

Effect of Acid Type and Buffer-assisted Stimulation on Pepsin Recovery from Skipjack Tuna (*Katsuwonus pelamis*) Stomach

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ABSTRACT

The global tuna processing industry generates substantial by-products, including stomach tissues that remain underutilized despite their potential as a source of industrial enzymes. This study evaluated the effects of acid type, acid concentration, and buffer-assisted acid stimulation on crude pepsin recovery from skipjack tuna (*Katsuwonus pelamis*) stomach. Acetic acid (CH_3COOH) provided better enzymatic performance than hydrochloric and sulfuric acids. Among the concentrations tested, 1 M CH_3COOH produced the highest pepsin activity ($1.03 \pm 0.05 \text{ U} \cdot \text{mL}^{-1}$) and specific activity ($0.115 \pm 0.005 \text{ U} \cdot \text{mg}^{-1}$). Buffer-assisted acid stimulation yielded higher pepsin activities than direct acid extraction. Direct extraction produced substantially higher total protein concentration, suggesting greater co-extraction of non-pepsin proteins. The recovered crude pepsin exhibited both optimal activity and stability at pH 2, while the highest pepsin activity was observed at 50 °C, with stability maintained at 30 °C or below. More than 80% of the activity was retained within moderately acidic and mid-temperature ranges. These findings demonstrate that acid selection, concentration, and extraction strategy strongly influence functional pepsin recovery. Buffer-assisted acid stimulation with acetic acid provides a simple yet promising approach for obtaining catalytically active crude pepsin from tuna stomach by-products, supporting their valorization as a sustainable source of acid proteases for potential food and biotechnological applications.

Keywords: Acidic protease, *Katsuwonus pelamis*, Pepsin, Skipjack tuna

INTRODUCTION

The tuna industry plays a central role in the international seafood market and contributes significantly to global food supply chains. Among capture fisheries commodities, tuna is one of the most highly valued groups, along with cephalopods, shrimps, and lobsters. Indonesia is one of the world's largest tuna producers, with skipjack, bigeye, and yellowfin tuna as major commodities (Ministry of Trade of Republic of Indonesia, 2020; FAO, 2025). Tuna supports extensive economic activity, provides employment opportunities across harvesting, processing, and distribution, and

generates substantial export revenues, estimated at around 9% of the total export value (McCluney *et al.*, 2019; FAO, 2022).

Given such scale, the industry inherently generates large amounts of solid and liquid processing by-products, including head, bones, viscera, gills, dark muscle, skin, and cooking juice. In fish canning industries, for example, up to 65 to 70 percent of the original material is discarded as waste. Depending on the processing method and final product, tuna by-products may represent approximately 50–55% or more of the initial raw material (Gamarro *et al.*, 2013; Fraterrigo Garofalo *et al.*, 2023; Precup *et al.*,

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2024). Tuna canning also generates protein-rich cooking juice that requires appropriate management or valorization (Hajihama and Youravong, 2013).

In recent decades, fisheries by-products have been increasingly recognized as valuable resources for high-value materials such as gelatin, collagen, lipids, protein derivatives, and enzymes (Amnuakitt *et al.*, 2022; Heidari and Rezaei, 2022; Razali *et al.*, 2023; Binh and Hong, 2025; Sari *et al.*, 2025). Proteases are a group of enzymes with wide industrial applications and constitutes nearly 60% of the global enzyme market (Mamo and Assefa, 2018). Among them, pepsin, an aspartic protease, has potential applications in protein hydrolysis, collagen and gelatin extraction, cheese processing, and other food and biotechnological processes.

Fish by-products, specifically stomachs, are a valuable yet underutilized source of pepsin. Their digestive enzymes often display unique and versatile characteristics (Brier *et al.*, 2007; Nalinanon *et al.*, 2010a; 2010b; Kuepethkaew *et al.*, 2022; Pérez-Velázquez *et al.*, 2024). Moreover, pepsin available on the global market has historically been derived predominantly from porcine materials. While it has high efficacy and is widely available (Borrajó *et al.*, 2020), it also presents limitations in regions where religious and cultural restrictions prohibit the use of porcine-derived products (Iqbal *et al.*, 2020; Anggarkasih and Resma, 2022; Muttaqin *et al.*, 2025).

Pepsin is synthesized as the inactive precursor pepsinogen and becomes active under acidic conditions. Accordingly, acidic extraction has commonly been applied to recover pepsin from fish stomachs (Nalinanon *et al.*, 2008; 2010b; Pasaribu *et al.*, 2018). However, extraction conditions can influence enzyme activation, recovery, and stability. Thus, the selection of an appropriate acid is a critical prerequisite before optimizing extraction conditions.

Therefore, this study aimed to: (1) compare the effects of different acid types on crude pepsin recovery from skipjack tuna stomach; (2) determine the optimum concentration of the selected acid; and (3) compare buffer-assisted acid-stimulated

extraction with direct acid extraction and characterize the pH and temperature activity and stability of the recovered crude pepsin. The optimized approach is expected to support the valorization of tuna-processing by-products as a sustainable source of acid proteases. From an industrial and environmental standpoint, the effective valorization of tuna waste through enzyme recovery could simultaneously address environmental concerns and improve resource efficiency within seafood supply chains and contribute to waste reduction and circular bioeconomy initiatives.

MATERIALS AND METHODS

Materials

Skipjack tuna (*Katsuwonus pelamis*) stomach samples were collected, cut into small pieces, and stored at -20 °C until use. Sample preparation was carried out in the laboratory of the Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand.

Skipjack tuna (*Katsuwonus pelamis*) stomachs were obtained from a processing plant in Thailand. Upon arrival, the stomach contents were removed, and the stomachs were thoroughly rinsed with cold distilled water to remove residual digesta and adhering materials. The samples were cut into small pieces, packed in polyethylene bags, and stored at -20 °C before analysis. Samples were subjected to no freeze-thaw cycles prior to enzyme extraction. For each extraction, 50 g of tuna stomach tissue was used. Sample preparation and experimental procedures were carried out at the Faculty of Fisheries, Kasetsart University, Bangkok, Thailand.

Extraction of crude enzyme extract: Effect of acid type

Crude enzyme extraction was performed using a modified method of Tohmadlae *et al.* (2020). Tuna stomach tissue (50 g) was mixed with 0.2 M phosphate buffer (pH 7.0) at a sample-to-buffer ratio of 1:3 (w/v). The mixture was homogenized using a Polytron Kinematica PT 10-35 homogenizer

for 1 min at 10,000 rpm and subsequently incubated for 3 h at 4 °C with agitation at 100 rpm. The homogenate was then centrifuged at $10,000 \times g$ for 30 min at 4 °C, and the resulting supernatant was collected as the crude enzyme extract.

To determine the optimum acid for stimulation, the crude enzyme extract was separately treated with 2 M acetic acid (CH_3COOH), 2 M sulfuric acid (H_2SO_4), or 2 M hydrochloric acid (HCl). The acid-treated extracts were incubated at 4 °C for 30 min before enzyme activity analysis. Pepsin activity, protein concentration, and specific enzyme activity were subsequently determined for each treatment.

Extraction of crude enzyme extract: Effect of acid concentration

Based on the acid type screening, CH_3COOH was selected for concentration optimization. Tuna stomach tissue was extracted as described in the previous section by homogenizing the stomach tissue with extraction buffer at pH 7 at a sample to buffer ratio of 1:3 (w/v). After a 3 h incubation at 4 °C, the mixture was centrifuged at $10,000 \times g$. The supernatant was collected and separately mixed with CH_3COOH at concentrations of 1 M, 2 M, and 3 M at a ratio of 1:1 (v/v) followed by incubation at 4 °C for 30 min. The crude extracts were analyzed for physical characteristics, enzyme activity, protein concentration, and specific activity.

Comparison of direct acid extraction and buffer-assisted acid stimulated extraction

Two extraction strategies were compared using the selected acid and concentration. The first strategy was buffer-assisted acid-stimulated extraction. A 50 g portion of stomach tissue was first homogenized with 150 mL of 0.2 M phosphate buffer (pH 7.0). The mixture was homogenized for 1 min at 10,000 rpm and incubated for 3 h at 4 °C with agitation at 100 rpm. The homogenate was then centrifuged at $10,000 \times g$ for 30 min at 4 °C, and the resulting supernatant was collected. A 5 mL aliquot of the supernatant was subsequently mixed

with 5 mL of 1 M CH_3COOH and incubated for 30 min at 4 °C to stimulate enzyme activation.

The second strategy was direct acid extraction. A 50 g portion of tuna stomach tissue was directly mixed with 150 mL of 1 M CH_3COOH , corresponding to a tissue-to-acid ratio of 1:3 (w/v). The mixture was homogenized for 1 min at 10,000 rpm, incubated for 3 h at 4 °C with agitation at 100 rpm, and centrifuged at $10,000 \times g$ for 30 min at 4 °C. The supernatants obtained from both strategies were collected as crude extracts and further analyzed for pepsin activity and specific activity.

Pepsin activity assay

Pepsin activity was quantified based on a spectrophotometric method using bovine hemoglobin (Sigma-Aldrich, St. Louis, USA) as a substrate (Anson, 1938; Nolasco-Soria *et al.*, 2020; Tohmadlae *et al.*, 2020). A 2% (w/v) bovine hemoglobin substrate solution at pH 2.0 was prepared using ultrapure water and HCl according to the standard manufacturer's procedure. The reaction mixture consisted of 200 μL of 2% acidified hemoglobin substrate and 200 μL of crude enzyme extract and was incubated at 50 °C for 20 min. The reaction was terminated by adding 200 μL of 50% (w/v) trichloroacetic acid (TCA) corresponding to a final TCA concentration of approximately 16.7% (w/v). After centrifugation at $6,000 \times g$ for 10 min at 4 °C, the resulting supernatant was subjected to Lowry color development using alkaline copper and Folin–Ciocalteu reagents (Lowry *et al.*, 1951).

A 50-fold dilution of the supernatant was reacted with alkaline copper reagent, whereby peptide bonds formed copper–peptide complexes under alkaline conditions. Folin–Ciocalteu phenol reagent was subsequently added, and the copper-treated peptides, together with reducing amino acid residues, particularly tyrosine and tryptophan, reduced the phosphomolybdic–phosphotungstic components of the Folin reagent, producing an intense blue color (Lowry *et al.*, 1951). After color development, absorbance was measured at 750 nm using a UV-1700 spectrophotometer (Shimadzu,

Japan) and converted to tyrosine equivalents using a tyrosine standard curve (0–1 μM). Pepsin activity and specific activity were calculated following standard formulas adapted from Maiz *et al.* (2019). One unit of pepsin activity was defined as the amount of enzyme required to release 1 μmol of tyrosine per minute under the assay conditions, and was calculated as follows:

Pepsin activity ($\text{U}\cdot\text{mL}^{-1}$)

$$= \frac{[(\text{Tyrosine})_{\text{Sample}} - (\text{Tyrosine})_{\text{Control}}] \times \text{Dilution factor}}{\text{Volume of Enzyme Solution (mL)} \times \text{Assay incubation time (min)}}$$

Protein concentration was determined according to the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as the standard. Pepsin specific activity was then estimated by dividing the enzyme activity value ($\text{U}\cdot\text{mL}^{-1}$) by the total protein concentration.

Effect of pH on pepsin activity and stability

The effect of pH on pepsin activity was evaluated by incubating the crude enzyme with hemoglobin substrate prepared in buffers ranging from pH 2 to 7. Reaction mixtures consisted of 200 μL of 2% substrate and 200 μL of crude enzyme were incubated in buffers ranging from pH 2 to 7. Incubation was performed at 50 °C for 20 min and was terminated with 50% TCA.

For pH stability, crude enzyme extracts were preincubated separately in buffers at each pH value from 2 to 7 on ice for 30 min. Then, 200 μL of 2% substrate at pH 4 was added, followed by incubation at 50 °C for 20 min. Residual activity was then determined under standard assay conditions. Relative activity was expressed as a percentage of the highest activity observed.

Enzyme activity was determined using the same spectrophotometric method described previously. These assays enabled the determination of both optimal activity conditions and the stability range of tuna pepsin across physiologically and industrially relevant pH levels.

Effect of temperature on pepsin activity and stability

The effect of temperature on pepsin activity was evaluated by incubating the enzyme-substrate mixture at various temperatures. A mixture of 200 μL of enzyme extract and 200 μL of 2% substrate at pH 4 was incubated at various temperatures (0–80 °C) for 30 min. After incubation, the reaction was stopped with 50% TCA, and the absorbance of the supernatant was measured at 750 nm.

For temperature stability, crude enzyme extracts were preincubated without substrate at each temperature for 30 min. Afterward, 200 μL of 2% substrate at pH 4 was added, and the mixture was incubated at 50 °C for 20 min. The reaction was stopped using 50% TCA, and the mixture was then centrifuged before analyzing the supernatant for residual activity. Relative activity was expressed as a percentage of the highest activity observed.

Statistical analysis

A completely randomized design (CRD) with three independent collections was used as the experimental design. Statistical tests were performed using IBM SPSS Statistics for Windows, version 30.0 (IBM Corp., Armonk, NY, USA). Before parametric analysis, data normality and homogeneity were evaluated using the Shapiro–Wilk test and Levene’s test, respectively. Data satisfying the assumptions were analyzed using one-way analysis of variance (ANOVA). The data were presented as mean $\pm$ standard deviation. A probability value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Extraction of crude enzyme: Effect of acid type

The physical characteristics of the crude pepsin extracts varied substantially depending on the acid used to activate pepsinogen. As presented in Table 1, samples treated with 2 M HCl and 2 M CH_3COOH exhibited greater clarity and lighter coloration, whereas treatment with 2 M H_2SO_4 produced extracts that were distinctly darker and exhibited greater turbidity.

The enzymatic activity of crude extracts activated with different acids varied significantly (Table 2). Protein concentrations were similar among all treatments, indicating that acid type did not substantially influence overall protein solubility. The highest enzyme activity ($0.92 \pm 0.04 \text{ U} \cdot \text{mL}^{-1}$) and greatest tyrosine release (0.0744 ± 0.005) were obtained with 2 M CH_3COOH . This result is noteworthy because HCl is commonly used in published studies for pepsin isolation or activation.

Specific activity results further confirmed the superior performance of 2 M CH_3COOH , which yielded a specific activity of $0.209 \pm 0.001 \text{ U} \cdot \text{mg}^{-1}$ protein, which was higher than the values obtained with both HCl and H_2SO_4 . The higher activity obtained with CH_3COOH suggests that this organic acid provided sufficient acidification for pepsinogen activation while preserving greater enzymatic activity than the stronger mineral acids under the tested conditions.

Crude enzyme extraction: Effect of CH_3COOH concentration

Based on the initial comparison, CH_3COOH was selected as the most effective acid for activating pepsinogen during pepsin extraction from tuna stomachs. To determine its optimal concentration, further extractions were conducted using 1 M, 2 M, and 3 M CH_3COOH . The clarity, color, and odor of the crude extracts were visibly affected by the concentration of CH_3COOH used during activation (Table 3).

Extracts treated with 1 M CH_3COOH remained clear, while those prepared with 2 M and 3 M CH_3COOH showed progressively darker coloration and increased turbidity.

The enzyme activity results revealed that 1 M CH_3COOH produced the highest activity ($1.03 \pm 0.05 \text{ U} \cdot \text{mL}^{-1}$), and the highest protein concentration

Table 1. Effect of acid type on the physical characteristics and final pH of crude pepsin extracts from skipjack tuna (*Katsuwonus pelamis*) stomach. Crude extracts were treated with 2 M hydrochloric acid (HCl), sulfuric acid (H_2SO_4), or acetic acid (CH_3COOH), and their physical characteristics were evaluated qualitatively after extraction.

Acid treatment	Physical characteristics			
	Color	Clarity	Odor	Final pH
2 M HCl	Clear to slightly yellowish	Relatively clear	Sharp and pungent	pH 1.5
2 M H_2SO_4	Darker or cloudy	Less clear	Less pungent than HCl, but still acidic	pH 1
2 M CH_3COOH	Slightly yellow, softer in tone	Clear and stable	Vinegary and acidic	pH 2

Table 2. Effect of acid type on the enzyme activity, protein concentration, and specific activity of crude pepsin extracts from skipjack tuna (*Katsuwonus pelamis*) stomach. Crude extracts were prepared using 2 M hydrochloric acid (HCl), sulfuric acid (H_2SO_4), or acetic acid (CH_3COOH).

Acid treatment	Enzyme activity ($\text{units} \cdot \text{mL}^{-1}$)	Protein concentration ($\text{mg} \cdot \text{mL}^{-1}$)	Specific enzyme activity ($\text{units} \cdot \text{mg}^{-1}$ protein)
2 M HCl	0.86 ± 0.04^b	4.92 ± 0.25^a	0.174 ± 0.009^a
2 M H_2SO_4	0.80 ± 0.04^a	4.93 ± 0.24^a	0.162 ± 0.008^a
2 M CH_3COOH	0.92 ± 0.04^c	4.40 ± 0.22^a	0.209 ± 0.001^b

Note: Values are presented as mean $\pm$ SD ($n = 3$). Different superscript letters within the same column indicate significant differences ($p < 0.05$).

($8.89 \pm 0.4 \text{ mg} \cdot \text{mL}^{-1}$) among the tested concentrations (Table 4). Enzyme activity decreased progressively at 2 M and 3 M CH_3COOH , with 3 M CH_3COOH yielding the lowest activity ($0.75 \pm 0.03 \text{ U} \cdot \text{mL}^{-1}$).

Specific activity followed a similar pattern, peaking at $0.115 \pm 0.005 \text{ U} \cdot \text{mg}^{-1}$ for 1 M CH_3COOH and decreasing at higher concentrations. The decrease in enzyme activity at higher CH_3COOH concentrations may be associated with increased acidity, which may have reduced functional enzyme recovery. However, further structural analyses are required to confirm whether conformational changes or aggregation occurred.

Comparison of direct acid extraction and buffer-assisted acid stimulated extraction

In the stimulated approach, a neutral buffer (pH 7) with sample-to-buffer ratio of 1:3 was used as the initial extraction buffer, followed by 1M CH_3COOH at a buffer extract-to-acid ratio of 1:1 for acid stimulation. In direct acid extraction, 1 M CH_3COOH was used directly as the extraction buffer at a sample-to-acid ratio of 1:3.

Based on Table 5, buffer-assisted acid stimulation with 1 M CH_3COOH produced significantly different extraction outcomes from the

Table 3. Effect of acetic acid concentration on the physical characteristics and final pH of crude pepsin extracts from skipjack tuna (*Katsuwonus pelamis*) stomach. Physical characteristics were evaluated qualitatively after extraction at acetic acid concentrations of 1–3 M acetic acid.

Concentration of acetic acid	Physical characteristics			
	Color	Clarity	Odor	Final pH
1 M	Clear	Relatively clear	Noticeable	3
2 M	Slightly yellow	Slightly turbid	Vinegary and Acidic	2
3 M	Yellowish/dark	More turbid	Very strong Acidic	1

Table 4. Effect of acetic acid concentration on enzyme activity, protein concentration, and specific activity of crude pepsin extracts from skipjack tuna (*Katsuwonus pelamis*) stomach.

Concentration of acetic acid	Pepsin activity ($\text{units} \cdot \text{mL}^{-1}$)	Protein concentration ($\text{mg} \cdot \text{mL}^{-1}$)	Specific enzyme activity ($\text{U} \cdot \text{mg}^{-1} \text{ protein}$)
1 M	1.03 ± 0.05^c	8.89 ± 0.4^c	0.115 ± 0.005^b
2 M	0.90 ± 0.04^b	7.95 ± 0.4^b	0.113 ± 0.006^a
3 M	0.75 ± 0.03^a	7.15 ± 0.3^a	0.104 ± 0.005^a

Note: Values are presented as mean $\pm$ SD (n = 3). Different superscript letters within the same column indicate significant differences among acetic acid concentrations ($p < 0.05$).

Table 5. Enzyme activity, protein concentration, and specific activity of crude pepsin extracts from skipjack tuna (*Katsuwonus pelamis*) stomach obtained using buffer-assisted acid activation (stimulated extraction) and direct extraction with 1 M acetic acid (non-stimulated extraction).

Treatment	Enzyme activity ($\text{units} \cdot \text{mL}^{-1}$)	Protein concentration ($\text{mg} \cdot \text{mL}^{-1}$)	Specific enzyme activity ($\text{units} \cdot \text{mg}^{-1} \text{ protein}$)
Stimulated	1.03 ± 0.05^a	8.89 ± 0.4^b	0.115 ± 0.005^a
Non-stimulated	0.96 ± 0.05^b	22.27 ± 1.11^a	0.043 ± 0.002^b

Note: Values are presented as mean $\pm$ SD (n = 3). Different superscript letters within the same column indicate significant differences between treatments ($p < 0.05$).

non-stimulated method ($p < 0.05$). Enzyme activity was higher with stimulated extraction ($1.03 \pm 0.05 \text{ U} \cdot \text{mL}^{-1}$) than with the non-stimulated process ($0.96 \pm 0.05 \text{ U} \cdot \text{mL}^{-1}$). More importantly, stimulated extraction produced much higher specific activity ($0.115 \pm 0.005 \text{ U} \cdot \text{mg}^{-1}$) than the non-stimulated extracts ($0.043 \pm 0.002 \text{ U} \cdot \text{mg}^{-1} \text{ protein}$). The significantly higher protein concentration measured in the non-stimulated extracts ($22.27 \pm 1.11 \text{ mg} \cdot \text{mL}^{-1}$) suggests that direct acid extraction co-extracted a greater amount of proteins other than functionally active pepsin, thereby lowering the specific activity of the crude extract. Although direct acid extraction resulted in a higher protein concentration, its lower specific activity supports the possibility of greater co-extraction of non-pepsin or less-active protein components. In contrast, buffer-assisted acid stimulation increased the proportion of functionally active enzyme in the crude extract.

Effect of pH on crude pepsin activity and stability

The effect of pH on crude pepsin activity is shown in Figure 1a. The maximum activity occurred at pH 2.0, consistent with the well-established optimal pH range (1.5–3.0) for fish pepsins (Jesús-Ramírez *et al.*, 2017; Nolasco-Soria *et al.*, 2020). Activity gradually declined as the pH increased toward neutrality. At pH 3 and 4, the enzyme retained more than 70% of its maximal activity, whereas activity decreased to less than

50% at pH 7. Figure 1b shows that tuna pepsin was most stable at pH 2.0 and retained moderate stability at pH 3.0–4.0. Stability declined gradually above pH 5 and decreased to approximately 29% at pH 7.0.

Effect of temperature on activity and stability of crude pepsin

The optimum temperature represents the point of highest catalytic activity, while thermal stability reflects the enzyme's ability to retain activity after exposure to different temperatures. This study evaluated pepsin at temperatures ranging from 0 to 80 °C at 10 °C intervals to determine its peak activity and thermal stability. Figure 2a shows that pepsin extracted under stimulated conditions using 1 M CH_3COOH reached maximum relative activity at approximately 50 °C, retained over 80% of its relative activity between 40 and 60 °C, and declined sharply at 70 °C and above.

The thermal stability of the enzyme is shown in Figure 2b. Pepsin maintained over 90% relative stability after preincubation at temperatures from 0 °C to 30 °C and retained approximately 80% at 50 °C. A notable decline occurred above 60 °C, indicating moderate thermostability. This was consistent with the optimum activity profile (Figure 2a), in which 50 °C was identified as the optimum temperature.

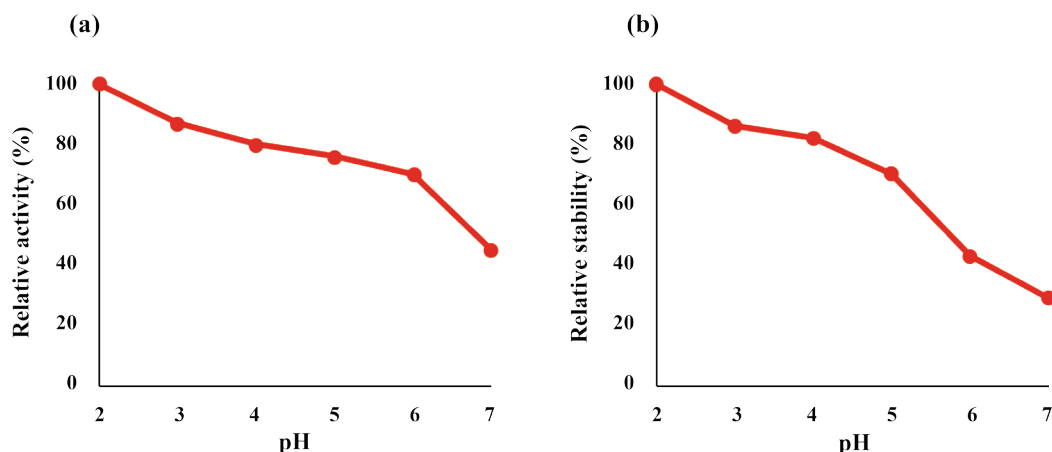


Figure 1. Effects of pH on the activity and stability of crude pepsin extracted from skipjack tuna (*Katsuwonus pelamis*) stomach: (a) relative activity determined at pH 2–7; (b) relative stability after pre-incubation of the crude enzyme at pH 2–7. Activity is expressed as a percentages to the maximum value, which was defined as 100%.

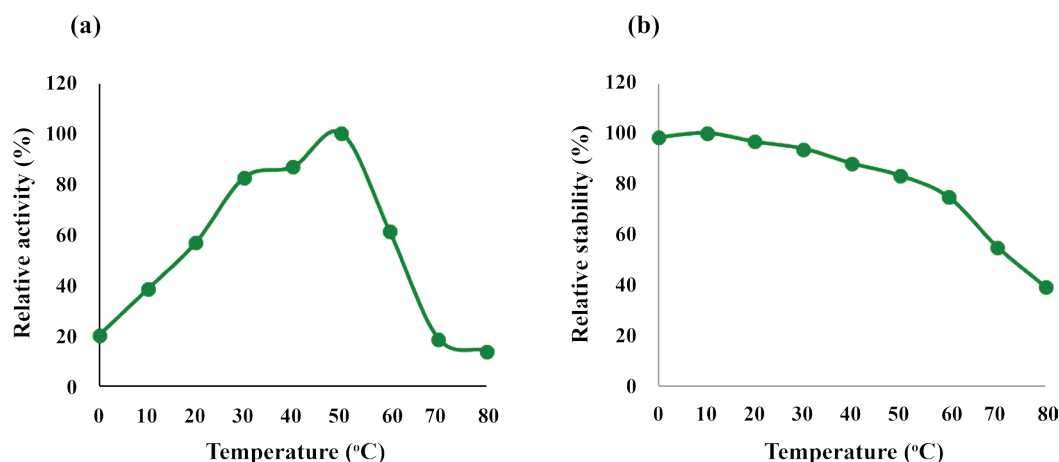


Figure 2. Effects of temperature on crude pepsin extracted from skipjack tuna (*Katsuwonus pelamis*) stomach: (a) relative activity measured at different reaction temperatures ranging from 0 to 80 °C, and (b) thermal stability expressed as relative activity after preincubation at different temperatures. Relative activity and stability are expressed relative to their respective maximum values, which were defined as 100%.

The present study demonstrates that extraction parameters, particularly acid type, acid concentration, and the use of buffer-assisted homogenization, played a decisive role in determining the yield, catalytic efficiency, and stability of pepsin derived from skipjack tuna (*Katsuwonus pelamis*) stomachs. Variations in these parameters directly influenced enzyme activity, specific activity, and extract clarity, highlighting the importance of controlled activation of pepsinogen during extraction.

Among the acids evaluated, acetic acid (CH_3COOH) consistently outperformed hydrochloric acid and sulfuric acid in preserving enzymatic functionality. Although strong mineral acids are commonly employed for pepsin activation, their strongly acidic conditions may destabilize the protein tertiary structure, leading to partial denaturation and reduced catalytic efficiency (Yu *et al.*, 2018; Gaikwad and Kim, 2024). In contrast, the superior activity and specific activity obtained with CH_3COOH indicate that this organic acid provided sufficient protonation for pepsinogen activation while minimizing structural damage. This observation supports previous reports suggesting that organic acids may better preserve protease conformation and solubility than strong inorganic acids (Delgado *et al.*, 2017; De Domenico *et al.*,

2019). The lower specific activity observed after direct acid extraction suggests that direct exposure of stomach tissue to acid may solubilize a broader range of proteins, including non-enzymatic or less-active proteins. In contrast, the buffer-assisted step may facilitate protein extraction before controlled acid activation, thereby improving the proportion of active pepsin in the crude extract.

Further optimization revealed that 1 M CH_3COOH was the most effective concentration for pepsin recovery. At this concentration, the enzyme exhibited the highest activity and specific activity, whereas higher acid concentrations resulted in darker, more turbid extracts and reduced enzymatic performance.

Advantage of buffer-assisted acid-stimulated extraction

A key finding of this study is the clear advantage of buffer-assisted acid-stimulated extraction over direct acid extraction. The stimulated method, which involved initial homogenization in a neutral buffer followed by controlled acid activation, produced extracts with significantly higher specific activity despite their lower total protein content. In contrast, the non-stimulated method yielded a substantially higher protein

concentration but markedly lower specific activity, suggesting substantial co-extraction of non-enzymatic or denatured proteins. This result aligns with previous findings that buffer systems may help preserve enzyme integrity by reducing premature denaturation and aggregation during extraction (De Domenico *et al.*, 2019; Asaduzzaman and Salmon, 2023). Highly acidic conditions may alter protein conformation and, depending on the protein and solution environment, promote denaturation and aggregation, thereby reducing enzymatic activity and functional stability (Fink *et al.*, 1994; Jahromi *et al.*, 2011). The observed data support the use of buffer-assisted extraction for preserving enzyme integrity and activity under the tested conditions.

Characteristics of pH and temperature

The optimum pH of the crude pepsin in this study was pH 2, which is within the commonly reported optimum range for fish pepsins, generally between pH 1.5 and 4.0. A previous study on pepsins from three sciaenid fish species, *Cynoscion othonopterus*, *Cynoscion xanthulus*, and *Cynoscion parvipinnis*, showed significantly greater activity at pH 2.0, with activity decreasing at pH 3.0 and falling to less than 40% at pH 4.0 or above (Pérez-Velázquez *et al.*, 2024). Similarly, three pepsin isoforms have been identified in lizardfish (*Saurida micropectoralis*); pepsin I and pepsin II were optimally active at pH 2.0, whereas pepsin III showed a higher optimum of pH 3.5 (Kuepethkaew *et al.*, 2022). Freshwater fish pepsins also exhibited acidic optima, although two reports indicated slightly higher values; snakehead (*Channa argus*) pepsins showed maximal activity at pH 3.0, 3.5, and 3.0 for the three isoforms, respectively (Chen *et al.*, 2009), while rice field eel (*Monopterus albus*) pepsins showed optimum activity at pH 3.0–3.5 (Weng *et al.*, 2010).

Among tuna-derived pepsins, the present result is supported by previous findings. In skipjack tuna, two purified pepsin isoforms were previously reported, with pepsin 1 showing optimum activity at pH 2.5 and pepsin 2 at pH 2.0 (Nalinanon *et al.*, 2010a). Similar optimum pH values have also been reported for albacore tuna (pH 1.5 and 3.0)

(Nalinanon *et al.*, 2010b) and yellowfin tuna (pH 2.0–3.5) (Pasaribu *et al.*, 2018), further supporting the acidic working range of tuna pepsin although the study reported a working pH range rather than a single sharply defined optimum. However, the yellowfin tuna study reported an optimum range rather than a single sharply defined value.

The optimum temperature of 50 °C observed in the present study is also comparable with that reported for several fish-derived pepsins, which commonly show maximum activity between 40 and 55 °C. These similarities support the classification of the recovered enzyme as an acid protease with typical pepsin-like behavior.

The optimum at pH 2.0 and the decline in activity and stability at near neutral pH are consistent with the general biochemical nature of pepsin as an acidic aspartyl protease. The reduction in activity under less acidic conditions may reflect changes in protonation state and enzyme conformation that disrupt the catalytic cleft, a phenomenon widely documented for pepsins exposed to pH values above 5 (Oliveira *et al.*, 2014; Jesús-Ramírez *et al.*, 2017; Razali *et al.*, 2023). However, the optimum pH for catalytic activity should be interpreted specifically as the pH at which the enzyme most efficiently hydrolyses the substrate during the assay, rather than a direct measure of enzyme stability.

Temperature profiling showed that the pepsin extracted from skipjack tuna stomach in the present study exhibited maximum proteolytic activity at approximately 50 °C. The results of this study showed a pattern similar to those previously reported for tuna pepsins (Nalinanon *et al.*, 2010a; 2010b). This result indicates that the enzyme exhibited relatively high catalytic efficiency at a moderately elevated temperature, which is consistent with the behavior of pepsins from various fish species, that exhibit optimum activity within the temperature range of 45–55 °C (Wald *et al.*, 2016; Mazumder *et al.*, 2018; Tohmadlae *et al.*, 2020; Kuepethkaew *et al.*, 2022; Fatiranes *et al.*, 2025). However, compared with optimum pH, the optimum temperatures of fish pepsins vary more widely among species and experimental conditions. For example, lizardfish pepsins III was optimally

active at 40 °C (Kuepethkaew *et al.*, 2022), similar to pepsin obtained from *C. parvipinnis* (Pérez-Velázquez *et al.*, 2024), snakehead (*Channa argus*) (Chen *et al.*, 2009), and rice field eel (*Monopterus albus*) pepsins I and II, while pepsin III was optimally active at 35 °C (Weng *et al.*, 2010). Japanese seabass (*Lateolabrax japonicus*), and some cold-water fish pepsins showed even lower temperature optima. These differences may reflect variation in enzyme conformation, intramolecular interactions, isoform composition, and evolutionary adaptation to the digestive physiology and environmental temperature of each species (Weng *et al.*, 2010; Cao *et al.*, 2011; Pérez-Velázquez *et al.*, 2024).

It is important, however, to distinguish between the optimum temperature for enzymatic activity and thermal stability. The optimum temperature for activity refers to the temperature at which the enzyme hydrolyses the substrate most efficiently during the reaction assay, whereas thermal stability refers to the ability of the enzyme to retain residual activity after prior exposure to a given temperature. In this study, the enzyme retained substantial stability up to approximately 50 °C, while its high residual activity at lower temperatures, particularly from 0 to 30 °C, indicated that the enzyme structure was well preserved under cold to mild conditions. This is consistent with previous findings for skipjack tuna pepsins, which were stable up to 50 °C with residual activity above 85%. However, after incubation at 60 °C for 30 min, residual activity declined sharply to 9.7% and 4.7% for pepsins 1 and 2, respectively (Nalinanon *et al.*, 2010a). Albacore tuna pepsin also showed similar thermal stability, retaining more than 85% activity up to 50 °C, followed by a marked decrease in activity above 50 °C (Nalinanon *et al.*, 2010b). These findings indicate that tuna pepsins can tolerate moderate heating but are susceptible to thermal inactivation when exposed to temperatures above 60 °C, consistent with previous studies on acid proteases (Precup *et al.*, 2024).

The biochemical properties observed in this study suggest that skipjack tuna pepsin is potentially suitable for industrial processes requiring strong acidity and moderate thermal stability, such as protein hydrolysis and pepsin-solubilised collagen

(PSC) production (Zhao *et al.*, 2018; Cadar *et al.*, 2024). Beyond its functional performance, the use of tuna stomachs as an enzyme source supports waste valorization and circular bioeconomy principles by converting underutilized by-products into high-value biomolecules.

Overall, the present findings provide systematic evidence that a simple organic acid-based, buffer-assisted stimulation strategy can improve the recovery and functional quality of pepsin from skipjack tuna stomachs. Unlike many previous studies that rely on direct extraction using strong mineral acids, this study shows that controlled activation using 1 M CH₃COOH yields a more catalytically efficient crude enzyme extract. By demonstrating how acid selection, concentration, and buffer use can be optimized to enhance enzymatic activity and stability, this research provides a valuable foundation for future investigations aimed at scaling up extraction processes, improving enzyme purity, and tailoring pepsin applications to meet specific industrial and market needs.

CONCLUSIONS

This study demonstrates that acid selection, concentration, and extraction strategy significantly influence the recovery and functional performance of pepsin from skipjack tuna stomach. The optimal extraction conditions involved the use of 1 M CH₃COOH under buffer-assisted stimulated conditions, resulting in higher enzyme activity and specific activity than those obtained by direct acid extraction. These findings indicate that simple, controlled acid activation enhances functional enzyme recovery while minimizing the extraction of non-functional proteins. The extracted pepsin exhibited optimal activity at pH 2 and 50 °C, while its highest pH and thermal stability were observed under strongly acidic conditions and at low-to-moderate temperatures, respectively. These characteristics support its potential suitability for processes conducted under acidic conditions and at moderately elevated temperatures. Overall, this optimized extraction approach supports the valorization of tuna-processing by-products through their conversion into sustainable source of industrial proteases and provides a practical basis for further application and scale-up studies.

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