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CHARACTERISTICS OF TRYPSIN ISOLATED FROM THE INTERNAL ORGANS OF YELLOWFIN TUNA AND STABILITY IN NaCl

KARAKTERISTIK TRIPSIN YANG DIISOLASI DARI ORGAN DALAM IKAN TUNA SIRIP KUNING DAN STABILITASNYA DALAM NaCl

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ABSTRACT

The demand for enzymes in Indonesia is extremely high, and they are still imported from other countries. Commercial trypsin is usually extracted from the pancreas of pigs and cattle, so other alternative sources are needed from fish, namely the intestines, liver, and spleen of tuna. The intestine, liver, and spleen are internal fish organs that contain trypsin with different characteristics. This study aims to determine the characteristics of trypsin in the internal organs of yellowfin tuna and its stability in NaCl. The method used was a Completely Randomized Design with the treatment of different types of intestines, liver, and spleen of tuna. Optimum trypsin activity was at 60°C and pH 8, with a specific activity value in the intestine of 0.948±0.114 U/mg, liver of 0.610±0.029 U/mg, and spleen of 0.605±0.159 U/mg. The maximum reaction speeds (V_{max}) showed the largest value for the intestine, liver, and spleen were 0.248 mmol/s, 0.138 mmol/s, and 0.096 mmol/s, respectively. The constant values (K_m) obtained for the intestine, liver, and spleen were 2.342, 2.268, and 1.276 mM, respectively. Trypsin has a molecular weight range of 20–30 of approximately 28 kDa. The trypsins extracted from the intestine and liver were relatively stable in up to 30% NaCl with a minimum relative activity of 60%, whereas the trypsin extracted from the spleen was relatively stable up to 20% NaCl with 54% relative activity. Based on their activity and characteristics, the internal organs of tuna, especially the intestines and liver, have the potential to be sources of trypsin.

Keywords: halal enzyme, NaCl stability, specific activity, trypsin characteristics, tuna viscera

ABSTRAK

Kebutuhan enzim di Indonesia sangat tinggi dan masih diimpor dari negara lain. Tripsin komersial biasanya diekstraksi dari pankreas babi dan sapi, sehingga diperlukan sumber alternatif lainnya yang bersumber dari ikan, yaitu usus, hati, dan limpa ikan tuna. Usus, hati, dan limpa merupakan organ dalam ikan yang mengandung tripsin dengan karakteristik yang berbeda. Penelitian ini bertujuan untuk menentukan karakteristik tripsin pada organ dalam ikan tuna sirip kuning dan kestabilannya dalam NaCl. Metode yang digunakan adalah Rancangan Acak Lengkap dengan perlakuan jenis jeroan (usus, hati, dan limpa) ikan tuna yang berbeda. Aktivitas tripsin optimum pada suhu 60°C dan pH 8, dengan nilai aktivitas spesifik pada usus sebesar 0,948±0,114 U/mg, hati 0,610±0,029 U/mg, dan limpa 0,605±0,159 U/mg. Kecepatan reaksi maksimum (V_{max}) menunjukkan nilai terbesar untuk usus yaitu 0,248 mmol/s, hati 0,138 mmol/s, dan 0,096 mmol/s untuk limpa. Nilai konstanta (K_m) yang diperoleh untuk usus, hati, dan limpa masing-masing adalah 2,342, 2,268, dan 1,276 mM. Tripsin memiliki kisaran berat molekul dari 20–30 yaitu sekitar 28 kDa. Tripsin yang diekstrak dari usus dan hati relatif stabil hingga 30% NaCl dengan aktivitas relatif minimum 60%, sedangkan tripsin yang diekstrak dari limpa relatif stabil hingga 20% NaCl dengan aktivitas relatif 54%. Berdasarkan aktivitas dan karakteristiknya, organ dalam ikan tuna, terutama usus dan hati, berpotensi menjadi sumber tripsin.

Kata kunci: aktivitas spesifik, enzim halal, jeroan tuna, karakteristik tripsin, stabilitas NaCl

INTRODUCTION

The demand for enzymes in Indonesia tends to increase annually, along with the demand for enzymes in the global market. The global enzyme market is valued at USD 11.47 billion in 2021 and will continue to increase based on a compound annual growth rate (CAGR) of 6.5% from 2022 to 2030 (GVR2020). The enzyme demand rate in Indonesia from 2012 to 2016 was 11.05% per year (CCI 2017). Enzymes play a role in various industries, including the food industry, and are used to minimize waste, improve product quality, and reduce energy consumption (Kuddus 2018). One type of enzyme is the protease group, which has an important function in the digestive process in the body. Enzymes can be isolated from various sources, including microorganisms, plants, and animals. Trypsin is an enzyme derived from animals (Chitte and Chaphalkar 2017).

Trypsin, which is found in the digestive tract, combines with proteases and other alkaline peptidases, such as chymotrypsin, aminopeptidase, and carboxypeptidase, to complement each other for acid digestion in the stomach. The combination of these enzymes can hydrolyze proteins and peptides into free amino acids and peptides, which are more easily absorbed in the intestines (Nolasco-Soria 2021). Therefore, trypsin enzyme activity can be used as an important indicator of the digestive capacity of fish and as a biomarker that has the benefit of assessing the nutritional content and physiological condition of the fish (Nazdar *et al.* 2018). Trypsin hydrolyzes peptide bonds by splitting the C-terminal into arginine or lysine (Olsen *et al.* 2004), and the molecular weight ranges from 20 to 30 kDa (Khantaphant and Benjakul 2010). Enzyme characterization is required to determine the stability of enzyme activity. Enzyme characterization is required to determine the stability of enzyme activity. Trypsin stability is affected by pH and temperature. Changes in the pH and temperature affect the activity of trypsin enzyme catalysts (Larassagita *et al.* 2018). Trypsin enzyme is included in alkaline proteinases and cannot work optimally under acidic conditions (dos-Santos *et al.* 2016).

Trypsin is a protease that hydrolyzes peptide bonds by cutting the C end (C-terminal) to arginine or lysine (Olsen *et al.* 2004). Trypsin is included in alkaline proteinases and cannot work optimally under acidic conditions (dos-Santos *et al.* 2016). Trypsin is stable under high salt conditions but varies depending on the source of the enzyme. The addition of salt affects enzyme stability. The salt causes a decrease in enzyme activity due to the salting-out process. High concentrations of NaCl compete with enzymes for binding water, resulting in stronger interactions between protein molecules (Bougatef *et al.* 2010). Commercial trypsin is typically extracted from pig and cattle pancreas. The use of pig pancreas raw material is a problem for Muslims because of its prohibition (Napitupulu *et al.* 2021), while the use of trypsin from pork has the potential to contaminate active compounds and infectious bacteria that harm humans when consumed (Zhang *et al.* 2020). Fish can be a solution for the supply of halal trypsin.

Trypsin can be extracted from several parts of the body of a fish. Trypsin can be obtained from the internal organs of fish, such as the intestines. Some studies have shown that trypsin is produced in the intestines of yellowfin tuna and red snapper (Arbajayanti *et al.* 2021), tuna intestine (Nurhayati *et al.* 2020), and the bigeye tuna intestines (Pamungkas *et al.* 2022). Trypsin is also produced from the internal organs of other fish, such as the spleen of yellowfin tuna (Klomklao *et al.* 2004) and the pancreas of the great spotted shark (Blanco *et al.* 2013). The specific activity of trypsin varies depending on its source. Among several sources of trypsin, the highest trypsin activity was produced from the intestine of yellowfin tuna at 4.908 U/mg, and the lowest was produced from the intestine of red snapper at 0.076 U/mg (Arbajayanti *et al.* 2021). Gudmundsdóttir *et al.* (2013) stated that trypsin from Atlantic cod has a greater ability to degrade substrate protein compared to bovine trypsin due to increased flexibility of the active site, so that it can make the substrate more easily degraded or hydrolyzed by enzymes. This is evidence that fish are able to produce good trypsin enzymes. Fish is a potential source of enzymes because of its high production rate, especially waste in the form of offal that has not been optimally utilized.

Tuna is a high-value export commodity (Hutapea *et al.* 2020), with production continuing to increase. Production in 2020 is expected to reach 139,300 tons (KKP 2023). The increase in tuna commodities resulted in improper utilization of fishery product waste. Approximately 36% of fish

waste is generated during processing. Fish waste consists of 17% head, 8% skin, 4% bones, 2% fins, and 5% innards (Sayana and Sirajudheen 2017). Research on trypsin from tuna innards could be an alternative to the use of commercial trypsin enzymes. The characteristics and stability of trypsin enzymes are thought to differ depending on the origin of the fish organs. Therefore, this study aimed to determine the characteristics of trypsin in the internal organs of yellowfin tuna and its stability in NaCl.

METHODS

Material

The materials used in this study were tuna offal enzymes (intestine, liver, and spleen), dimethyl sulfoxide (Merck, Darmstadt, Germany), 4% SDS, glycerol (Merck, Darmstadt, Germany), βmercaptoethanol (βME), Coomassie Briliant Blue R-250 (AppliChem, Darmstadt, Germany), Separating gel (Sigma-Aldrich, Missouri, United States), Stacking gel (Sigma-Aldrich, Missouri, United States), Bio-rad protein marker (Bio-Rad Laboratories, Inc. United States), tris base (Sigma-Aldrich, Missouri, United States), hydrochloric acid (Merck, for analysis, Darmstadt, Germany)), acetic acid (Merck KGaA Darmstadt, Germany), N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Sigma-Aldrich, Missouri, United States), Bovine Serum Albumin (BSA) (AppliChem, Darmstadt, Germany), distilled water, $CaCl_2 \cdot 2H_2O$ (Merck, Darmstadt, Germany), and NaCl (Merck, Darmstadt, Germany).

Trypsin enzyme extraction from the viscera of yellowfin tuna

Viscera used as a source of enzymes consisted of intestine, liver, and spleen with a total weight of 10.33±6.66 g, 28.00±6.25 g, and 7.33±1.53 g, respectively. Trypsin was extracted from the intestine, spleen, and liver of tuna. The samples used had been stored for 1 year in the freezer at -13°C. The extraction procedure was based on the method of Bougatef *et al.* (2008) with modifications. The viscera sample was cut into pieces (±1-1.5 cm), and liquid nitrogen was added and pulverized using a mortar. The sample was then suspended in buffer (50 mM Tris–HCl, pH 8.0) at a ratio of 1:4 (w/v) between sample and buffer. The mixture was homogenized and centrifuged at 9,500 g for 30 min at 4°C. The supernatant (crude extract of the trypsin enzyme) was separated to measure enzyme activity using the method described by Khantaphant and Benjakul (2008), with modifications and protein concentration (Bradford 1976).

Determination of optimum temperature

The optimum temperature test on trypsin enzyme was conducted with 0.05 mL of trypsin enzyme mixed with 2.5 mL of BAPNA pH 8 solution. The mixture was incubated at different temperatures (30, 40, 50, 60, and 70°C) for 10 min, followed by the addition of 30% acetic acid to a volume of 1 mL. The sample was incubated again for 10 min, and the absorbance of the sample was measured using a spectrophotometer at a wavelength of 410 nm.

Determination of optimum pH

Optimum pH measurements were performed at pH values of 6, 7, 8, 9, and 10. The analysis began by mixing BAPNA solution (pH 6–10 in buffer Tris-HCl 0.05 M, $CaCl_2.H_20$ 0.02 M) with 0.05 mL of the enzyme sample and then incubating at the optimum temperature (60°C) for 10 min. The solution was added to 1 mL of 30% acetic acid and measured using a 410 nm wavelength spectrophotometer.

Kinetics of the trypsin enzyme

The kinetic parameters of the enzyme were determined by assaying the protease under varying BAPNA concentrations (1–3.5 mmol L−1) (under optimized assay conditions). Kinetic constants, including the apparent Michaelis–Menten constant (K_m) and maximum velocity (V_{max}), were calculated from the Lineweaver–Burk plot.

Trypsin molecular weight measurement (SDS-PAGE)

Trypsin molecular weights were determined using SDS-PAGE, starting with the pretreatment stage. A sample of 10 µL was mixed with a sample buffer consisting of 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol (βME). Ten microliters of each sample buffer were added. The solution was vortexed, centrifuged for 1 min, and stored at 0°C. The next step was the preparation of gel stock at a concentration of 4% and separating gel at

15%. Samples with a protein concentration of 0.394 mg/ml were added to the wells at a volume of 15 µL. The next step was to run the sample with a current of 34 mA/gel at a voltage of 70 V for 15 min, followed by a voltage of 170 V until the band on the marker reached the lower limit of the gel. Samples were gel-stained with 0.1% Coomassie Brilliant Blue R250 in 50% methanol and 7% acetic acid. Rinsing was performed using 7% acetic acid until the bands were visible (Laemmli 1970).

Addition of NaCl with different concentrations

This method refers to Nurhayati *et al.* (2020). Testing the effect of NaCl on enzyme activity begins with the addition of NaCl (5, 10, 15, 20, 25, and 30% (b/v)) into 0.05 mL of the enzyme samples at room temperature. The next step was to mix the BAPNA solution with an optimum pH of 8 and then incubate it at an optimum temperature of 60°C. Acetic acid (30 mL, 1 mL) was added to the sample and measured using a 410 nm wavelength spectrophotometer.

Data analysis

Data analysis was conducted on enzyme activity analysis, protein concentration, specific activity, and enzyme characteristics testing, consisting of optimum temperature, optimum pH, substrate concentration, and NaCl concentration. Quantitative data from the research results were processed using the Microsoft Excel application to calculate the mean value and standard deviation. The data were shown in tabular form and explained descriptively. The effect of different types of samples (intestine, liver, and spleen) of tuna on enzyme activity, protein concentration, and specific activity was tested using the Completely Randomized Design (CRD) one-way ANOVA (Analysis of Variance) method, which was previously tested for normality first. Data processing was carried out using SPSS 25.

RESULTS AND DISCUSSION

Specific activity of the trypsin enzyme

Specific enzyme activity is the result of the number of units of enzyme activity per milligram of protein (Djarkasi *et al.* 2017). Trypsin enzyme activity was performed using N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) substrate dissolved in Tris-HCl, pH 8, and incubated at 60°C. Protein levels were measured using the Bradford method, which was then measured using a spectrophotometer with a wavelength of 595 nm. The principle of protein content analysis is that the protein in the sample will bind to the Coomassie Brilliant Blue G-250 dye, which binds to arginine and lysine residues (Walker 1996). The results of the trypsin enzyme-specific activity measurement can be seen in Table 1.

The results shows that the different organ treatments had no significant effect on enzyme activity and protein concentration, while specific activity showed a significant effect. The results of Duncan's further test shows that the specific activity of intestinal organs significantly influenced the liver and spleen. The specific activity of trypsin, shown in Table 1, obtained the highest intestinal value of 0.948±0.114 U/mg. The results of organ-specific activity of the intestine and spleen in this study were lower than those Arbajayanti (2022), which amounted to 4.98±0.52 U/mg in the intestine and 2.67±0.10 U/mg in the spleen. Research conducted by Nurhayati *et al.* (2021) obtained the activity value of trypsin crude extract from the intestine of yellowfin tuna of 0.050±0.001 U/mL, protein content of 0.195±0.000 mg/mL, and specific activity of 0.256±0.0005 U/mg. Trypsin was also studied by Nurhayati *et al.* (2020) using tuna intestine, which obtained trypsin crude extract activity of 0.205 U/mL, protein content of 0.700 mg/mL, and specific activity of 0.29 U/mg. Suhito (2016) stated that enzyme work is influenced by several factors, including pH, temperature, substrate concentration, activators, and inhibitors. These factors are related to habitat and genetics in fish species (Silva *et al.* 2011).

Optimum temperature

Enzymes, as one of the main biological macromolecular components, have maximum activity when impacted by optimal temperature conditions (Zamani *et al.* 2023). Sulistyowati *et al.* (2016) stated that temperature is one of the main factors that can determine trypsin enzyme activity. Enzymes function above the optimum temperature to denature the enzyme (Kishimura *et al.* 2008). Enzymes, which are

proteins, can be denatured by heat, similar to other proteins. This means their structure is disrupted, causing them to lose their functional ability. Denaturation is more likely to happen at temperatures exceeding the enzyme's optimal range. When an enzyme becomes denatured, its active site—the region that binds to the substrate—alters in shape, rendering it ineffective in catalyzing the reaction. The effects of temperature on trypsin enzyme activity are shown in Figure 1.

The optimum incubation temperatures were determined to be 30, 40, 50, 60, and 70°C. The optimum enzyme activity occurs at 60°C and decreases at 70°C, which can occur due to protein denaturation. Fish habitats can affect the optimum temperature of the enzyme. Fish habitat consists of tropical zone fish, temperate zone fish, and frigid zone fish. Tuna fish are included in the tropical zone fish because they inhabit all warm oceans worldwide (Rohit *et al.* 2012).

Optimum pH

Determination of the optimum pH was analyzed using 0.05 M tris-HCl buffer pH 6–10. pH testing can be used to determine the optimum conditions for enzyme activity. Nurhayati *et al.* (2020) explained that the tris-HCl buffer serves to maintain pH stability. Trypsin activity at various pHs (6–10) can be seen in Figure 2.

Determination of the optimum pH of the intestine, liver, and spleen of tuna was determined using 0.05 M tris-HCl buffer at pH 6, 7, 8, 9, and 10. The function of HCl was to maintain the pH stability. The intestine, liver, and spleen organs were optimum at pH 8 and experienced a decrease in activity at pH 9.

Kinetics of trypsin enzyme

Enzyme kinetics are determined by the Michaelis-Menten curve graph, which is a graph of the relationship between the substrate concentration [S] and enzyme activity (V) (Fayyaz *et al.* 1995). The maximum speed value (V_{maks}) is a condition in which speed can no longer be added. The results of the measurement of substrate concentration using trypsin enzyme kinetics are shown in Figures 3 and 4.

The trypsin enzyme activity values present at substrate concentrations of 3.5 mM, which, in the intestine, liver, and spleen were 0.156 U/mL, 0.083 U/mL, and 0.075 U/mL, respectively. The activity of the trypsin enzyme, which increases with increasing substrate concentration, is in accordance with the research of Nurhayati *et al.* (2021). The activity of the trypsin enzyme in the intestine of yellowfin tuna increased from a substrate concentration of 0.1 mM to 2 mM, then became constant at a concentration of 3 mM, and decreased at a concentration of 4 mM.

The calculation of reaction speed (V_{max}) is determined by the Lineweaver-Burk equation (Figure 5). The Vmax value is obtained in mmol/s, while the K_m value is obtained in mM. The factor that needs to be considered in order for the enzyme to work properly is the amount of substrate used to achieve the reaction speed value. This situation is expressed by the Michaelis-Menten constant (K_m), which is the substrate concentration required for the enzyme to reach half its maximum speed (Radzicka and Wolfenden 1995). The reaction velocity (V_{max}) generated in this study was obtained at an intestine value of 0.248 mmol/s, a liver value of 0.138 mmol/s, and a spleen value of 0.096 mmol/s, while the constant value (K_m) obtained was 2.342 mM for intestine, 2.268 mM for liver, and 1.276 mM for spleen. These results indicate that K_m values in the intestine, liver, and spleen are higher than V_{max}. A higher K_m value compared to V_{max} proves that the affinity of the enzyme to the substrate is low, so that a high substrate concentration is needed to increase the reaction speed. The value of enzyme kinetics in this study is greater than the research conducted by Nurhayati *et al.* (2020), who obtained the K_m value of tuna intestinal trypsin of 1.12 mM.

Molecular weight (SDS-PAGE)

Measurement of the molecular weight of the trypsin enzyme was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This method is an electrophoretic method used for separation (Arndt *et al.* 2012). The analysis was carried out using protein markers of 10–250 kDa. SDS-PAGE electrophoresis results were visualized using Coomassie Brilliant Blue dye. Staining was performed after removing the gel from the two glass plates. Molecular weight data are shown in Figure 5.

Table 1. Specific activity of the trypsin enzyme.

Sample	Enzyme activity (U/mL) (U/mg)	Protein concentration (mg/mL)	Enzyme specific activity (U/mg)	Enzyme specific activity (U/mg)*
Intestines	0.149±0.021[a]	0.155±0.006[a]	0.948±0.114[b]	4.98±0.52
Liver	0.102±0.021[a]	0.164±0.023[a]	0.610±0.029[a]	0.28±0.28
Spleen	0.099±0.029[a]	0.162±0.015[a]	0.605±0.159[a]	2.67±0.10

*Arbajayanti (2022). Different superscript letters in the same column indicate a significant effect (α=0.05).

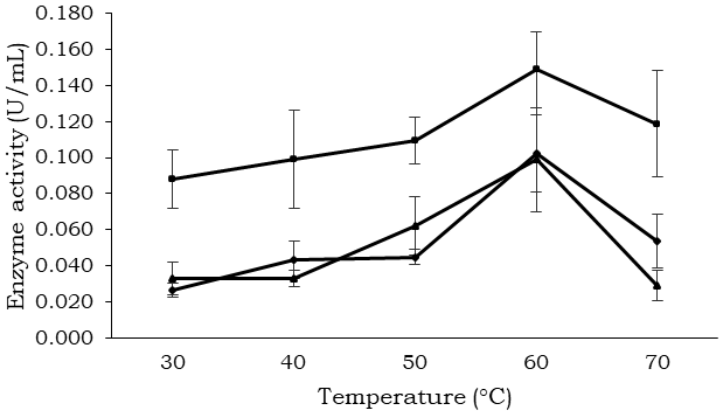

Figure 1. Trypsin enzyme activity at different incubation temperatures in the intestine (■), liver (♦), and spleen (▲) of tuna.

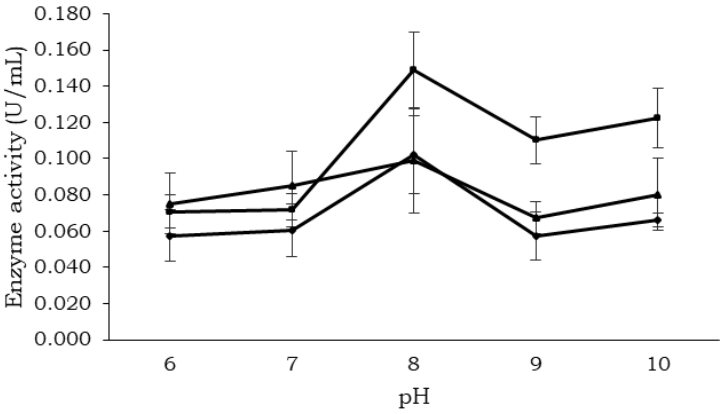

Figure 2. Trypsin enzyme activity at different pH in the intestine (■), liver (♦), and spleen (▲) of tuna.

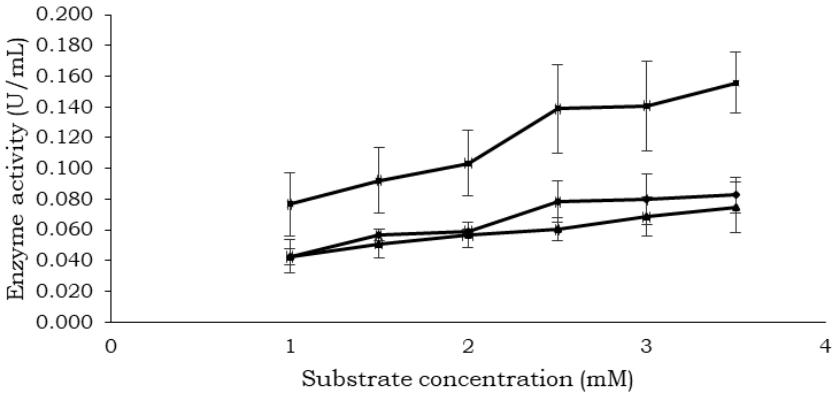

Figure 3. Trypsin enzyme activity at different substrate concentrations in the intestine (■), liver (♦), and spleen (▲) of tuna.

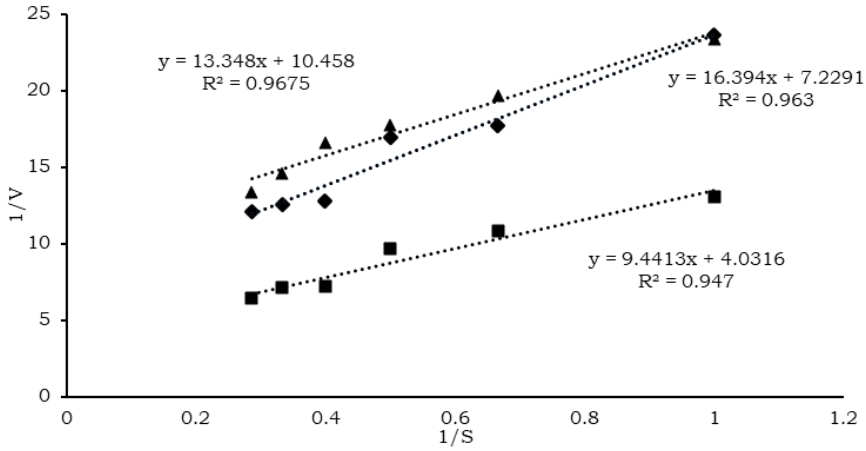

Figure 4. Reaction speed of trypsin enzyme in intestine (■), liver (♦), and spleen (▲) of tuna.

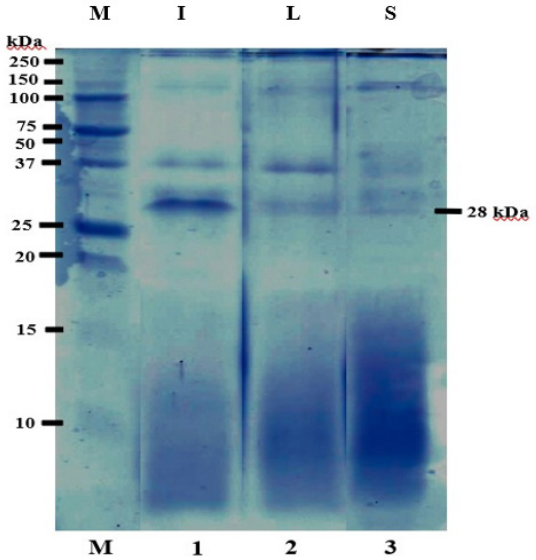

Figure 5. Polyacrylamide gel of trypsin enzyme by the SDS-PAGE method. M: marker, I: intestine, L: liver, S: spleen.

Several proteins with varying molecular weights from each part of the fish viscera were identified based on the SDS-PAGE results. The analysis was conducted using protein markers of 10–250 kDa. The molecular weight measurement data from each sample were obtained for the intestine at 29.347±0.510 kDa, liver at 27.438±0.196 kDa, and spleen at 27.951±0.148 kDa. Zamani *et al.* (2023) explained that the difference in trypsin molecular weight is due to the influence of various factors such as habitat variation, climate, autolytic degradation, and genetic diversity among fish species.

Stability of trypsin enzyme in NaCl

The salt concentration also affected trypsin enzyme activity. Trypsin enzyme stability analysis in NaCl was performed at NaCl concentrations of 5–30% at 5% intervals. The effects of different NaCl concentrations on trypsin enzyme activity are shown in Figure 6.

The intestines and liver were stable up to 30% NaCl because they maintained their activity above 50%, while the spleen was stable only at 20% NaCl concentration, resulting in a relative activity value of 56%. Bahrens *et al.* (2023) stated that the liver is a very vital organ against damage because this organ is passed by blood carried from all over the body, especially in the digestive system. Joyce and Axelsson (2021) mentioned the location of the spleen adjacent to the liver, which plays an important role in storing red blood cells.

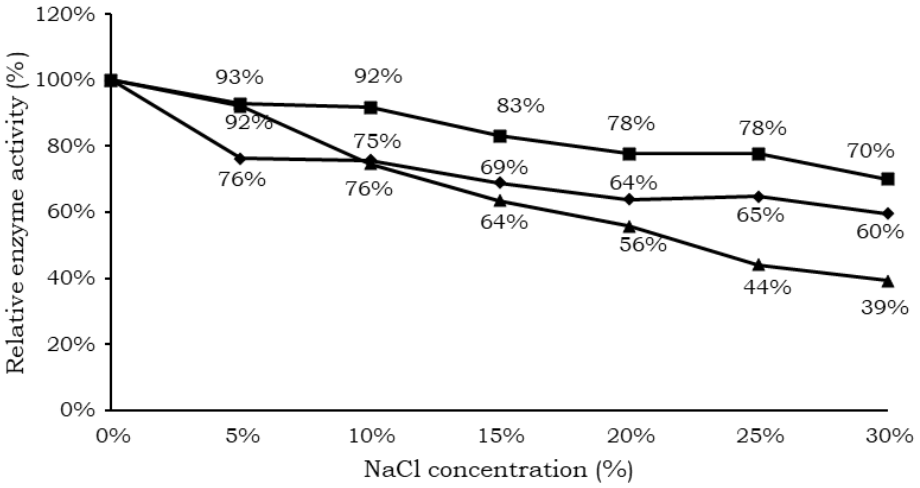

Figure 6. Relative activity of trypsin enzyme with 5–30% NaCl concentration in intestine (■), liver (♦), and spleen (▲) of tuna.

Discussion

Many studies have focused on fish-derived protease enzymes such as trypsin. However, there are differences from the findings of other studies owing to a number of factors, including the use of other methodologies, different species, feeding conditions (fed fish as opposed to starved fish), and the type of enzyme preparation (Alarcon *et al.* 1998). The high level of purification of the trypsin enzyme can also influence the optimum level of the enzyme involved in the hydrolysis process (Klomklao *et al.* 2006).

The rate at which enzyme-catalyzed reactions occur is influenced by temperature. Typically, as the temperature rises, enzyme activity also increases until it reaches an optimal level. Beyond this optimal temperature, extreme temperatures, whether too high or too low, can lead to the denaturation and inactivation of enzymes. Since fish are ectothermic, meaning their body temperature varies with their surroundings, this affects their metabolic rates and enzyme activity (Robinson 2015). The results obtained show similarities in the optimum conditions for the trypsin enzyme, which comes from the trypsin enzyme extract from the intestinal tract of sturgeon fish, which is approximately 60°C (Zamani *et al.* 2023). In another study, trypsin isolated from the pancreas of catshark (*Scyliorhinus canicular*) showed optimum activity at 55°C (Blanco *et al.* 2013). Catshark (*Scyliorhinus canicular*) is a temperate zone fish found in subtropical waters of the Mediterranean Sea (Navarro *et al.* 2018). Research on frigid zone fish or fish living in Antarctic waters has shown that Walleye pollock (*Theragra chalcogramma*) has an optimal trypsin temperature of 50°C. The difference in the optimum conditions for the trypsin enzyme from cold-water fish compared to other types of fish is lower, approximately 40–45°C (Kishimura *et al.* 2008). Heat-induced denaturation of the enzyme generally results in decreased activity at temperatures higher than 70°C (Prihanto *et al.* 2019). The location, species' living environment, and its body components can all contribute to this diversity. A wide range of ideal temperatures has been reported for protease enzyme function. This discovery suggests that the fish enzyme is thermostable, which might be attributed to the presence of Ca^{2+} in the Bougatef extraction buffer (Bougatef 2013). Trypsin has a high activity value at an alkaline pH between 8.5 and 11 (Namjou *et al.* 2019). Enzymes operate most efficiently within specific pH ranges. Straying from these ranges can diminish enzyme activity or even lead to denaturation, which is the loss of their structure and function. Fish residing in various aquatic habitats encounter different pH levels, affecting how their enzymes perform. In general, these enzymes are irreversibly denatured under very acidic and alkaline conditions. Low activity of this enzyme has also been observed at very acidic and alkaline pH (Khangembam *et al.* 2012). Different fish species determine the optimum pH for the production of the trypsin enzyme. Some examples include the enzyme trypsin, which is produced from sardine viscera in alkaline water (*Sardinops sagax caerulea*) with an optimum pH of 8.0

(Castillo-Yanez *et al*. 2005), silver mojarra fish (*Diapterus rhombeus*) (Silva *et al*. 2011), and jumbo squid (*Dosidicus gigas*), which are found at a pH of 8.5 (Villalba-Villalba *et al*. 2017). The optimum activity at alkaline pH is in accordance with Benjakul and Morrissey (1997), who stated that trypsin enzymes include alkaline proteases, so that they bind well to substrates under alkaline conditions.

Enzyme activity increases with the addition of substrate concentration, but after a certain point, further increases in substrate concentration lead to a rise in enzyme activity only until a constant rate is reached. This state, where the rate can no longer be increased, is known as the maximum speed (V_{max}). Enzyme kinetics, commonly referred to as the rate of enzyme activity, can function as a guide for maximum absorption of an enzyme and its concentration on a particular substrate (Srinivasan 2022). The Michaelis-Menten constant (K_m) refers to the substrate concentration at which an enzyme reaches half of its V_{max}. It is typically associated with the affinity of an enzyme for a substrate. A greater K_m value represents reduced affinity of the enzyme for the substrate (Chrisman *et al*. 2023).

Nurhayati *et al*. (2020) reported that the intestine of tuna had activity at a substrate concentration of 1 mM of 0.2 U/mL, then continued to increase up to 3.5 mM of 0.308 U/mL. The K_m value of trypsin varies according to the fish species. Khandagale *et al*. (2017) obtained a K_m value of 0.0206 mM for a sample of *Sardinella longiceps* innards. Research conducted by Costa *et al*. (2013) reported that the pyloric caeca of *Caranx hippos* obtained a K_m value of 0.69 mM. *Rastrelliger kanagurta* fish obtained a K_m value of 0.430 mM (Khandagale *et al*. 2013). Blanco *et al*. (2013) from the *Scyliorhinus canicular* sample obtained a K_m value of 0.104 mM. The concentration of the substrate is a key factor influencing the activity of the trypsin enzyme. As the substrate concentration increases, the test tube becomes turbid, with a slight sedimentation observed during the study. This indicates the impact of substrate concentration on enzyme activity levels. With the enzyme concentration held constant, adding more substrate can enhance trypsin activity until it reaches its maximum capacity. Once the trypsin enzyme is saturated with substrate, further additions do not increase its activity.

Khantaphant and Benjakul (2010) explained that fish trypsin has a molecular weight of 20–30 kDa. SDS-PAGE results showed that the protein separated and lost its original conformation and was negatively charged, so that the protein moved towards the positive pole and separated based on its size (Arndt *et al*. 2012). Khandagale *et al*. (2017) reported that trypsin derived from *Sardinella longiceps* has a molecular weight of 24 kDa. The molecular weight obtained from the spleen of tongol tuna (*Thunnus tonggol*) is 24 kDa (Klomklao *et al*. 2004). Trypsin from purified Kurisi fish (*Acanthopagrus latus*) viscera has a molecular weight of 23 kDa (Namjou *et al*. 2019). A sample from *Catla catla* showed a single band with a molecular weight of 20.2 kDa (Khangembam *et al*. 2012)

Research carried out by Reza *et al*. (2020) explained that gourami (*Trichogaster fasciata*) treated with salt water concentrations (3, 6, and 9 ppt) for 30 days experienced liver function disorders, such as bleeding and necrosis of liver tissue. The decrease in the relative activity of trypsin enzyme also occurred in the study by Khandagale *et al*. (2017), who used sardine offal (*Sardinella longiceps*) with a relative activity of 40% at a 30% NaCl concentration. Research conducted by Aissaoui *et al*. (2017) on *Scorpaena notata* samples showed a relative activity of 53.32% at 30% NaCl. The relative activity of trypsin from shark fish decreased by 91% at 15% NaCl (Nurhayati *et al*. 2024).

The decrease in trypsin activity was caused by enzyme denaturation due to the salting-out effect. Bougatef *et al*. (2008) explained that high concentrations of NaCl will compete with enzymes in binding water so that stronger protein interactions occur. Scopes (1987) stated that salt includes compounds that are easily ionized in polar solvents; if added to the enzyme solution, it can reduce water activity, so that hydrophobic interactions between non-polar amino acid residues on enzyme protein molecules will be stronger. Wheaton and Lawson (1985) stated that most enzymes are damaged and inactive in saturated salt solutions.

In the food industry, trypsin enzymes that remain stable in NaCl are employed. According to Rianingsih *et al*. (2016), trypsin is used to enhance the quality of fish sauce. When 0.3% of the enzyme is added along with 20% salt concentration, it leads to a greater degree of hydrolysis and reduces TVB, TMA, and pH levels, allowing microorganisms responsible for spoilage to produce volatile bases. Permanasari *et al*. (2014) also found

that incorporating 0.3% trypsin enzyme and 25% salt concentration into fish sauce improves the product's quality, with lower TVBN and TMA values compared to fish sauce made without trypsin enzyme.

CONCLUSION

The optimum conditions for trypsin enzyme activity were 60°C, pH 8, and substrate concentration of 3.5 mM, and the highest V_{max} and K_m values were found in the intestine. Enzymes that are more stable under NaCl conditions are found in the intestines. The intestine, liver, and spleen can be used as producers of trypsin.

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