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Characteristics of Crude Pepsin Enzyme from Catfish Stomach (*Clarias* sp.)

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Abstract: Fish stomach is a by-product of the fishing industry that has potential as a natural source of pepsin enzyme, particularly from catfish (*Clarias* sp.). This study aimed to characterize crude pepsin extracted from catfish stomachs. The extraction was carried out by homogenizing the stomach with 10 mM Tris-HCl buffer (pH 7.5), followed by centrifugation at 10,000 g for 15 min at 4°C. The obtained pepsinogen was activated using 3 N HCl at pH 2 and neutralized to pH 2.75 with 2 N $NaHCO_3$. Enzyme activity was determined using the hemoglobin assay at 280 nm, and protein concentration was measured by the Bradford method at 595 nm. The crude pepsin showed an activity of 33.50 ± 0.87 U/mL, protein concentration of 0.358 ± 0.005 mg/mL, total activity of 1,608 ± 41.57 U, and specific activity of 93.52 U/mg. The enzyme exhibited optimal activity at 50°C and pH 4, with relative activity toward NaCl, $ZnCl_2$, and $FeCl_3$ of 81.08%, 49.10%, and 128.15%, respectively, indicating Fe^{3+} acted as an activator. These results demonstrate that catfish stomachs can serve as a potential halal-compatible pepsin source, supporting enzymology advancement and fish waste valorization.

Introduction

Catfish is one of the most popular and widely consumed freshwater fish species among Indonesians from all walks of life. The total national catfish production from 2017 to 2023 was reported to reach 5,283,755 tons (1). For the city of Pekanbaru alone, total catfish production from 2018 to 2022 reached 35,692 tons (1). The FAO (Food and Agriculture Organization) Department of Fisheries and Aquaculture ranks catfish third as an economically important fish species after carp and tilapia (2). In addition to being affordable, catfish also has various advantages, such as being easy to cultivate and rich in nutrients (3-5). Catfish contains 12.82% protein, 3.70% fat, 2.70% ash content, 2.60% carbohydrates, 5.59% calcium, and 72.59% water content (6).

The increase in catfish production generates considerable waste, including skin, head, bones, and internal organs. Internal organs represent approximately 9–11% of total fish weight, equivalent to about 892.3 tons annually in Pekanbaru. These by-products are commonly used for animal feed and fertilizer, yet their biochemical potential remains underutilized. Transforming this waste into value-added biomaterials through enzyme biotechnology offers both economic and environmental benefits (7, 8). Among fish-derived enzymes, pepsin is of particular interest because it plays a critical role in protein hydrolysis and is widely applied in fish sauce production, collagen extraction, and gelatin manufacture (9).

Currently, most commercial pepsin is sourced from porcine tissues, which raises religious and ethical limitations for Muslim consumers (10, 11). Consequently, fish stomachs are being explored as alternative sources. Pepsin enzymes from aquatic species exhibit distinctive catalytic properties such as wider temperature and pH tolerance due to physiological adaptations to their habitats (9, 12). Comparative studies have shown species-specific differences in enzyme characteristics: pepsin from smooth hound (*Mustelus mustelus*) displayed optimal activity at 45 °C (13), while yellowfin tuna (*Thunnus albacares*) pepsin showed higher activity at 50 °C (14, 15). These variations suggest that pepsin from catfish (*Clarias* sp.) may possess unique enzymatic profiles linked to its tropical freshwater environment, justifying further investigation.

Therefore, this study aimed to extract and characterize crude pepsin enzyme from catfish stomachs using Tris-HCl buffer, contributing to the broader review and understanding of fish-derived pepsin enzymes as potential halal and sustainable alternatives for industrial applications.

Experimental Section

Materials

Fresh catfish (*Clarias sp.*) were obtained from a local aquaculture farm in Pekanbaru. The fish used were healthy adults aged 6–8 months, with an average weight of 400–600 g, and fasted for 24 h before sampling to minimize digestive variability. All procedures were conducted under chilled conditions to prevent enzymatic degradation. Other materials used included Tris (Vivantis, Malaysia), hemoglobin (CDH, India), trichloroacetic acid (TCA) 99% (Labotiq,

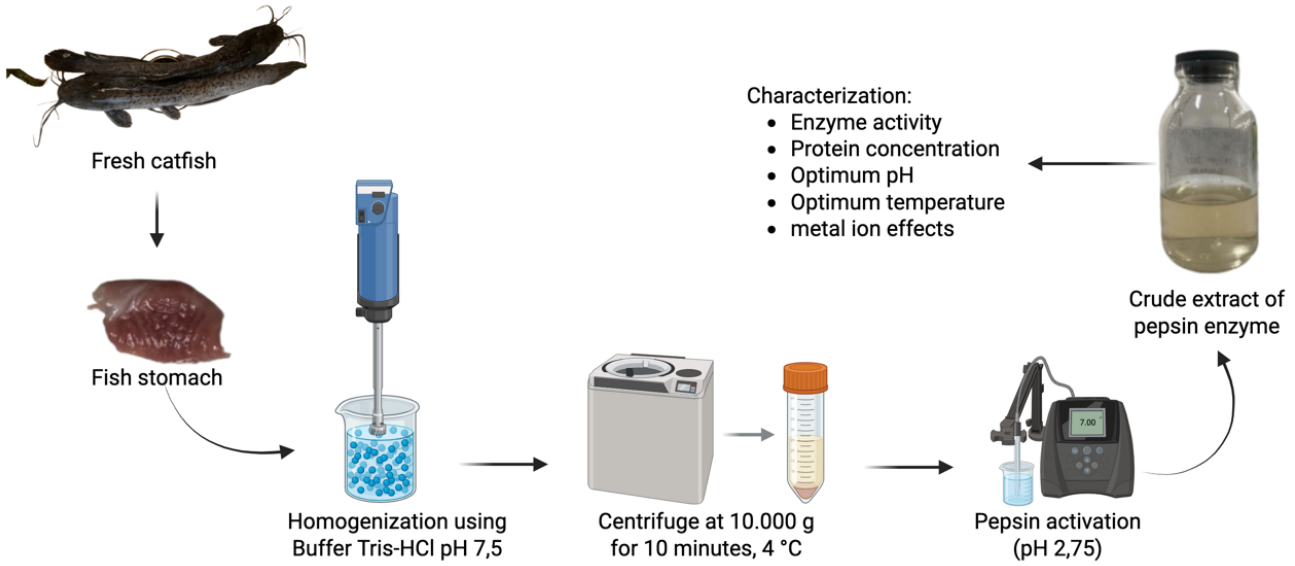

Figure 1. Extraction of pepsin enzyme from catfish stomach (*Clarias* sp.).

Indonesia), bovine serum albumin (AppliChem, Germany), Coomassie Brilliant Blue (Himedia, India), hydrochloric acid 37% (Emsure, Germany), sodium chloride (Xijiang Science, China), zinc chloride (Merck, Germany), ferric chloride (Loba Chemie), acetic acid (Labotiq, Indonesia), sodium bicarbonate (Himedia, India), phosphoric acid (Labotiq, Indonesia), ethanol 96% (Emsure, Germany), and Whatman No. 1 filter paper. The equipment included a refrigerated centrifuge (Centurion Scientific K3 series, temperature accuracy ±0.5 °C), a homogenizer (IKA T25 digital ULTRA-TURRAX), a thermostatic incubator (±0.2 °C precision), a spectrophotometer (Biobase BK-UV1800), micropipette (Dragon Lab), digital scale (ACAS SF-400C), and pH meter (Tryte Technologies).

Preparation of Catfish Stomach Samples

The sample used was catfish (Clarias sp.). The stomachs were separated from the fresh fish, washed thoroughly under running water to remove residual feed and mucus, and stored in sterile polyethylene bags at 4 °C prior to extraction. Each sample was weighed and recorded for morphometric data. The catfish samples were adult individuals (average length 42.8 ± 0.5 cm; average weight approximately 500 g), consisting of mixed sexes and obtained from a local freshwater aquaculture farm in Pekanbaru, Indonesia. All fish were healthy and maintained under standard feeding conditions using commercial floating pellets twice daily before sampling.

Extraction of Pepsin Enzyme from Catfish Stomach

The cleaned stomach tissue was minced and homogenized in a 10 mM Tris-HCl buffer (pH 7.5) at 1:2 (w/v) using the IKA homogenizer for 3 min at 12,000 rpm. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C using a refrigerated centrifuge with automatic temperature control (±0.5 °C). The resulting supernatant was collected as crude pepsinogen extract (17). Activation was performed by adjusting the pH to 2.0 using 3 N HCl and maintaining the solution in a temperature-controlled water bath (25 °C) for 10 min, then raising the pH to 2.75 with 2 N $NaHCO_3$ and precipitating for 6 h at 4 °C. The obtained supernatant was used as the crude pepsin enzyme extract. The resulting solution was the crude pepsin enzyme extract (**Figure 1**).

Pepsin Enzyme Activity Test

Pepsin enzyme activity was determined using 2% hemoglobin substrate at pH 2 following (18). A total of 1 mL substrate was mixed with 0.2 mL enzyme extract and incubated for 10 min at 37 °C in a thermostatic incubator (±0.2 °C). After incubation, 2 mL of 5% TCA was added to stop the reaction, followed by a 50-minute precipitation at 25 °C. The mixture was filtered, and absorbance was read at 280 nm using a spectrophotometer. One unit of enzyme activity was defined as a 0.001 increase in absorbance per minute.

Protein Concentration of Pepsin Enzyme

Protein concentration was analyzed by the Bradford method (19). A reaction tube containing 0.1 mL enzyme sample was mixed with 5 mL Bradford reagent, incubated 5 min at 25 °C and measured at 595 nm, with BSA standard curve (0.01–0.20 mg/mL)

Analysis of the protein concentration of the crude pepsin enzyme extract began with the addition of 5 mL of Bradford reagent to a reaction tube containing 0.1 mL of sample solution. Incubation was carried out for 5 min, and measurements were taken using a spectrophotometer at a wavelength of 595 nm. The Bradford reagent was prepared by dissolving 25 mg of Coomassie Brilliant Blue in 12.5 mL of 95% ethanol, adding 25 mL (v/v) of 85% phosphoric acid, and adding distilled water until the volume reached 250 mL. The solution was filtered using Whatman No. 1 filter paper. The preparation of standard solutions began by dissolving Bovine Serum Albumin (BSA) at a concentration of 2 mg/mL. The stock solution was diluted to a standard range of 0.01–0.20 mg/mL. The standard solutions were measured for absorbance using a spectrophotometer at a wavelength of 595 nm.

$$\mathrm{SE\ (U/mg)} = \frac{\mathrm{EA\ (Units)}}{\mathrm{PW\ (mg)}}$$

Equation 1 | *SE* = specific enzyme activity of pepsin, *EA* = total enzyme activity, and *PW* = total protein weight.

Specific Activity of Pepsin Enzyme

Enzyme specific activity is a direct measurement to assess enzyme purity based on the number of active enzyme molecules per milligram of protein (20). The higher the enzyme specific activity, the higher the enzyme purity (21). **Equation 1** is used for calculating pepsin enzyme specific activity.

Total enzyme activity is expressed in units and is obtained by multiplying the enzyme activity per unit volume (U/mL) by the total volume of the enzyme solution (mL). Total protein concentration (mg) is calculated by multiplying the protein concentration (mg/mL) by the total volume of the enzyme solution (mL).

Determination of the Optimum Temperature and pH of Pepsin

Optimum temperature was tested using a precision-controlled incubator (±0.2 °C) at 30, 40, 50, and 60 °C for 10 min, and optimum pH was tested at pH 2–5 using 0.25 M acetate buffers.

Determination of the influence of metal ions

The effects of monovalent (NaCl), divalent ($ZnCl_2$), and trivalent ($FeCl_3$) metal ions were examined by adding 0.1 mL of each ion solution to 1 mL of 2% hemoglobin substrate (pH 4) and 0.1 mL enzyme extract, incubated at 50 °C for 15 min in a precision-controlled incubator. Relative activity was expressed as a percentage of control (without ions).

Results and Discussion

Proportion and Morphometry of Catfish Stomach

The proportion is used to estimate the percentage of body weight that can be utilized (22). A catfish with an average length of 42.80 ± 0.5 cm has a stomach proportion of 0.70% of the total body weight, with an average stomach weight of 3.08 ± 0.5 g. The stomach of a catfish is pale red in color, cone-shaped, and curved. The stomach has a smooth surface with a slightly slimy texture and is elastic. The elastic nature of the stomach serves to accommodate food entering the body. The size of the stomach adapts to the size, age, and type of fish, and the volume of nutrients consumed by the fish can influence the production of pepsin (23, 24).

The stomach consists of four layers: the mucosa, submucosa, muscle, and serosa. (25) states that the entire surface of the stomach is covered by mucus cells containing mucopolysaccharides to protect the stomach wall from the action of hydrochloric acid. Hydrochloric acid is secreted by the stomach glands (oxintic glands) and functions to break down food. The outermost layer of the stomach is called the serous layer. The serous layer is very thin and composed of fibroblasts (26). The gastric mucosa contains tubular glands, namely oxyntic (gastric) glands and pyloric glands. Oxyntic glands function to produce acid by secreting mucus, hydrochloric acid (HCl), and pepsinogen (27). The gastric mucosa in the stomach produces protease enzymes that work at low pH levels. Hydrochloric acid (HCl) is secreted by the oxintic glands/gastric mucosa to create a low pH environment. Hydrochloric acid is formed from hydrogen ions (H+) and chloride ions (Cl-) transported by different pumps in the plasma membrane of parietal cells (28). Morphometry is a method of measuring and analyzing the size and shape of fish bodies. Morphometric analysis of catfish stomachs as crude pepsin enzyme samples was conducted by measuring the weight, length, and width of the stomach before pepsinogen was isolated.

Table 1. Morphometry of catfish stomach.

No	Parameters	Units	Mean
1	Weight	g	3.08 ± 0.5
2	Length	cm	3 ± 0.5
3	Width	cm	3 ± 0.5

The morphometric results of the catfish stomach showed weight, length, and width (**Table 1**). The weight of the catfish stomach is directly proportional to the thickness of the stomach, while the diameter of the stomach is inversely proportional to the thickness of the stomach. The stomach in fish differs from that in mammals. Pepsinogen in mammals is secreted by chief cells, and hydrochloric acid is secreted by parietal cells (29). This is different in fish, where pepsinogen and hydrochloric acid are secreted together by parietal cells in the stomach lining or mucosa, which contains oxintic glands.

Crude Extract of Pepsin Enzyme from Catfish Stomach

The crude extract obtained from centrifuging the homogenized fish stomach solution and Tris-HCl buffer at pH 7.5 consists of 48 mL of pinkish-brown pepsinogen (#780700). Pepsinogen is the inactive form of pepsin; when exposed to hydrochloric acid (HCl), the stomach fluid with a pH of 1.5 to 2.0, it releases 44 amino acids through autocatalysis, thereby activating it into pepsin (30). Centrifugation at 10,000 g for 15 min at 4 °C was conducted using a refrigerated high-speed centrifuge (Eppendorf 5810R) to maintain enzyme stability.

Centrifugation was performed at 10,000 g for 15 min at 4 °C using a refrigerated high-speed centrifuge (Eppendorf 5810R) to ensure constant temperature and prevent enzyme degradation. The extracted pepsinogen from the catfish stomach is activated, causing it to change color to pale yellow (#c2b883) in liquid form. Enzyme activity testing, protein concentration, and the specific activity value of the pepsin enzyme from the catfish stomach were then conducted.

Based on the data presented in **Table 2**, it can be seen that the average enzyme activity performed in 3 replicates

Table 2. Crude extract of pepsin enzyme.

Testing	Pepsin enzyme
Volume	48 mL
Enzyme activity	33.50 ± 0.87 U/mL
Total enzyme activity	16.08 ± 41.57 U
Protein concentration	0.36 ± 0.005 mg/mL
Total protein	17.19 ± 0.22 mg
Specific activity	93.54 ± 2.56 U/mg

was 33.500 ± 0.866 U/mL. The value of pepsin enzyme activity is influenced by several factors, such as the habitat of the fish and the size of the fish's stomach. The weight capacity of the fish's stomach will affect the size of the fish's stomach. The size of the stomach affects the protease enzyme components within it, including pepsin (31). The reported enzyme activity (33.500 ± 0.866 U/mL) was re-evaluated to ensure accuracy and no calculation error was found. The value was confirmed based on the hemoglobin assay calibration curve, and normalization was performed per milliliter of crude extract, not per milligram of protein. The relatively high activity likely reflects the catalytic efficiency of tropical freshwater species such as Clarias sp., which have been reported to exhibit higher pepsin activities compared to temperate fish (32).

The activity of crude pepsin extract from tuna, according to (33), in samples of Yellowfin Tuna Stomach (Thunnus albacares) yielded a crude extract activity of 1.27 U/mL. The use of a buffer can influence the enzyme activity obtained. The total pepsin enzyme activity in the sample was 1.608 ± 41.569 U. The study (34) using Yellowfin Tuna (Thunnus albacares) samples yielded a total enzyme activity of 1.235 U. A study conducted (16) using smooth hund (Musletus musletus) samples isolated pepsin using tris-HCl buffer, resulting in enzyme activity of 183.51 U. These differences may be due to variations in the samples used, but may also stem from differences in the analytical methods applied (35).

Optimum Temperature of Pepsin Enzyme in Catfish Stomach

Temperature affects the catalytic rate of enzymatic reactions. As temperature increases to a certain threshold, it can enhance enzymatic activity up to the optimal temperature condition; however, excessive temperature increases can cause enzyme activity to decrease due to enzyme denaturation. Enzyme activity can also be influenced by other factors such as Potential of Hydrogen (pH), substrate concentration, enzyme concentration, inhibitor compounds, and cofactors (36). The results of the temperature effect test on pepsin activity can be seen in **Figure 2**.

Based on the data presented in **Figure 2**, it is evident that the enzyme activity of pepsin from catfish stomach is influenced by incubation temperature. The test results show that 50°C has better activity compared to 30 °C, 40 °C, and 60 °C. This is also reported in (35), which states that the optimal pepsin temperature for yellowfin tuna (Thunnus albacares) is 50 °C and decreases drastically at 60 °C. Zhao *et al*. (37) found that in samples of albacore tuna (Thunnus alalunga), optimal activity occurred at 50 °C and decreased when the temperature was increased to 60 °C. Wu T *et al*. (38) conducted a study on pepsin from sea eels, with an optimal temperature range of 30 °C to 50 °C.

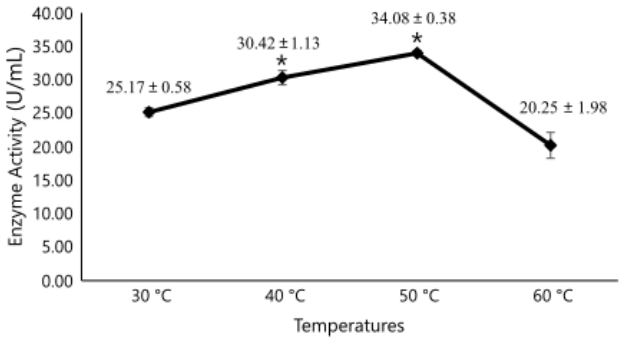

Figure 2. Enzyme activity of crude pepsin extract from catfish stomach at different temperatures. All values are presented as mean ± standard deviation (n = 3). (*, $p < 0.05$) shows significant difference to group 30 and 60 °C.

The optimal activity of pepsin in catfish stomachs occurs at 50 °C and decreases at 60 °C. Brier S *et al*. (39) states that the optimal temperature for pepsin varies depending on the type and habitat of the fish. This is because pepsin in fish stomachs adjusts to the temperature of the fish's habitat for metabolic processes. Generally, the optimal temperature for pepsin in fish ranges from 30-55 °C. Fish living in tropical waters have an optimal temperature range of 40-50 °C, while fish living in cold waters are sensitive to high temperatures and thus have a lower optimal pepsin temperature.

Pepsin activity increases with rising temperature. This is because thermal energy enhances enzyme-substrate interactions, thereby increasing reaction rate and enzyme activity. Enzyme activity continues to increase until the optimal temperature is reached. However, excessively high temperatures can reduce pepsin activity due to enzyme denaturation (40). Nurhayati T *et al*. (34) states that denaturation causes the enzyme structure on the surface to open, altering the active site of the enzyme (aspartic acid) and resulting in reduced enzyme activity. Conversely, if the temperature is too low, no bond will form between the enzyme and substrate, causing the enzyme reaction rate to slow down and activity to decrease.

The increase in enzyme activity up to 50 °C reflects Arrhenius behavior, in which the reaction rate rises exponentially with temperature due to greater molecular collision frequency and improved enzyme–substrate interaction. Beyond this optimum, partial unfolding of the enzyme's tertiary structure near the catalytic aspartic residues (Asp32–Asp215) leads to a sharp decrease in activity. The apparent activation energy (Ea) estimated from the Arrhenius region is approximately 32 kJ/mol, comparable to mesophilic proteases. From a kinetic perspective, the rise in temperature up to 50 °C may also reduce the apparent Km value, indicating enhanced substrate affinity before denaturation occurs. These findings are consistent with reports in pepsin from tropical fish species such as yellowfin tuna (*Thunnus albacares*) and Nile tilapia (*Oreochromis niloticus*) (41).

Optimum pH of Pepsin Enzyme in Catfish Stomach

An optimal and constant acidity level (pH) will produce stable enzymes and significantly affect the activity of the pepsin enzymes formed (42). Salelles L *et al*. (43) states that in general, pepsin is optimal at a pH of 2 to 4. Pepsin activity will decrease when the pH deviates from its optimal value.

Based on the data presented in **Figure 3**, it is evident that differences in pH values of pepsin from catfish stomachs affect pepsin enzyme activity. Pepsin with a pH of 4 exhibits better activity compared to pH 2.3 and 5. Pepsin is an acidic protease enzyme whose stability is related to protein denaturation at pH conditions above 6.0 (44). Stanforth KJ *et al*. (44) investigated the effect of pH on rattail pectora, showing that pepsin A and pepsin B have optimal stability within a pH range of 2 to 6, with a significant decrease in activity when pH exceeds 6, and a drastic decrease when pH exceeds 6. stated that pepsin in albacore tuna has stability under pH conditions of 2 to 5.

Enzyme pH activity describes the pH conditions at which

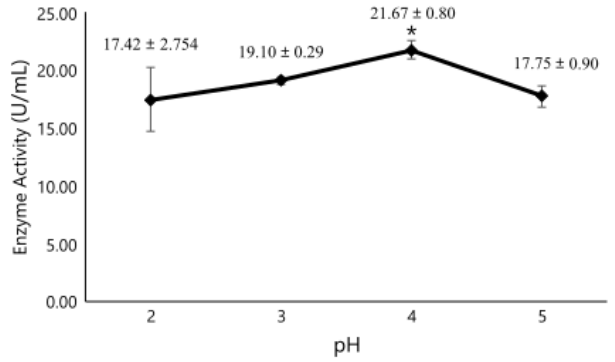

Figure 3. Activity of crude pepsin enzyme extract from catfish stomach at different pH levels. All values are presented as mean ± standard deviation (n = 3). (*, $p < 0.05$) shows significant difference to other groups.

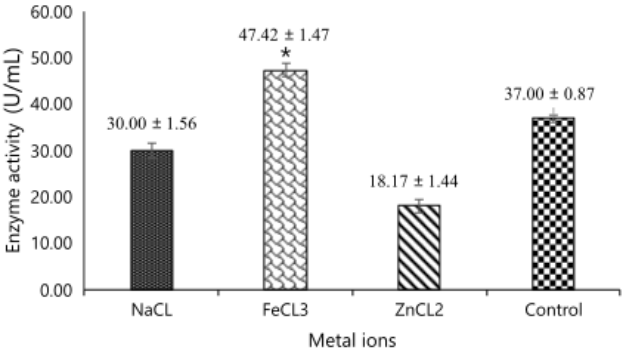

Figure 4. Activity of crude pepsin enzyme extract from catfish stomach with the addition of different metal ions. Statistical analysis using one-way ANOVA followed by Tukey's test ($p < 0.05$) showed that $FeCl_3$ addition significantly increased enzyme activity compared to NaCl and $ZnCl_2$ treatments.

Tabel 3. Relative activity of pepsin enzyme from catfish stomach.

Metal ions	Relative activity (%)
NaCL	81.081
$FeCL_3$	128.153
$ZnCL_2$	49.099
Kontrol	100

the proton donor and acceptor sites on the catalytic side of the enzyme reach their optimum ionization levels. Certain pH levels, such as extreme values, can cause denaturation, resulting in the loss of the enzyme's biological activity (45).

From a kinetic viewpoint, at the optimum pH (4.0), the ionization state of both aspartic residues in the catalytic center is balanced, leading to maximal catalytic turnover (kcat) and minimal substrate dissociation (Km). At higher pH values, partial deprotonation disrupts charge balance within the active site, lowering catalytic efficiency and substrate affinity (46).

Determination of the Influence of Metal Ions

Enzyme activity is influenced by metal ions in two ways: as activators or as inhibitors. These reactions cannot be distinguished chemically, but can be distinguished after interaction occurs when metal ions are added to the enzyme. As activators, ions can bind to enzymes, thereby affecting the rate of enzyme reactions, whereas as inhibitors, the interaction causes a decrease in the rate of reaction (47). Metal ions can assist in the binding between enzymes and substrates. Additionally, they can directly bind to enzymes, stabilizing the active conformation of the enzyme, and can also bind to enzyme inhibitors, thereby affecting the inhibitor's ability to inhibit the enzyme. Testing the effect of metal ions on enzyme activity shows an influence that causes an increase or decrease in enzyme activity. The results of testing the effect of inhibitors on pepsin enzyme activity are shown in **Figure 4**. Specifically, the results of testing metal ions on the relative activity of pepsin enzymes can be seen in **Table 3**.

Based on the data presented in **Figure 4** and **Table 3**, it can be seen that FeCl3 metal ions have high activity values and act as enzyme activators that can increase the reaction rate of pepsin enzymes. Meanwhile, NaCL and ZnCl2 metal ions have low activity values and act as enzyme inhibitors that can decrease the reaction rate of pepsin enzymes. Pepsin is an enzyme classified as an aspartic protease. da Silva-López RE *et al*. (48) states that metal ions such as $FeCl_2$ and $ZnSO_4$ have relatively high activity toward aspartic proteases from Aspergillus foetidus, similar to pepsin from catfish stomachs. Some enzymes and inhibitors require specific ions to maintain their activity stability. These ions can act as inhibitors at certain concentrations but also as activators at different concentrations.

The activating effect of Fe^{3+} may be attributed to its biochemical role in maintaining the tertiary structure of the enzyme and stabilizing the electrostatic environment of the catalytic site. Fe^{3+} can interact with negatively charged residues (Asp32 and Asp215) in the active site of pepsin, reducing repulsive forces and preserving the correct spatial arrangement for proton transfer during catalysis. This charge stabilization promotes proper folding and reduces local flexibility around the active cleft, preventing denaturation at acidic pH. In contrast, Zn^{2+} may form stronger coordinate bonds with carboxylate groups, distorting the active conformation and thereby acting as an inhibitor. Additionally, similar Fe^{3+}-mediated activation effects have been reported for pepsin-like proteases from *Oreochromis niloticus* and *Thunnus albacares*, suggesting a conserved mechanism across teleost species (49).

Conclusion

Rough pepsin enzyme extract from catfish stomach can be extracted and tested, yielding an enzyme activity of 33.50±0.87 U/mL and a protein concentration of 0.358±0.005 mg/mL. The characteristics of crude pepsin enzyme extract from catfish stomach show optimal activity at 50°C and optimal activity at pH 4. The addition of $FeCl_3$ metal ions can increase the reaction rate of pepsin enzyme from catfish stomach, while the addition of NaCl and $ZnCl_2$ metal ions can decrease the reaction rate of pepsin enzyme from catfish stomach.

These findings fill an important knowledge gap in enzymology and fish waste valorization by demonstrating that catfish (*Clarias sp.*) stomach an underutilized by-product can serve as a potential alternative source of pepsin enzyme with comparable biochemical characteristics to commercial and marine-derived pepsins.

It is recommended that future research focus on optimizing purification methods, kinetic modeling (e.g., *Km* and *Vmax* estimation), and the application of catfish-derived

pepsin in industrial processes such as collagen hydrolysis or halal food production to enhance its commercial and sustainable potential.

Declarations

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Conflict of Interest

The authors declare no conflicting interest.

Data Availability

The unpublished data is available upon request to the corresponding author.

Ethics Statement

Not applicable.

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Additional Information

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