme at a concentration of 3.5 mM was 0.123 U/mL. The trypsin enzyme level in the intestines of sharks and flatfish tends to be constant, which differs from that in rabbitfish. Enzyme samples from sharks had enzyme activities ranging from 0.043 to 0.055U/mL, and those from flatfish ranged from 0.051 to 0.080 U/mL. According to Fahisyah et al. (2019), the

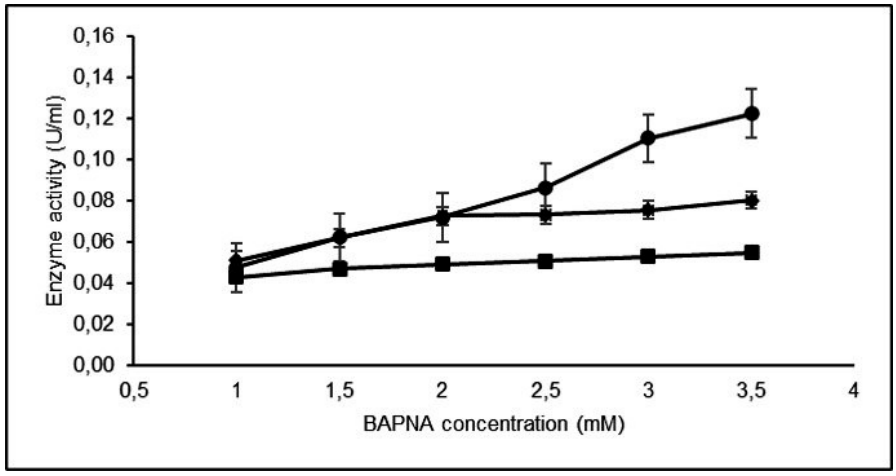

Figure 3. Effect of substrate concentration on trypsin enzyme activity in the intestine of (•) rabbitfish, (▪) shark, and (♦) flatfish

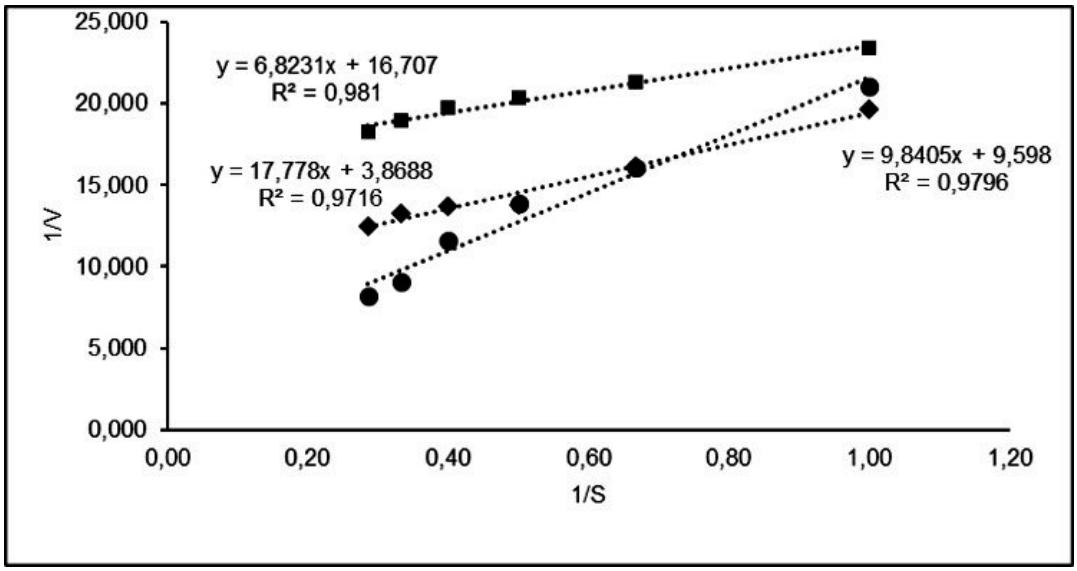

Figure 4. Reaction rate on trypsin enzyme activity in the intestine of (•) rabbitfish, (▪) shark, and (♦) flatfish.

substrate concentration increases the enzyme reaction rate; however, no reaction occurs at a specific substrate concentration.

The Lineweaver-Burk equation was used to calculate the V_{max} and K_m of the sample. The highest rate that can be achieved in a reaction is the reaction rate (V_{max}). V_{max} values of trypsin in rabbitfish, shark, and flatfish were 0.2585, 0.0599, and 0.1042 mmol/s, respectively. Enzyme kinetics, often known as the rate of enzyme activity, guide the maximal application of a particular enzyme and its concentration for a specific substrate (Srinivasan, 2022). The highest K_m value obtained in this study was obtained with shark trypsin (0.4084 mM). Trypsin enzymes from flatfish and rabbitfish had K_m values of 1.0253 and 4.5952 mM, respectively. Chrisman et al. (2023) stated that the lower the K_m value, the better the enzyme-substrate complex. This is because enzymes have a strong affinity or ability to bind to substrates. Using specific substrate concentrations at certain temperatures and pH values causes enzymes to have different V_{max} and K_m values. Proteases with higher and more efficient substrate-binding capacities could be important biotechnological tools for peptide synthesis and the food industry.

The Effect of NaCl on Enzyme Activity

Testing of the trypsin enzyme in an environment that had been added with different concentrations of NaCl was carried out to see its effect on the activity of the trypsin enzyme. The results of the tests on the effect of NaCl on the activity of trypsin enzyme from the intestines of rabbitfish, shark, and flatfish are presented in Figure 5.

The objective of testing the stability of the trypsin enzyme against salt concentration, specifically NaCl, was to assess the extent of the influence of salt on trypsin enzyme activity. The results depicting trypsin enzyme activity across different NaCl concentrations are shown in Figure 5. The trypsin activity in all fish species fluctuated in response to varying salt concentrations. Moreover, analysis of variance revealed that differences in fish types did not significantly affect trypsin enzyme activity at various NaCl concentrations, thus necessitating further Duncan tests.

Duncan's test results demonstrated that NaCl concentration did not significantly affect trypsin enzyme activity across all three fish species, with the highest activity observed in the intestines of rabbitfish. Furthermore, the relative activity remained notably high in fresh rabbit intestine trypsin, maintaining activity levels above 50%. The trypsin enzyme activity of rabbitfish intestines when not treated with salt was 0.17 U/mL, and when treated with different salts, 5-35% was still stable at 0.11-0.15 U/mL. Trypsin from sharks had the lowest relative activity value, dropping to 91% at an NaCl concentration of 15%. Enzyme activity decreased slightly with increasing NaCl concentration (Lamas et al., 2017). The decrease in activity may be due to the denaturation effect of the salt on the enzymes. Owing to relatively high salt levels, the low value of unstable enzyme activity can be caused by enzyme salting out. The increase in ionic strength can be caused by high salt levels and the use of high temperatures, thereby increasing protein denaturation, which causes the trypsin enzyme activity to decrease.

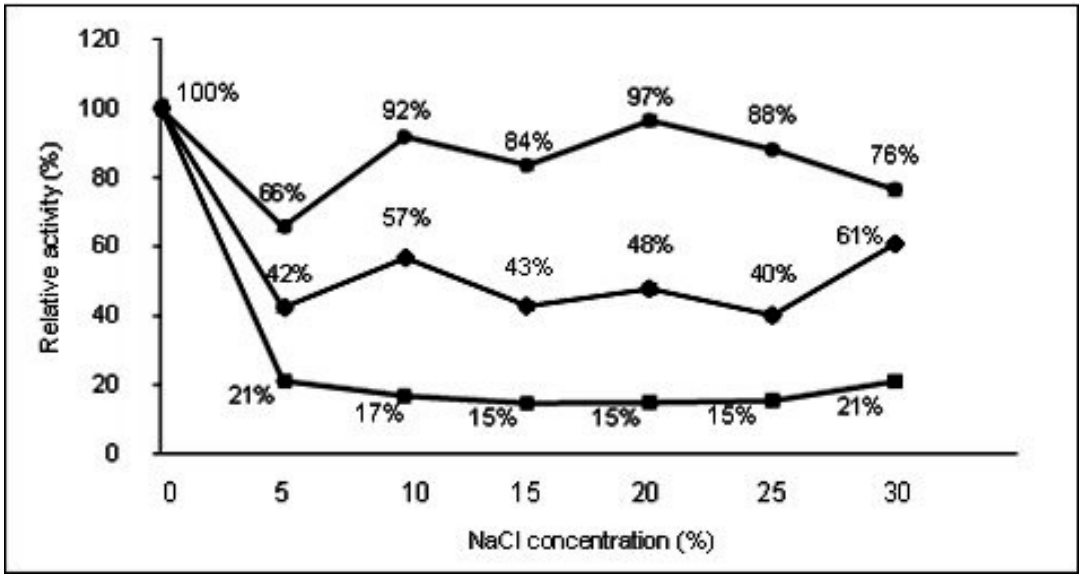

Figure 5. Effect of NaCl concentration on trypsin enzyme relative activity in the intestine of (•) rabbitfish, (▪) shark, and (♦) flatfish.

Trypsin Enzyme Molecular Weight (SDS-PAGE)

Qualitative testing of protein content or molecular weight of the trypsin enzyme can use electrophoresis. The electrophoresis method used is sodium dodecyl sulfate–polyacrylamide gel electrophoresis known as the SDS-PAGE method with Coomassie brilliant blue (CBB) staining. The results of testing the molecular weight of trypsin enzyme from the intestines of rabbitfish, sharks, and flatfish are shown in Figure 6.

The molecular weight of the trypsin enzyme subjected to SDS-PAGE was determined using the photocap software. Enzyme molecules migrate from the negative pole to the positive pole under the influence of electricity. Enzyme molecules migrate based on their molecular weight and migration rate in an electric field. The molecular weight of trypsin in each sample was measured by SDS-PAGE. The SDS-PAGE results showed that shark intestinal trypsin was the trypsin with the most protein bands detected (10 bands). The trypsin enzyme from flatfish intestines had nine bands. The trypsin enzyme from the flatfish intestine had the fewest bands, namely six bands. Trypsin enzymes from rabbitfish, sharks, and flatfish intestines were tested in each replicate. The average molecular weights of the three trypsin enzymes were 26.8, 27.2; and 21.9 kDa. Khangembam & Chakrabarti (2015) reported that the molecular weight of *Cirrhinus mrigala* trypsin is 21.7 kDa. SDS-PAGE analysis demonstrated that the molecular weight of trypsin enzymes extracted from catshark (*Scyliorhinus canicula*) was 28 kDa (Blanco et al., 2014). The molecular weight of trypsin from golden gray mullet (*Liza aurata*) was determined to be 23 kDa (Bkhairia et al., 2016). Based on these results, the molecular weight of the trypsin enzyme from rabbitfish, shark, and flatfish intestines was determined.

These data are different from the results of a study by Nurhayati et al. (2023), who reported that the molecular weights of trypsin enzymes extracted from yellowfin and bigeye tuna were identical, accounting for 29.192 and 29.346 kDa, respectively. The molecular weight of trypsin was found to be 27 kDa in other studies performed on catfish (*Luphiosilurus alexandri*) (Dos Santos et al., 2016), Oil Sardine (*Sardinella longiceps*) (Khandagale et al., 2017), and *Thunnus albacares* (Arbajayanti et al., 2021). The differences in the results were due to several factors, such as differences in species and purification treatments. Variations in habitat, climate, autolytic degradation, and genetic diversity among fish species may account for the differences in the molecular weights of trypsins from different sources (Zamani et al., 2023). This property improves the performance of the subsequent ammonium sulfate fractionation and gel filtration chromatography stages. Heating during purification affected the results obtained. Heating eliminates thermolabile proteins and promotes hydrolysis of thermostable contaminating proteins. Poonsin Poonsin et al. (2019) also found that genetic variations may cause different molecular weights in fish trypsin.

Principal Component Analysis (PCA) of Fish Types on Trypsin Enzyme Activity

Principal component analysis (PCA) is an analysis that aims to determine the relationship between each type of fish and trypsin enzyme activity. This analysis

Table 2. Correlation matrix (Pearson)

Variable	rabbitfish	Shark	flatfish
rabbitfish	1	0,066	0,681
Shark	0,066	1	0,546
flatfish	0,681	0,546	1

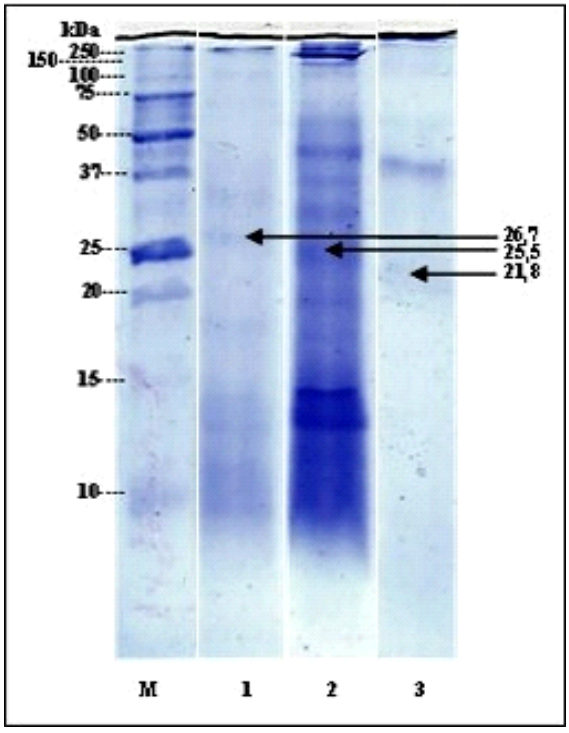

Figure 6. Trypsin enzyme molecular weight (SDS-PAGE). (M) marker, (1) rabbitfish intestine trypsin, (2) shark intestine trypsin, and (3) flatfish intestine trypsin.

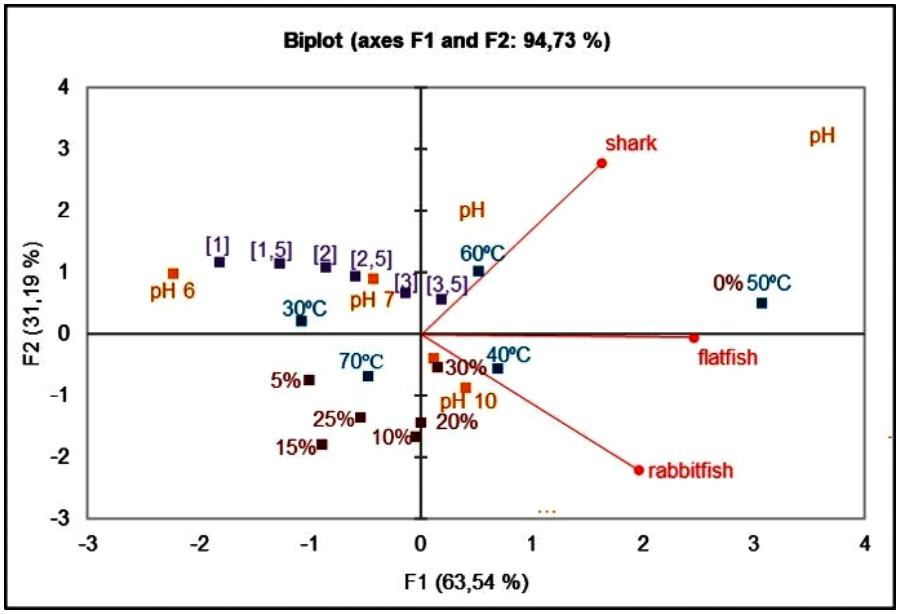

Figure 7. The biplot model for the relationship between fish species and enzyme activity parameters (based on temperature, pH, substrate concentration and NaCl concentration).

can help to determine the main factors that dominate the identification of these fish. The correlation of each type of fish in this PCA analysis can be seen from the size of the resulting correlation matrix, which is close to 1; the closer the value, the stronger the relationship. The Pearson's correlation matrix (Pearson) is presented in Table 2.

Correlation matrix values based on Table 1 show that rabbitfish correlate highly with flatfish fish. Sharks also showed high correlation with flatfish. The fish with the highest correlation matrix value were rabbitfish to flatfish (0.681), followed by shark to flatfish (0.546). This shows that flatfish are closely related to rabbitfish and sharks because flatfish are included in the omnivorous fish group. Omnivorous fish can eat both herbivorous and carnivorous fish; therefore, the close relationship between flatfish is stronger than that between rabbitfish and shark. The biplot model for the relationship between fish species and enzyme activity parameters (based on temperature, pH, substrate concentration, and NaCl concentration) is shown in Figure 7.

The biplot model in Figure 7 shows that the shark is a carnivorous fish with good enzyme activity at temperatures of 50 °C and 60 °C, substrate concentration of 3.5 mM, NaCl concentration of 0%, and pH 8 because it is in the same quadrant, namely, quadrant 1. Omnivorous fish, namely flatfish, and herbivorous fish, namely rabbitfish, have the same characteristic parameters in quadrant 2. The types of omnivorous fish are almost in a straight line with the positive x-axis; therefore, the parameters of this omnivorous fish can influence the types of fish in both quadrants 1 and 2. This indicated that the trypsin activity of omnivorous fish was stable.

The results of this study can be used as a trypsin enzyme candidate for application in products that are not influenced by its specific activity. In the future, this crude trypsin enzyme could be applied to food products, such as soy sauce, meat tenderizers, and the leather industry. To maximize and improve the quality, enzymes need to be further processed through purification to increase the effectiveness of their use and specific activity to compete with commercial enzymes and meet market needs.

Conclusion

Trypsin from rabbitfish, sharks, and flatfish intestines showed optimum activity at a temperature of 50 °C, pH of 8, and substrate concentration of 3.5 mM. The trypsin enzyme in the intestines of flatfish has a higher enzyme activity value than that in the intestines of rabbitfish and sharks, but the trypsin enzyme in rabbitfish can maintain its relative activity at NaCl concentrations above 50%. The average molecular weights of the three trypsin enzymes from the intestines of rabbitfish, sharks, and finfish were 26.8, 27.2; and 21.9 kDa. Differences in the type of fish affected trypsin enzyme activity. Flatfish are omnivorous, and rabbitfish, as herbivores, have better enzyme activity values than sharks, as carnivores. The results of this study could potentially become enzyme candidates to be developed and applied to food products and the leather industry.

Supplementary Materials

Supplementary materials is not available for this article

References

Arbajayanti, R. D., Nurhayati, T., & Nurilmala, M. (2021). komponen asam amino dan aktivitas enzim tripsin dari usus tuna sirip kuning (*Thunnus albacares*) dan kakap merah (*Lutjanus campechanus*). *Jurnal Pengolahan Hasil Perikanan Indonesia*, *24*(1), 97–106. https://doi.org/10.17844/jphpi.v24i1.33878

Arbajayanti, R. (2022). Kajian Keterkaitan antara Morfometrik Spesies Ikan dengan Komposisi Kimia, Asam Amino dan Aktivitas Enzim Tripsin [Master thesis, IPB University. IPB Repository. https://repository.ipb.ac.id/

Bkhairia, I., Ben Khaled, H., Ktari, N., Miled, N., Nasri, M., & Ghorbel, S. (2016). Biochemical and molecular characterisation of a new alkaline trypsin from *Liza aurata*: Structural features explaining thermal stability. *Food Chemistry*, *196*, 1346–1354. https://doi.org/10.1016/j.foodchem.2015.10.058

Blanco, M., Simpson, B. K., Pérez-Martín, R. I., & Sotelo, C. G. (2014). Isolation and partial characterization of trypsin from pancreas of small-spotted catshark (*Scyliorhinus canicula*). *Journal of Food Biochemistry*, *38*(2), 196–206. https://doi.org/10.1111/jfbc.12038

Bradford, M. M. (1976). A rapid and sensitive method for quantitating microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248–254. https://doi.org/10.1006/abio.1976.9999.

Charu, B., & Ragini, G. (2020). Optimization and characterization of trypsin of *Labeo rohita* from its visceral waste. *GSC Advanced Research and Reviews*, *3*(2), 039–047. https://doi.org/10.30574/gscarr.2020.3.2.0041

Chrisman, M. A, Goldcamp, M. J., Rhodes, A. N., & Riffle, J. (2023). Exploring Michaelis–Menten kinetics and the inhibition of catalysis in a synthetic mimic of catechol oxidase: An experiment for the inorganic chemistry or biochemistry laboratory. Journal of Chemical Education, 100, 893–899.

Deeth, H. C. (2021). Heat-induced inactivation of enzymes in milk and dairy products. A review. *International Dairy Journal*, *121*, 105104. https://doi.org/10.1016/j.idairyj.2021.105104

dos Santos, C. W. V., da Costa Marques, M. E, de Araújo Tenório, H., de Miranda, E. C., & Pereira, H. J. V. (2016). Purification and characterization of trypsin from Luphiosilurus alexandri pyloric cecum. Biochemistry and Biophysics Reports, 8: 29–33. https://doi.org/10.1006/abio.1976.9999.

Elgendy, A., & Abdelrasool. (2016). A Literature Review on Trypsin Enzyme A literature review on Trypsin Enzyme. Qatar University College of Arts and Sciences Department of Chemistry and Earth Sciences.

Fahisyah, R. N., Naim, N., & Armah, Z. (2019). Pengaruh variasi lama penyimpanan reagen enzim 1a terhadap hasil pemeriksaan ureum darah metode berthelot. *Jurnal Media Analis Kesehatan*, *10*(1), 21. https://doi.org/10.32382/mak.v10i1.980

Gioda, C. R., Pretto, A., Freitas, C. de S., Leitemperger, J., Loro, V. L., Lazzari, R., Lissner, L. A., Baldisserotto, B., & Salbego, J. (2017). Diferentes hábitos alimentares influenciam a atividade das enzimas digestivas em peixes de água doce. *Ciencia Rural*, *47*(3), 1–7. https://doi.org/10.1590/0103-8478cr20160113

Grand View Research. (2020). Enzymes market size, share & trends analysis report by product (lipases, polymerases & nucleases, carbohydrase), by type (industrial, specialty), by source (plants, animals), by region, and segment forecasts, 2022-2030. Indonesia. https://www.grandviewresearch.com/industry-analysis/enzymes-industry.

Hani, Y. M. I., Marchand, A., Turies, C., Kerambrun, E., Palluel, O., Bado-Nilles, A., Beaudouin, R., Porcher, J. M., Geffard, A., & Dedourge-Geffard, O. (2018). Digestive enzymes and gut morphometric parameters of threespine stickleback (*Gasterosteus aculeatus*): Influence of body size and temperature. *PLoS ONE*, *13*(4), 1–21. https://doi.org/10.1371/journal.pone.0194932

Harini, G., Prabu, D., & Balakrishnan, R. N. (2021). Trypsin and chymotrypsin usage in reduction of pain following the surgical extraction of mandibular third molars. Journal of Contemporary Issues in Business and Government, 27(2): 564–570. https://doi.org/10.47750/cibg.2021.27.02.069.

Jesús-de la Cruz, K., Álvarez-González, C. A., Peña, E., Morales-Contreras, J. A., & Ávila-Fernández, Á. (2018). Fish trypsins: potential applications in biomedicine and prospects for production. *3 Biotech*, *8*(4), 1–14. https://doi.org/10.1007/s13205-018-1208-0

Ketnawa, S., Benjakul, S., Martínez-Alvarez, O., & Rawdkuen, S. (2017). Fish skin gelatin hydrolysates produced by visceral peptidase and bovine trypsin: Bioactivity and stability. *Food Chemistry*, *215*, 383–390. https://doi.org/10.1016/j.foodchem.2016.07.145

Khandagale, A., Mundodi, L., & Sarojini, B. (2017). Isolation and characterization of trypsin from fish viscera of Oil Sardine (*Sardinella longiceps*). *International Journal of Fisheries and Aquatic Studies*, *5*(2), 33–37. www.fisheriesjournal.com

Klomklao, S., Kishimura, H., & Benjakul, S. (2014). Anionic trypsin from the pyloric ceca of pacific saury (*Cololabis saira*): Purification and biochemical characteristics. *Journal of Aquatic Food Product Technology*, *23*(2), 186–200. https://doi.org/10.1080/10498850.2012.708387

Kulsum, Y., & Nugrahapraja, H. (2023). Biochemical and molecular characterization of eel fish trypsin (*Anguilla bicolor* McClelland) as potential candidates protease enzyme. *BIO Web of Conferences*, *75*. https://doi.org/10.1051/bioconf/20237502001

Laemmli, U. (1970). © 1970 Nature Publishing Group. *Nature Publishing Group*, *228*, 726–734. http://www.mendeley.com/research/discreteness-conductance-chnge-n-bimolecular-lipid-membrane-presence-certin-antibiotics/

Lamas, D. L., Yeannes, M. I., & Massa, A. E. (2017). Alkaline trypsin from the viscera and heads of *Engraulis anchoita*: Partial purification and characterization. *Biotechnologia*, *98*(2), 103–112. https://doi.org/10.5114/bta.2017.68309

Larassagita, A. F., Hana, H., & Susilo, U. (2018). Aktivitas tripsin dan kimotripsin pada ikan sidat tropik (*Anguilla bicolor* McClelland). *Scripta Biologica*, *5*(1), 55. https://doi.org/10.20884/1.sb.2018.5.1.660

Lassoued, I., Hajji, S., Mhamdi, S., Jridi, M., Bayoudh, A., Barkia, A., & Nasri, M. (2015). Digestive alkaline proteases from thornback ray (*Raja clavata*): Characteristics and applications. *International Journal of Biological Macromolecules*, *80*, 668–675. https://doi.org/10.1016/j.ijbiomac.2015.07.038

Lehninger A. L. (1997). Dasar-Dasar Biokimia. Thenawidjaja M, translator; Suwarman, editor. Jakarta: Erlangga. Translation of: Principle of Biochemistry.

Manni, L., Zouine, N., Ouahidi, I., Bouraqqadi, M., & Ananou, S. (2024). Purification of trypsin from sardine (*Sardina pilchardus*) viscera and its application in preparation of antioxidative fish protein hydrolysates. *Tropical Journal of Natural Product Research*, *8*(1), 5881–5888. https://doi.org/10.26538/tjnpr/v8i1.25

Mao, Y. H., Krischke, M., Hengst, C., & Kulozik, U. (2018). Comparison of the influence of pH on the selectivity of free and immobilized trypsin for beta-lactoglobulin hydrolysis. Food Chemistry, 253:194–202. https://doi.org/10.1016/j.foodchem.2018.01.151.

Muallifah, A. Y. (2018). Mengurai hadis tahnik dan gerakan anti vaksin. *Jurnal Living Hadis*, *2*(2), 253. https://doi.org/10.14421/livinghadis.2017.1334

Namjou, F., Yeganeh, S., Madani, R., & Ouraji, H. (2019). Extraction, purification, and characterization of trypsin obtained from the digestive system of yellowfin seabream (*Acanthopagrus latus*). *Archives of Razi Institute*, *74*(4), 405–411.

Nurhayati, T., Ibrahim, B., Rahmawati, R., Pamungkas, I. D., & Qanitia, P. A. (2023). Characterization of trypsin enzyme extracted from intestines of yellowfin tuna (*Thunnus albacares*) and bigeye tuna (*Thunnus obesus*) from Indonesian seawater. *Food Research*, *7*(6), 160–167. https://doi.org/10.26656/fr.2017.7(6).020

Nurhayati, T., Nugraha, R., & Lihuana, D. N. (2020). Characterization of ammonium sulphate fraction tripsin isolated from intestine of little tuna. *Jurnal Pengolahan Hasil Perikanan Indonesia*, *23*(2), 372–382. https://doi.org/10.17844/jphpi.v23i2.32221

Nurkhotimah, N., Yuliati, E., & Rahmawati, A. (2017). Pengaruh suhu dan pH terhadap aktivitas enzim fosfatase bakteri termofilik sungai gendol pasca erupsi merapi. *Kingdom (The Journal of Biological Studies)*, *6*(8), 465–471. https://doi.org/10.21831/kingdom.v6i8.7891

Pamungkas, I. D., Nurhayati, T., Ibrahim, B., Abdullah, A., & Nurjanah. (2022). Pengaruh penambahan tripsin terhadap karakteristik surimi ikan nila merah (*Oreochromis niloticus*). *Jurnal Ilmu Dan Teknologi Kelautan Tropis*, *14*(2), 243–258. https://doi.org/10.29244/jitkt.v14i2.33622

Patil, U., Baloch, K. A., Nile, S. H., Kim, J. T., & Benjakul, S. (2023). Trypsin from pyloric caeca of Asian Seabass: Purification, characterization, and its use in the hydrolysis of acid-soluble collagen. *Foods*, *12*(15). https://doi.org/10.3390/foods12152937

Poonsin, T., Simpson, B. K., Benjakul, S., Visessanguan, W., Yoshida, A., Osatomi, K., & Klomklao, S. (2019). Anionic trypsin from the spleen of albacore tuna (*Thunnus alalunga*): Purification, biochemical properties and its application for proteolytic degradation of fish muscle. *International Journal of Biological Macromolecules*, *133*, 971–979. https://doi.org/10.1016/j.ijbiomac.2019.04.122

Rianingsih, L., Ibrahim, R., & Anggo, A. D. (2016). Effect of different concentration salt and trypsin on the physicochemical properties of fish sauce made from sea catfish (*Arius* sp.) viscera. *Jurnal Teknologi*, *78*(4–2), 99–104. https://doi.org/10.11113/jt.v78.8188

Rungruangsak, T. K. (2014). Atlantic Salmon, & Salmo salar L.: Food Utilization, Protein Growth Efficiency and Maturation. Chapter 7. Nova Science Publishers.

Siddik, M. A. B., Howieson, J., Fotedar, R., & Partridge, G. J. (2021). Enzymatic fish protein hydrolysates in finfish aquaculture: a review. *Reviews in Aquaculture*, *13*(1), 406–430. https://doi.org/10.1111/raq.12481

Solovyev, M. M., Kashinskaya, E. N., Rogozhin, E. A., & Moyano, F. J. (2021). Seasonal changes in kinetic parameters of trypsin in gastric and agastric fish. *Fish Physiology and Biochemistry*, *47*(2), 381–391. https://doi.org/10.1007/s10695-020-00919-0

Srinivasan, B. (2022). A guide to the Michaelis–Menten equation: steady state and beyond. *FEBS Journal*, *289*(20), 6086–6098. https://doi.org/10.1111/febs.16124

Stefansson, B., Sandholt, G. B., & Gudmundsdottir, Á. (2017). Elucidation of different cold-adapted Atlantic cod (Gadus morhua) trypsin X isoenzymes. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1865:11–19. https://doi.org/10.1016/j.bbapap.2016.10.005.

Trismillah, Sumaryono, W., Malik, A., & Sadikin, M. (2014). Isolasi dan karakterisasi protease serupa tripsin (PST) dari *Lactobacillus plantarum* FNCC 0270. *Ilmu Kefarmasian Indonesia*, *12*(1), 57–66.

Vannabun, A., Ketnawa, S., Phongthai, S., Benjakul, S., & Rawdkuen, S. (2014). Characterization of acid and alkaline proteases from viscera of farmed giant catfish, Food Bioscience, 6, 9-16.

Villalba-Villalba, A. G., Márquez-Ríos, E., Esquerra-Brauer, M. J., & Castillo-Yáñez, F. J. (2017). Trypsin from jumbo squid (*dosidicus gigas*) hepatopancreas: Purification and characterization. *Interciencia*, *42*(3), 168–174.

Zamani, A., & Benjakul, S. (2016). Trypsin from unicorn leatherjacket (*Aluterus monoceros*) pyloric caeca: Purification and its use for preparation of fish protein hydrolysate with antioxidative activity. *Journal of the Science of Food and Agriculture*, *96*(3), 962–969. https://doi.org/10.1002/jsfa.7172

Zamani, A., Khajavi, M., Abedian Kenari, A., Haghbin Nazarpak, M., Solouk, A., Esmaeili, M., & Gisbert, E. (2023). Physicochemical and biochemical properties of trypsin-like enzyme from two sturgeon species. *Animals*, *13*(5), 1–15. https://doi.org/10.3390/ani13050853

Zamani, A., Rezaei, M., Madani, R., & Habibi Rezaie, M. (2014). Trypsin enzyme from viscera of common kilka (*Clupeonella cultriventris caspia*): Purification, characterization, and its compatibility with oxidants and surfactants. *Journal of Aquatic Food Product Technology*, *23*(3), 237–252. https://doi.org/10.1080/10498850.2012.712630

Zhang, Y., Zhang, Y., Zhang, Y., Liang, Q., Zhang, C., Zhang, J., Du, G., Du, G., Kang, Z., & Kang, Z. (2020). Improving production of *Streptomyces griseus* trypsin for enzymatic processing of insulin precursor. *Microbial Cell Factories*, *19*(1), 1–11. https://doi.org/10.1186/s12934-020-01338-9