Egyptian Journal of Aquatic Biology and Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(4): 1593 – 1613 (2025)
www.ejabf.journals.ekb.eg


# Isolation and Characterization of a Novel Acid Protease from Striped Marlin (*Kajikia audax*) Stomach with Potential as a Rennet Substitute in Dairy Processing

**Endang Ismail[1, 3], Asep Awaludin Prihanto[2], Sukoso[2*], Hartatik Kartikaningsih[2], Nurul Huda[2,4], Ilham Misbakudin Al Zamzami[1, 5]**

[1]Doctoral Program of Fisheries and Marine Science, Universitas Brawijaya, Malang 65145, Indonesia
[2]Department of Fishery Product Technology, Faculty of Fisheries and Marine Sciences, Brawijaya University, Jl Veteran, Malang, East Java, 65145, Indonesia
[3]Secondary Fisheries Business School, Kotaagung, Lampung, Jl Pantai Harapan, Way Gelang, Kotaagung Barat, Tanggamus, Lampung, 35384, Indonesia
[4]Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan, 90509, Sabah, Malaysia
[5]Coastal and Marine Research Center, Brawijaya University, Jl Veteran, Malang, East Java, 65145, Indonesia

[*]**Corresponding Author: alislah@ub.ac.id**

## ARTICLE INFO

**Article History:**
Received: May 19, 2025
Accepted: July 16, 2025
Online: July 24, 2025

**Keywords**:
Cheese production,
Milk-clotting agent,
Protease,
Proteolytic activity,
Striped marlin

## ABSTRACT

This study investigated the isolation, characterization, and optimization of acid protease from the stomach of the Striped Marlin (*Kajikia audax*). The gastrointestinal tract—including the stomach, pyloric caeca, pancreas, and intestine—was analyzed for size, weight, volume, and enzymatic activity. The stomach was selected for further study based on its high enzymatic potential. Subsequent analyses included proximate composition, acidified gastric extract (AGE), protease activity, total protein, and the effects of pH, temperature, metal ions, SDS-PAGE, and milk-clotting ability. Proximate analysis of the stomach showed crude protein (16.39%), crude lipid (4.89%), moisture (73.96%), ash (1.05%), and carbohydrates (3.71%). The extraction yield was 33.33%, and protease activity was significantly higher (1,013.823 ± 2.541 U/mL, $P < 0.05$) than in other organs. A 30% ammonium sulfate fraction yielded optimal specific activity (3.7116 ± 0.023 U/mg), with a 2.3-fold purification and 45% yield. Biochemical characterization revealed peak activity at pH 2.0 and 50 ± 1°C, with the enzyme retaining over 80% activity between 45–55°C. At 5 mM concentration, $MgCl_2$ enhanced activity (443% ± 12%, $P< 0.01$), while NaCl and $CaCl_2$ were inhibitory (37% and 48% relative activity, respectively). Milk-clotting activity, evaluated using 12% (w/v) reconstituted skim milk, was optimal at pH 3.0–4.0 and 50°C, with a clotting/proteolytic ratio of 1.8. The dialyzed enzyme maintained >70% activity after 1 hour at 45°C and showed consistent milk-clotting, suggesting its potential as a rennet substitute. This marine-derived protease offers promising applications in cheese production.

## INTRODUCTION

Striped marlin (*Kajikia audax*) is a marine teleost belonging to the family Istiophoridae, with a geographic distribution encompassing the Indo-Pacific region, predominantly in tropical and temperate waters. This epipelagic predator exhibits highly migratory behavior and is vulnerable under conditions of overfishing **(Mamoozadeh *et al.*, 2020; Lam *et al.*, 2022)**.

Fishermen's awareness of catch preservation has led to the implementation of weeding and gutting treatments prior to landing. However, it is important to note that the waste generated from these practices still holds significant economic value due to its nutrient-rich content. Post-harvest handling typically involves evisceration and mechanical processing, generating biological waste rich in nutrients (15–30% crude protein, 0–25% lipids, and 50–80% moisture content by weight), though this waste remains commercially underutilized **(Ideia *et al.*, 2023)**.

The teleost digestive system contains various enzymes (proteolytic, lipolytic, and amylolytic). Among these, proteolytic enzymes, particularly those in the acid protease group, exhibit the highest activity **(de Oliveira *et al.*, 2022)**, suggesting potential for biotechnological applications under controlled processing conditions.

Currently, no precise data exists regarding the catch volume of striped marlin due to its extensive distribution across tropical and subtropical waters. In addition to recreational fishing **(Collette *et al.*, 2022)**, the species is also reported as bycatch in the tuna fishing industry **(Peatman *et al.*, 2023)**.

Obtaining milk coagulants from non-ritually slaughtered ruminants poses significant legislative and commercial challenges in Muslim-majority countries **(Nurilmala *et al.*, 2022)**. Alternative microbial coagulants often present limitations, including lower substrate specificity, higher thermal stability coefficients, and suboptimal milk-clotting activity (MCA) to proteolytic activity (PA) ratios compared to bovine rennet. Moreover, both microbial and plant-derived coagulants tend to produce more bitter peptides **(Jioe *et al.*, 2023; López Ruiz *et al.*, 2023)**.

While genetic engineering offers potential improvements, recombinant porcine and human genes introduce additional challenges **(Ju *et al.*, 2025)**. Furthermore, regulatory restrictions on genetically modified organisms hinder broader commercial implementation **(Catherine *et al.*, 2024)**.

The formation of milk clots, essential for developing unique dairy flavors, is influenced by MCA and PA **(Nitu *et al.*, 2021)**. The enzyme extract remains stable up to 70°C, with MCA decreasing as pH declines but increasing with higher enzyme concentrations and $CaCl_2$ supplementation. Proteolytic activity improves with increased enzyme concentrations and longer hydrolysis durations **(Bande-De Leon *et al.*, 2023)**.

Previous studies have identified several fish species as enzyme sources, including the yellowfin tuna (*Thunnus albacares*) **(Pasaribu *et al.*, 2018; Nurjanah *et al.*, 2021)**, various tuna species (*Euthynnus affinis*) **(Nurhayati *et al.*, 2020a)**, and the yellow pike

conger (*Congresox talabon*) **(Aboudamia *et al.*, 2024)**. However, these studies were limited to enzyme activity and characterization. In contrast, the present study extended this work by isolating, partially purifying, and characterizing proteases from striped marlin gastric tissue to assess their potential as milk-coagulating agents.

## MATERIALS AND METHODS

### Materials

The striped marlin (*Kajikia audax*, formerly *Tetrapturus audax*) was collected from the Regional Technical Implementation Unit (UPTD) Cold Storage in Karangsong, Indramayu Regency, West Java, Indonesia (6°19'N, 108°22'E). The specimen measured 167cm in length and weighed 54kg in its frozen state at -40°C, with a storage period of less than 3 months.

Gastric tissue was surgically excised from the striped marlin specimen and subjected to sequential thermal processing. Initial cleaning was performed using deionized water, followed by primary cooling in polyethylene bags at 0°C with a sample-to-ice mass ratio of 1:2 (w/w). The samples were rapidly frozen at -20°C and subsequently transferred to insulated polystyrene containers with a gel ice:dry ice:sample ratio of 2:1:1 to maintain the frozen condition.

Specimens were transported under controlled temperature conditions to the Advanced Laboratory, Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang, East Java, Indonesia (7°57'S, 112°37'E). The transport duration was standardized to 6 hours. The storage period before extraction was limited to a maximum of 90 days at -20°C to preserve enzymatic integrity.

### Proximate analysis

The proximate composition (moisture, protein, fat, and ash) of the stomach was analyzed following AOAC official methods **(AOAC, 2005)**. Moisture content was determined gravimetrically by drying the samples at 105°C for 24 hours. Ash content was measured after incineration at 550°C overnight in a furnace. Total nitrogen content was determined using the macro-Kjeldahl method, and crude protein was calculated by multiplying total nitrogen by a factor of 6.25 **(Egerton *et al.*, 2020)**. Fat content was determined using the Soxhlet method **(Rahman *et al.*, 2023)**.

### Acidified gastric extract preparation

Gastric tissue stored at -20°C was thawed using running water until it reached 0°C. The tissue was cut into ~1 $cm^2$ pieces.

Defatting followed a modified protocol **(Kuepethkaew *et al.*, 2023)** using acetone (Sigma-Aldrich, Emplura 8.22251) at a 3:1 solvent-to-sample ratio (v/v). Homogenization was done at 4°C for 60 seconds, followed by filtration through Whatman No. 42 paper. The residue was re-homogenized in acetone at a 2:1 ratio, and

the final filtrate was lyophilized to obtain defatted stomach powder, which was stored at -20°C.

For extraction, the powder was homogenized in 0.05 M Tris-HCl buffer (pH 7.0), supplemented with 0.2% (v/v) Tween 20, at a 1:9 (w/v) biomass-to-buffer ratio. The homogenate was stirred at 4°C for 30 minutes and filtered through Whatman No. 42. The filtrate was referred to as gastric extract (GE). Protease activation was induced by adjusting the pH to 2.0 with 1 M HCl, followed by 30-minute incubation at 4°C. The mixture was centrifuged at 10,000 × g for 20 minutes at 4°C, and the supernatant was designated as acidified gastric extract (AGE).

**Partial purification**

**Ammonium sulfate precipitation**

Solid ammonium sulfate ($(NH_4)_2SO_4$, Sigma-Aldrich 7783-20-2) was incrementally added to AGE to achieve saturation levels of 20%–80% (w/v) **(Manni *et al.*, 2024)**. After each addition, the solution was vortexed for 3 minutes and equilibrated at 4°C for 60 minutes. Precipitated proteins were recovered by centrifugation at 7,500 × g for 20 minutes at 4°C. The resulting precipitate was resuspended in 0.05 M Tris-HCl buffer (pH 7.0) at a 1:1 (w/v) ratio. The fraction with maximal proteolytic activity was selected for further analysis.

**Dialysis**

The active ammonium sulfate fraction was dialyzed against 0.05 M Tris-HCl buffer (pH 7.0) using a 12 kDa MWCO dialysis membrane. Dialysis was performed at a 1:50 (v/v) ratio at 4°C for 24 hours, with buffer changes every 8 hours to maintain a concentration gradient **(Anati *et al.*, 2021)**.

**Protease activity assay**

Protease activity was determined using a modified Kunitz method **(Gupta *et al.*, 2024)**. The reaction mixture included 0.4 mL of AGE and 2 mL of casein substrate (5 mg/mL in 0.05 M Tris-HCl buffer, pH 8.0). Reactions were incubated at 37°C for 30 minutes and terminated by adding 2 mL of 5% TCA. The mixture was incubated at 4°C for 30 minutes, centrifuged at 5,000 × g for 10 minutes, and filtered. Absorbance was measured at 280 nm using a Genesys 50 spectrophotometer.

**Total protein**

Protein concentration was determined using the Bradford assay **(Reinmuth-Selzle *et al.*, 2022)** with bovine serum albumin (BSA) as the standard. Standard curves were established using a concentration range from X to Y mg/mL (note: specify X and Y).

## Characterization

### Effect of pH

pH optimum was determined by assaying activity across pH 2.0–7.0 using the following buffers: HCl-KCl (pH 2.0), citrate (pH 3.0–6.0), and Tris-HCl (pH 7.0). Stability was tested by pre-incubating the enzyme at each pH for 2 hours at 25°C with agitation at 150 rpm, followed by standard activity assay.

### Effect of temperature

Temperature effects were evaluated across 40 to 70°C (5°C intervals) using casein as the substrate. Activity was expressed as relative activity (%) compared to the maximum observed. Thermal stability was assessed by pre-incubating aliquots for 2 minutes at each temperature before performing the activity assay under optimal conditions.

### Effect of metal ions

The effect of various metal ions and chelators (50 mM) was tested by pre-incubating AGE with NaCl, $CaCl_2$, KCl, $MgCl_2$, $ZnCl_2$, $CuSO_4$, $FeCl_3$, EDTA, and sodium molybdate at 37°C for 60 minutes. Enzyme activity was determined via the standard casein assay. Results were expressed as a percentage relative to the control (no additive), set at 100%.

### Milk clotting ctivity assay

MCA was determined based on a modified method **(Gagaouna *et al.*, 2017)**. A 12% (w/v) skim milk solution (pH 6.4 ± 0.1) was prepared with 0.01 M $CaCl_2$. One mL of the substrate was pre-incubated at 37 ± 0.5°C for 10 minutes. The reaction was initiated with 0.1 mL of AGE, and clotting was monitored by rotating test tubes every 30 seconds until visible protein aggregation occurred.

One milk clotting unit (MCU) was defined as the amount of enzyme required to coagulate 10mL of substrate in 40 minutes (2400 seconds) at 37°C. MCA was calculated using the following equation:

$$MCA\ \left(\frac{U}{mL}\right) = \frac{2.400\ x\ V_{milk}}{t\ x\ v_{enzyme}}$$

Where:

- Vmilk is the substrate volume (mL)
- Venzyme is the enzyme volume (mL)
- t is the clotting time (seconds)

The effect of calcium ions on MCA was evaluated using $CaCl_2$ concentrations ranging from 0 to 0.06M. Temperature dependence was assessed between 30- 80°C at

5°C intervals. The pH profile was determined using appropriate buffers (specify buffer systems) 50mM citrate (pH 3.0-6.0), and 50mM Tris-HCl (pH 7) at 0.5 pH unit intervals.

***SDS page analysis***

Protein molecular mass was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli. The gel system consisted of a 10% (w/v) resolving gel (pH 8.8) and a 4% (w/v) stacking gel (pH 6.8) **(Koshkina *et al.*, 2023)**. Protein samples (5 µL per well) were denatured in 5µL of Laemmli sample buffer (0.5M Tris-HCl, pH 6.8 – 3.75mL; 50% glycerol – 15.0mL; 1.0% bromophenol blue – 0.3 mL; 10% SDS – 6.0 mL; deionized water to 30mL; and 50µL β-mercaptoethanol added to 950µL of the buffer) at 95 °C for 5 minutes prior to loading. Electrophoresis was conducted at 4°C using a constant voltage of 180V until the tracking dye reached the bottom of the gel.

Protein bands were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue (CBB) G-250 in a solution containing 50% (v/v) ethanol and 10% (v/v) acetic acid, mixed with deionized water to a final volume of 100mL. After SDS-PAGE, the gel was soaked in the staining solution for 30 minutes to 1 hour with gentle shaking. Ensure the gel remains fully covered by the staining solution. Destaining was performed using several changes of destaining solution (same as the staining solution without CBB G-250) until optimal band resolution was achieved.

Molecular mass standards ranging from 10 to 180 kDa (Jena Bioscience 81614-Germany) were run in parallel for calibration. Molecular mass estimation was performed using two methods:

1. Direct comparison with molecular mass standards
2. Relative mobility (Rf) analysis using a semi-logarithmic plot

For the second method, Rf values were calculated as:

**Rf = (distance of protein migration) / (distance of tracking dye migration)**

The molecular mass calibration curve was constructed by plotting $\log_{10}$(MW) versus Rf values, yielding a linear regression equation:

$$\log_{10}(MW) = mRf + b$$

Where:

- MW is the molecular mass in kDa
- m is the slope of the regression line
- Rf is the relative mobility
- b is the y-intercept

The correlation coefficient ($R^2$) was calculated to assess the linearity of the calibration curve **(Wiesner *et al.*, 2021)**.

### Statistical analysis

Enzyme activity data from multiple replicates were statistically analyzed by calculating the mean. Standard deviation (SD) was used to describe the variability of the measurements.

## RESULTS AND DISCUSSION

### Proximate composition analysis

The proximate composition analysis of the striped marlin stomach contents revealed the following constituents: crude protein (16.39%), crude lipid (4.89%), moisture content (73.96%), ash (1.05%), and total carbohydrates (3.71%).

### Crude enzyme extraction

The resulting supernatant, representing the crude enzyme extract, appeared clear and yielded an extraction efficiency of 33.33%. Protein quantification indicated a concentration of 280.01mg/ mL, with a specific enzymatic activity of 3.62U/ mg.

### Ammonium sulfate fractionation and specific activity analysis

Protein precipitation was carried out through the stepwise addition of ammonium sulfate ($(NH_4)_2SO_4$) at saturation levels ranging from 20 to 80% (w/v). The specific activity profile of the fractionated enzyme at each ammonium sulfate concentration is illustrated in Fig. (1). The highest specific activity (U/mg) was observed in the 30% saturation fraction.

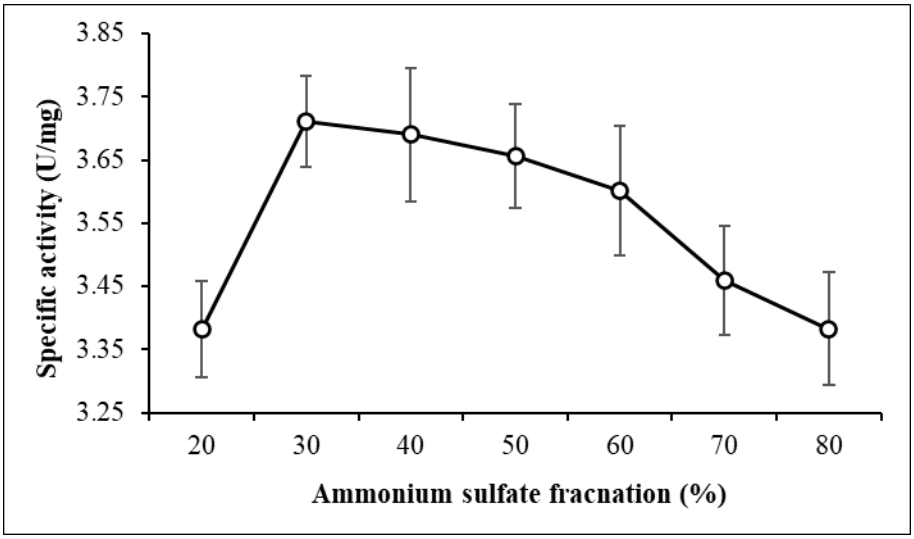


**Fig. 1.** Specific activity profile og AGE protease across ammonium sulfate saturation fractions

## Characterization of enzymatic properties

### *pH-dependent activity profile*

The pH-activity profile of the isolated protease showed maximum catalytic activity at pH 2.0 and maintained stability at pH 4.0. A progressive decline in enzymatic activity was observed from pH 5.0 to 7.0 (Fig. 2).

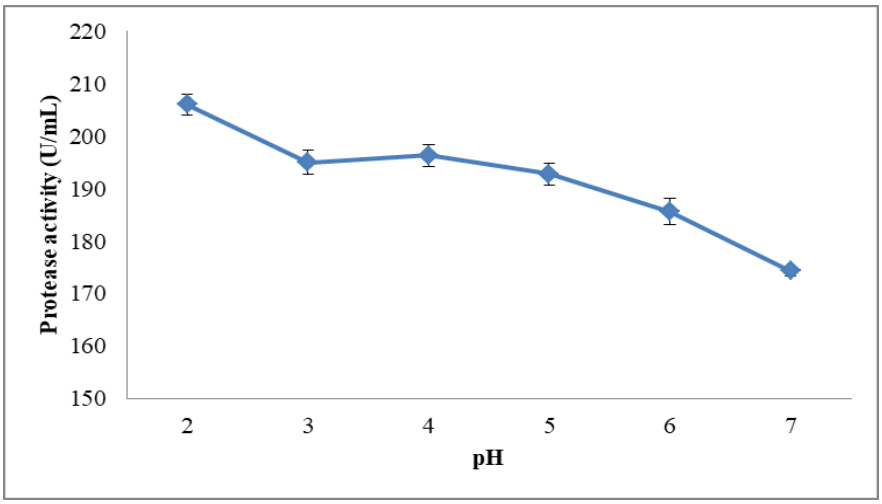


**Fig. 2.** pH-dependent activity profile of the purified protease

### *Temperature-dependent activity profile*

Thermal activity characterization revealed an optimal enzymatic activity at 50°C, with substantial activity retained between 30 and 70 °C (Fig. 3). However, exposure to temperatures above 60°C led to a significant decline in catalytic activity.

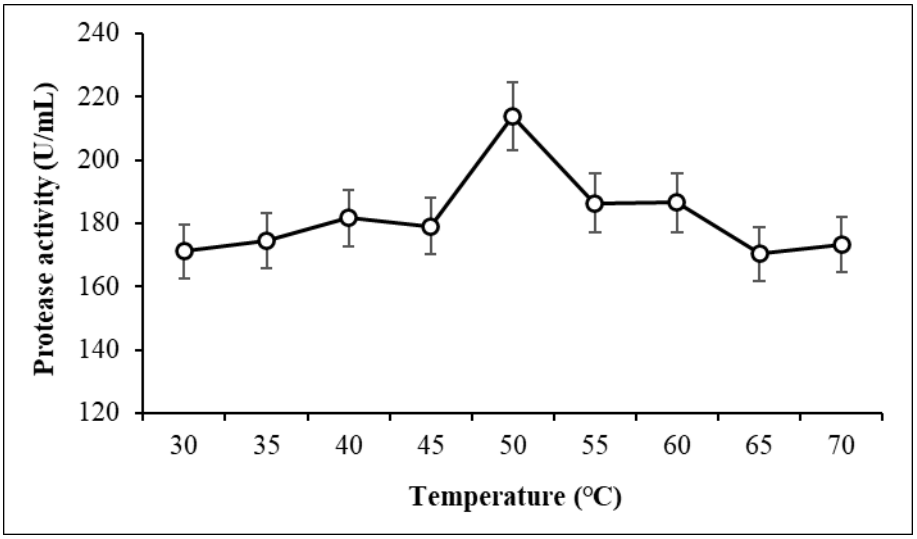


**Fig. 3.** Temperature-dependent activity profile of the purified protease

### *Effects of metal ions and inhibitors on enzymatic activity*

The influence of various metal ions on protease activity in the acidified gastric extract was investigated. At a concentration of 0.05M, both monovalent and divalent cations exhibited differential inhibitory effects. Specifically, $Na^{+}$, $Ca^{2+}$, and $K^{+}$ reduced enzymatic activity by 63, 52, and 1.4%, respectively.

The modulation of protease activity by metal ions demonstrated both concentration-dependent and ion-specific effects. Similar inhibitory patterns have been reported in previous studies, with the enzyme showing sensitivity to the metalloprotease inhibitor EDTA as well as to several metal ions, including $Ca^{2+}$, $Fe^{2+}/Fe^{3+}$, $Zn^{2+}$, $Cu^{2+}$, and $Mg^{2+}$ **(Amal *et al.*, 2024)**. Notably, NaCl and $CaCl_2$ at concentrations as low as 1 mM significantly suppressed protease activity.

### SDS-PAGE analysis

SDS-PAGE analysis revealed a distinct protein band at approximately ~17.32 kDa in both the crude AGE extract and the 30% ammonium sulfate fraction (Fig. 4).

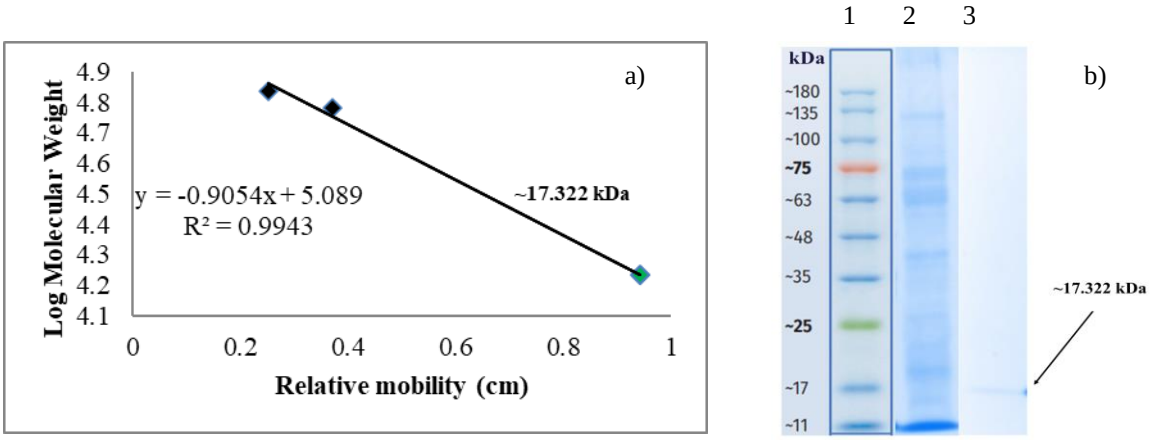


**Fig. 4.** Molecular weight profile of striped marlin gastric protease, a. Relative mobility value, b. line 1. marker, line 2. Crude AGE extract, and line 3. 30% $(NH_4)_2SO_4$ fraction

### Milk clotting mechanism and pH dependence

#### *pH-dependent milk clotting activity*

The milk-clotting activity of protease extracted from striped marlin exhibited maximum efficiency within the pH range of 5 (Fig. 5).

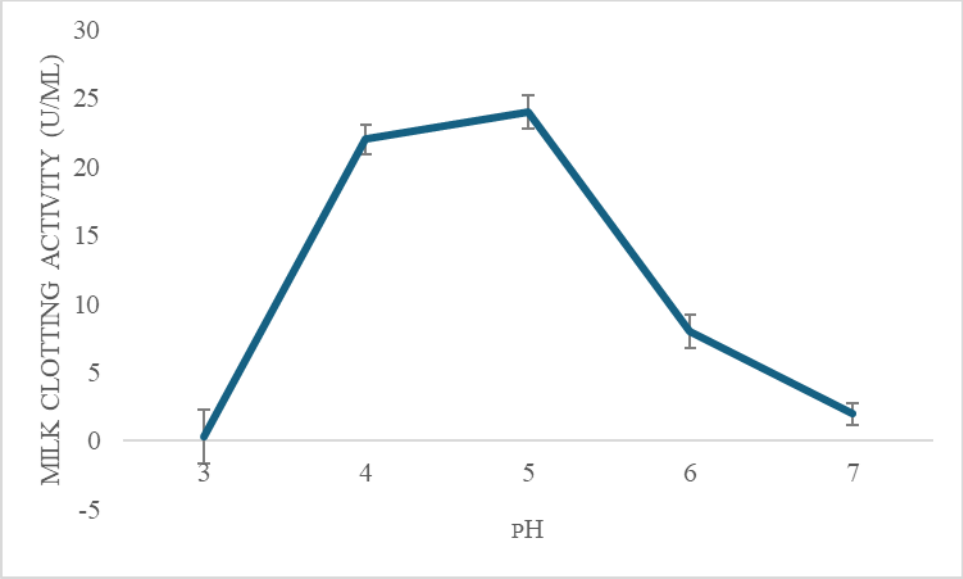


**Fig. 5.** pH-dependent milk clotting activity

***Temperature-dependent milk clotting activity***

Thermal optimization studies revealed maximum milk-clotting activity at 50°C (Fig. 6).

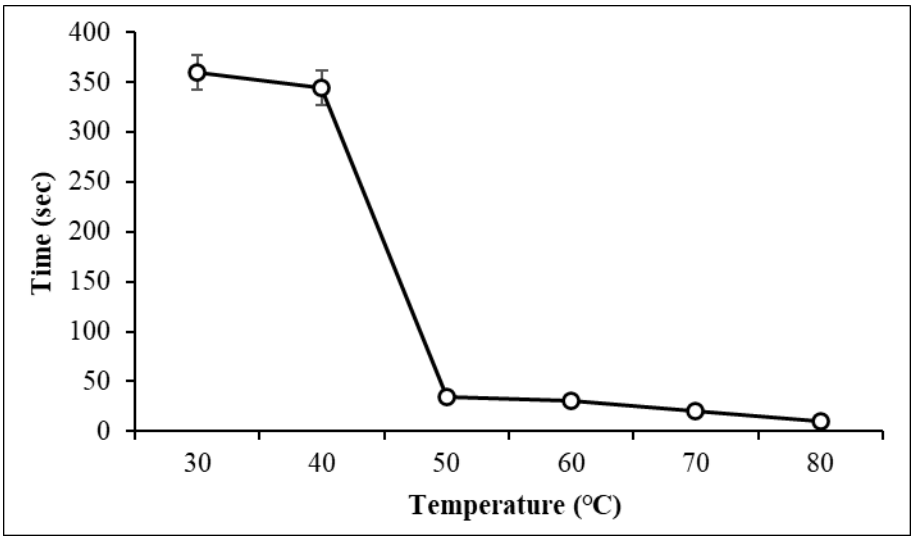


**Fig. 6.** Temperature-dependent milk clotting activity

***Effect of $CaCl_2$ concentration on milk clotting activity***

Optimal milk clotting activity of the striped marlin protease was observed at a $CaCl_2$ concentration of 0.3M (Fig. 7).

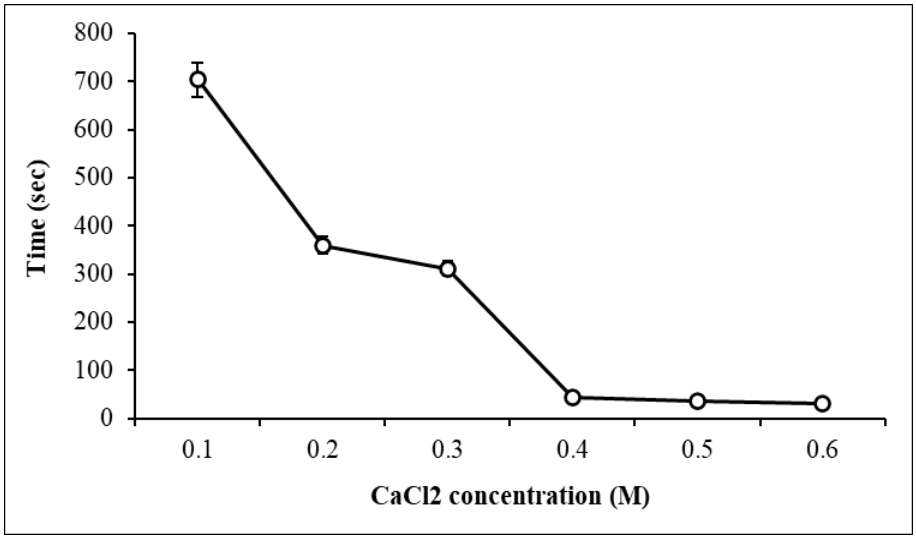


**Fig. 7.** Effect of $CaCl_2$ concentration on milk clotting activity

As a carnivorous species, the striped marlin employs a selective feeding strategy to optimize nutrient intake, which directly influences the composition of food in its digestive tract. This dietary adaptation is reflected in the anatomy of its digestive system, which features a relatively short transit time—necessitating a protein-rich diet to ensure efficient nutrient absorption **(Young *et al.*, 2018)**. The typical nutritional composition includes moderate fat content (4–8%), protein levels between 16–17%, and ash content ranging from 2–6%, aligning with values reported for other marine fish species **(Egerton *et al.*, 2020)**.

During the protease isolation process, lipid constituents—particularly triglycerides—can form barriers around enzyme-containing cells or particles. These lipid layers hinder the penetration of solvents and other reagents, thereby obstructing essential steps such as filtration or centrifugation during protease separation **(Kuepethkaew *et al.*, 2021; Jamalluddin *et al.*, 2022)**.

**Table 1**. Comparison of chemical composition of stomach parts of three carnivorous fish species

| Species | Moisture % (w/w) | Protein % (w/w) | Lipid % (w/w) | Ash (w/w) | References |
|---|---|---|---|---|---|
| Yellowfin Tuna | 76.02 | 18.96 | 0.54 | 0.59 | **Pasaribu *et al.* (2018)** |
| Obtuse Barracuda | 78,20 | 21.39 | 1.21 | 1.19 | **Meshram *et al.* (2022)** |
| Striped Marlin | 73.96 | 16.39 | 4.89 | 1.05 | **Ismail *et al.* (2025)** |

The proximate value of the striped marlin is not significantly different from that of the barracuda and yellowfin tuna, as shown in Table (1), where water content is the highest, followed by protein, fat, and ash content.

The selection of an appropriate buffer system is critical for optimal enzyme extraction and stability maintenance. Previous studies have demonstrated that buffer pH values between 7.0–7.5 are optimal for pepsin extraction and stability **(Zhu *et al.*, 2024)**. Comparative studies using various extraction media have shown that Tris-HCl buffer systems yield superior enzyme activity compared to distilled water and coagulating agents in gastric tissue extractions from diverse fish species, including the milkfish and catfish **(Nurhayati *et al.*, 2019)**.

This result is not much different from previous studies by **Pasaribu *et al.* (2018)**, who reported optimal specific activity (4.274 U/mg) in the 30–40% ammonium sulfate fraction of tuna stomach extract. Similarly, **Prihanto *et al.* (2019)** demonstrated peak specific activity (4.04 U/mg) at 40% saturation when isolating proteases from tilapia offal. These variations in optimal precipitation conditions can be attributed to differences in protein solubility profiles, as noted by **Burgess *et al.* (2009)**. Furthermore, **Silva *et al.* (2022)** established that protease precipitation typically occurs between 30–60% ammonium sulfate saturation, with peak activity frequently observed in the 30% fraction.

This pH optimum aligns with previously characterized aspartic proteases, such as those from *Rhizomucor miehei* and *Trichoderma asperellum* **(Wang *et al.*, 2021; Xue *et al.*, 2024)**, which demonstrate optimal activity at pH 3.0. **(Navarro-Guillen *et al.*, 2022)**. Comparative studies on fish gastric pepsins have revealed similar pH optima, with greater amberjack pepsin showing maximum activity at pH 2.5 and maintaining stability for 60 minutes across pH 3.0–8.0 **(Osuna-Ruiz *et al.*, 2019)**. Additionally, investigations of sea catfish (*Bagre panamensis*) proteases identified optimal activity within pH 2.0–3.0. These findings are consistent with the general characteristics of aspartic proteases, particularly pepsin, which predominantly functions in the acidic gastric environment of vertebrates, with peak activity observed between pH 3.0–4.0 **(Gurumallesh *et al.*, 2019)**.

These thermal properties parallel those reported for other piscine proteases, such as those isolated from rainbow trout (*Oncorhynchus mykiss*), which exhibit optimal activity and stability at 55°C **(Andevari *et al.*, 2019)**. Similar thermal characteristics were observed in proteases from peacock bass (*Cichla ocellaris*), with maximum activity at 55°C **(Oliveira *et al.*, 2020)**. The thermal stability profiles of these proteases appear to correlate with the ecological niches of their source organisms, particularly in carnivorous fish from tropical habitats, where efficient protein degradation at elevated temperatures is physiologically advantageous.

In contrast, $MgCl_2$ demonstrated a marked stimulatory effect, enhancing protease activity by 9.59 times compared to the control. This observation aligns with previous findings for aspartate protease from *Trichinella spiralis*, where $MgCl_2$ concentrations ranging from 1 to 100mM resulted in increased enzymatic activity **(Xu *et al.*, 2020)**. Conversely, the addition of 0.05 M $KCl_2$ to the enzyme-limited assay (ELA) resulted in a

1.42% decrease in activity. A comparable inhibitory effect was noted in a study by **Azadi *et al.* (2021)** involving aspartate protease extracted from amphipod (*Gammarus bakhteyaricus*), wherein 0.04 M $KCl_2$ resulted in a 40% decrease in enzyme activity.

The presence of $Na^+$, $Ca^{2+}$, and $K^+$ ions at a concentration of 0.05M reduced enzymatic activity by 63, 52, and 1.4%, respectively. In another study, no effect of $Ca^{2+}$ and $Mg^{2+}$ metallic ions on the acid proteolytic activity of the partially purified protease was observed **(Osuna-Ruiz *et al.*, 2019)**. As a general principle, Mg-binding proteins should have more polar and fewer charged groups (to reduce polarizability and electrostatic interaction) or suboptimal geometries that weaken electrostatic interaction. Ca-binding proteins should have multiple charged residues and rigid geometry; highly charged and flexible binding pockets favor large divalent ions **(Jing *et al.*, 2018)**.

The relatively low molecular mass detection may be attributed to the high dilution factor (1:9, crude extract:buffer ratio) employed during the extraction process. Consistent with previous studies, aspartic proteases typically exhibit optimal activity in the acidic pH range of 3.0–5.0 **(Anema, 2021)**. Various marine-derived aspartic proteases, including pepsin, chymosin, and gastricin, have been characterized with molecular masses ranging from 17.8 to 38.6 kDa **(Osuna-Ruiz *et al.*, 2019)**.

The observed molecular mass variation can be correlated with the enzyme's activation state, as demonstrated in *Mustelus mustelus* (smooth hound), where pepsinogen and its activated form pepsin exhibit molecular masses of 40 and 35kDa, respectively **(Bougatef *et al.*, 2008)**. Comparative analyses in other marine species, such as *Ommastrephes bartramii* (neon flying squid), identified a proteolytic band at 45kDa through casein zymography **(Zhang *et al.*, 2019)**. Furthermore, studies on *Sardinella aurita* (sardinelle) visceral proteases reported a purified enzyme with a molecular mass of 17 kDa, which aligns with our findings **(Khaled *et al.*, 2011)**. Nonetheless, the protease precipitated with ammonium sulfate exhibited several bands on the SDS-PAGE analysis **(Bhuimbar *et al.*, 2024)**. In this case, it is possible that the extract concentration in the sample was too low.

The peak milk clotting activity (MCA) was observed at pH 5, measuring 24 U/mL, in contrast to the maximal protease activity (PA) recorded at pH 2, totaling 206 U/mL. The disparity in optimal pH between PA and MCA arises from variations in their mechanisms of action. PA is assessed by the enzyme's capacity to cleave casein, while MCA pertains to the enzyme's ability to cleave $\kappa$-casein specifically, resulting in milk coagulation. MCA does not require the complete breakdown of casein, only selective cleavage. At pH 2.0, despite high proteolytic activity, the casein structure and milk ions may not promote efficient coagulation, as calcium and phosphoproteins behave differently at low pH. A pH of 5.0 is near the isoelectric point of casein (approximately

pH 4.6–5.2), where casein is least soluble and readily precipitates, thereby facilitating milk coagulation.

Milk protein coagulation occurs across distinct pH ranges through different mechanisms. At pH 2.0–3.0, insoluble casein salts precipitate. Isoelectric precipitation occurs at pH 4.7, and calcium caseinate complexes form around pH 6.5. Rennet-induced enzymatic coagulation reaches peak efficiency at pH 6.0, correlating with full conversion of casein to paracasein. Rennet proteolysis has a broad pH range with maximum activity at pH 6.2, whereas acid-induced coagulation shows a narrow range centered at pH 4.7. As the pH drops to 5.3, acid precipitation begins while rennet activity diminishes. Pepsin-mediated coagulation displays a distinct pH-dependent pattern, decreasing in efficiency until pH 2.7, then reactivating at lower pH values **(Anema *et al.*, 2021)**. Process optimization shows that pH reduction and increased temperature accelerate rennet coagulation while maintaining gel firmness **(Hovjecki *et al.*, 2022)**.

Comparative studies reveal varying pH optima among species and enzyme sources. For example, **Tavares *et al.* (1997)** found that the milk-clotting enzyme from Atlantic tuna (*Thunnus obesus*) showed peak activity at pH 6.4. Similarly, **Ghais *et al.*, (2019)** reported optimal activity at pH 4.0 in proteolytic enzymes from the longtail tuna (*Thunnus tonggol*). In microbial sources, **Qasim *et al.* (2022)** characterized *Mucor* spp. enzymes with maximum activity at pH 4.6. These differences reflect the structural and functional diversity of proteases across biological sources **(Arbita *et al.*, 2023)**.

Fig. (6) shows that the peak MCA at 50°C exceeds conventional cheese production temperatures (30–40°C), though it remains suitable for specific cheese types. Modern, fast-fermenting cheeses utilize thermotolerant enzymes at higher temperatures to expedite coagulation **(Lazárková *et al.*, 2025)**. The 50°C MCA peak can be adjusted by modifying time and temperature prior to reaching maximum activity to achieve desired cheese characteristics. This observation aligns with other findings. **Mozzon *et al.* (2020)** documented peak clotting at 55°C for *Onopordum tauricum* extract. While, **Ghais *et al.* (2019)** reported a 60°C optimum for *Streblus asper* extract. Additionally, **Nolli *et al.* (2022)** demonstrated optimal MCA at 50°C for proteases from *Aspergillus* sp. and *Pleurotus albidus*.

In AGE, the optimum calcium concentration for coagulation is 0.3M, consistent with **Vishwanatha *et al.* (2010)**, who found that $Ca^{2+}$ concentrations above 0.2% enhance milk clotting. The MCA of striped marlin AGE protease at elevated temperatures indicates potential to denature fats and proteins. However, regulating time and temperature before reaching the peak allows for controlled cheese production, suggesting that this protease could serve as a viable alternative in cheesemaking.

## CONCLUSION

This study explores the isolation, characterization, and optimization of an acid protease enzyme from the stomach of the striped marlin (*Kajikia audax*) for potential use in cheese production. Among various digestive organs tested, the stomach showed the highest protease activity and extraction yield. The enzyme was partially purified using ammonium sulfate fractionation and dialysis, achieving optimal specific activity at 30% saturation. Biochemical characterization revealed that the enzyme is most active at pH 2.0 and 50°C, maintaining over 80% activity between 45–55°C. $MgCl_2$ significantly enhanced enzymatic activity, while NaCl and $CaCl_2$ had inhibitory effects. SDS-PAGE analysis identified a molecular weight of ~17.3 kDa. The enzyme demonstrated effective milk-clotting properties, with optimal activity at pH 5.0, 50°C, and a $CaCl_2$ concentration of 0.3M, suggesting its potential as a rennet alternative in cheese production. Further studies are recommended to evaluate its stability, sensory impact, and industrial applicability.

**Acknowledgments**

The authors express their sincere appreciation to the Department of Fishery Product Technology, Faculty of Fisheries and Marine Sciences, Brawijaya University for providing essential research facilities and technical infrastructure. We would also like to acknowledge the valuable support of the laboratory staff during the experimental phase.

**Funding sources**

This work was supported by the Professor Grant Program (Grant No. 3708/UN10.F06/KS/2024) from the Faculty of Fisheries and Marine Sciences, Brawijaya University, Indonesia.

**Conflicts of interest**

We have no conflicts of interest to disclose.

## REFERENCES

**Aboudamia, F. Z.; Amerany, F. E. and Jaouad, A**. (2024). Strategies to Reduce/Manage Fish Waste. In Fish Waste to Valuable Products. Singapore: Springer Nature Singapore, 441-460.

**Amal, K.; Ourida, A. and Verkhoturov, V.V.** (2024). Sunflower cake protease as a promising biocatalyst for cheese fermentation processes: isolation, purification, properties. Azerbaijan Chemical Journal, 1: 29-43.

**Anati, N. J.; Siti Roha, A. M. and Normah, I.** (2021). African catfish (Clarias gariepinus) visceral protease: its specific activity and molecular weight at different purification stages. Food Research, 5(2):120-128.

**Andevari, G. T.; Rezaei, M.; Tabarsa, M. and Rustad, T.** (2019). Extraction, partial purification and characterization of alkaline protease from rainbow trout (Oncorhynchus Mykiss) viscera. Aquaculture, 153: 1-9.

**Anema, S. G.** (2021). Heat-induced changes in caseins and casein micelles, including interactions with denatured whey proteins. International Dairy Journal., 122: 1-27.

**Anema, S. G**. (2021). Heat-induced changes in caseins and casein micelles, including interactions with denatured whey proteins. International Dairy Journal, 122: 105136.

**Arbita, A. A. and Zhao, J.** (2024). Milk clotting enzymes from marine resources and their role in cheese-making: A mini review. Critical Reviews in Food Science and Nutrition, 64(27): 10036-10047.

**Azadi, M. A.; Hemmati, R.; Homaei, A. and Khalaji-Pirbalouty, V. (2022).** A psychrophilic caseinolytic aspartic protease from the freshwater amphipod Gammarus bakhteyaricus for application in milk coagulation. LWT., 153, 112536.

**Bande-De León, C.; Buendía-Moreno, L.; Abellán, A.; Manzi, P.; Al Mohandes Dridi, B.; Essaidi, I.; Aquilanti, L. and Tejada, L.** (2023). Clotting and Proteolytic Activity of Freeze-Dried Crude Extracts Obtained from Wild Thistles Cynara humilis L. and Onopordum platylepis Murb. Foods, 12(2325): 1-14.

**Bhuimbar, M. V.; Jalkute, C. B.; Bhagwat, P. K. and Dandge, P. B**. (2024). Purification, characterization and application of collagenolytic protease from Bacillus subtilis strain MPK. Journal of Bioscience and Bioengineering, 138(1): 21–28.

**Bougatef, A.; Balti, R.; Zaied, S. B.; Souissi, N. and Nasri, M.** (2008). Pepsinogen and pepsin from the stomach of smooth hound (Mustelus mustelus): Purification, characterization and amino acid terminal sequences. Food Chemistry, 107(2): 777-784.

**Burgess, R. R.** (2009). Protein precipitation techniques. Methods in enzymology, 463: 331-342.

**Catherine, K. N.; Mugiira, B. R. and Muchiri, N. J.** (2024). Public perception of genetically modified organisms and the implementation of biosafety measures in Kenya. Advances in Agriculture, 2024(1): 5544617.

**Collette, B.; Natale, W. Di; Fox, J.; Graves, J.; Jorda, M. J.; Schratwieser, J. and Pohlot, B**. (2022). Kajikia audax, striped Marlin. The IUCN Red List of Threatened Species, 8235: 1–17.

**de Oliveira, C. G.; López-Olmeda, J. F.; Costa, L. S.; do Espirito Santo, A. H.; dos Santos, F. A. C.; Luz, R. K. and Ribeiro, P. A. P**. (2022). Gastrointestinal emptying and daily patterns of activity of proteinolytic enzymes in Nile tilapia (Oreochromis niloticus). Aquaculture, 546(August 2021): 737338.

**Egerton, S.; Mannion, D.; Culloty, S.; Whooley, J.; Stanton, C. E. and Ross, R. P.** (2020). The proximate composition of three marine pelagic fish. Irish Journal of

Agricultural and Food Research, 59(1): 185-200.

**Gagaoua, M.; Ziane, F.; Rabah, S. N.; Boucherba, N.; El, A. A. K. E. H., Bouanane-Darenfed, A. and Hafid, K.** (2017). Three phase partitioning, a scalable method for the purification and recovery of cucumisin, a milk-clotting enzyme, from the juice of Cucumis melo var. reticulatus. International journal of biological macromolecules, 102: 515-525.

**Ghais, S.; Bhardwaj, V. and Kumbhar, P**. (2019). A new milk coagulating enzyme from Thunnus tonggol (Bleeker, 1851) tuna fish waste as rennet substitute. IJAR., 5(12): 36-40.

**Gupta, M.; Choudhury, B. and Navani, N. K.** (2024). Production and characterization of an organic solvent activated protease from haloalkaliphilic bacterium Halobiforma sp. strain BNMIITR. Heliyon, 10(3): 1-14.

**Gurumallesh, P.; Alagu, K.; Ramakrishnan, B. and Muthusamy, S.** (2019). A systematic reconsideration on proteases. International journal of biological macromolecules., 128: 254-267.

**Hovjecki, M.; Miloradovic, Z.; Barukčić, I.; Blažić, M. and Miocinovic, J. (2022).** Rheological properties of goat milk coagulation as affected by rennet concentration, pH and temperature. Fermentation, 8(6): 1-14.

**Ideia, P.; Pinto, J.; Ferreira, R.; Figueiredo, L.; Spínola, V. and Castilho, P. C.** (2020). Fish processing industry residues: A review of valuable products extraction and characterization methods. Waste and Biomass Valorization, 11: 3223-3246.

**Ismail, E.; Prihanto, A. A.; Sukoso, S. and Kartikaningsih, H.** (2025). Mapping and characterization of dominant hydrolase enzymes in the digestive tract of Striped marlin (Kajikia audax Philippi, 1887). Biodiversitas Journal of Biological Diversity, 26(3): 1156-1163.

**Jamalluddin, N. A.; Ismail, N.; Mutalib, S. R. A. and Sikin, A. M**. (2022). Sc-$CO_2$ extraction of fish and fish by-products in the production of fish oil and enzyme. Bioresources and Bioprocessing, 9(1).

**Jing, Z.; Liu, C.; Qi, R. and Ren, P.** (2018). Many-body effect determines the selectivity for $Ca^{2+}$ and $Mg^{2+}$ in proteins. Proceedings of the National Academy of Sciences, 115(32): E7495-E7501.

**Jioe, I. P. J.; Shiesh, C. C. and Lin, H. L.** (2023). Bitterness of papaya milk is related to protein and free amino acid contents, with phenylalanine and tyrosine/tryptophan levels being the most important. HortScience, 58(3): 261-267.

**Ju, W. S.; Kim, S.; Lee, J. Y.; Lee, H.; No, J.; Lee, S.; Oh, K**. (2025). Gene Editing for Enhanced Swine Production: Current Advances and Prospects. Animals, 15(3): 422.

**Khaled, H. B.; Ghorbel-Bellaaj, O.; Hmidet, N.; Jellouli, K.; Ali, N. E. H.; Ghorbel, S. and Nasri, M.** (2011). A novel aspartic protease from the viscera of Sardinelle

(Sardinella aurita): Purification and characterisation. Food Chemistry, 128(4): 147-853.

**Koshkina, M. K.; Shelomov, M. D.; Pometun, A. A.; Savin, S. S.; Tishkov, V. I. and Atroshenko, D. L.** (2023). Speeding up SDS–PAGE: Theory and experiment. Electrophoresis, 44: 1155-1164.

**Kuepethkaew, S.; Damodaran, S. and Klomklao, S**. (2021). A Novel Rapid Ultrasonic Treatment for Acid Protease Extraction from Threadfin Bream and Lizardfish Stomachs and Their Biochemical Characterization. Chiang Mai Journal Of Science, 48(5): 1322–1337.

**Kuepethkaew, S.; Klomklao, S.; Zhang, Y. and Simpson, B. K.** (2023). Partitioning and recovery of proteases from lizardfish (Saurida micropectoralis) stomach using combined phase partitioning systems and their storage stability. RSC advances, 13(22): 14931-14942.

**Lam, C. H.; Tam, C. and Lutcavage, M. E.** (2022). Striped marlin in their Pacific Ocean milieu: Vertical movements and habitats vary with time and place. Frontiers in Marine Science, 9: 879503.

**Lazárková, Z.; Lorencová, E.; Pětová, M.; Novotný, M. and Salek, R. N**. (2025). Sterilized Processed Cheese: Principles, Technological Aspects, and Properties: A Review. Foods, 14(6).

**López Ruiz, Á. L.; Ruiz Morales, F. D. A.; Ruiz Pérez-Cacho, P. and Galán-Soldevilla, H.** (2023). Effect of Lactose-Reduction in Murciano-Granadina Semi-Hard Goat Cheese on Physicochemical and Sensory Characteristics. Foods, 12(5): 996.

**Mamoozadeh, N. R.; Graves, J. E. and McDowell, J. R.** (2020). Genome-wide SNPs resolve spatiotemporal patterns of connectivity within striped marlin (Kajikia audax), a broadly distributed and highly migratory pelagic species. Evolutionary applications, 13(4): 677-698.

**Manni, L.; Zouine, N.; Ouahidi, I.; Bouraqqadi, M. and Ananou, S.** (2024). Purification of Trypsin from Sardine (Sardina pilchardus) Viscera and Its Application in Preparation of Antioxidative Fish Protein Hydrolysates. Tropical Journal of Natural Product Research., 8(1):5881-5888.

**Meshram, M. M.; Rajesh, M.; Suyani, N. K. and Rajesh, K. M.** (2022). Diet composition, feeding dynamics and proximate composition of obtuse barracuda *Sphyraena obtusata* (Cuvier, 1829) in the southeastern Arabian Sea. Egyptian Journal of Aquatic Research, 48(2): 163-168.

**Mozzon, M.; Foligni, R.; Mannozzi, C.; Zamporlini, F.; Raffaelli, N. and Aquilanti, L.** (2020). Clotting properties of Onopordum tauricum (Willd.) aqueous extract in milk of different species. Foods, 9(6): 692.

**Navarro-Guillén, C.; Yúfera, M. and Perera, E.** (2022). Biochemical features and modulation of digestive enzymes by environmental temperature in the greater amberjack, Seriola dumerili. Frontiers in Marine Science, 9: 1-17.

**Nitu, S.; Geicu-Cristea, M. and Matei, F**. (2021). Milk-clotting enzymes obtained from plants in cheesemaking-a review. Scientific Bulletin Series F. Biotechnologies, *25*(1): 66-75.

**Nolli, M. M.; Contato, A. G., Brugnari, T.; Buzzo, A. J. D. R.; Aranha, G. M.; Inácio, F. D. Peralta, R.M. and Souza, C. G. M. D**. (2022). Evaluation of the milk clotting potential and characterization of proteases from Aspergillus sp. and Pleurotus albidus. Acta Sci Technol., 44: 1-10.

**Nurhayati, T.; Ambarsari, L.; Nurilmala, M.; Abdullah, A. and Rakhmawati, I. A. I.** (2020). Pepsin activity from gastric of milkfish and catfish in Indonesian Waters. In IOP conference series: earth and environmental science., 1(404): 1-8.

**Nurhayati, T.; Nugraha, R. and Lihuana, D.** (2020). Characterization of ammonium sulphate fraction trypsin isolated from intestine of little tuna. Jphpi., 23: 372-82.

**Nurilmala, M.; Suryamarevita, H.; Husein Hizbullah, H.; Jacoeb, A. M. and Ochiai, Y**. (2022). Fish skin as a biomaterial for halal collagen and gelatin. Saudi Journal of Biological Sciences, 29(2): 1100–1110.

**Nurjanah, B, T. I. and Nurhayati, T.** (2021). Collagen extraction of yellowfin tuna (Thunnus albacares) skin using pepsin and papain. Jurnal Pengolahan Hasil Perikanan Indonesia, 24(2):174-187.

**Oliveira, V.M; Cunha, M. N. C.; Assis, C. R. D.; Batista, J. M. S.; Nascimento, T. P.; Santos, J. F.; Lima, C. A.; Marques, D. A. V., Bezerra, R. S. and Parto, L.F.** (2020). Separation and partial purification of collagenolytic protease from peacock bass (Cichla ocellaris) using different protocol: Precipitation and partitioning approaches. Biocatalysis and Agricultural Biotechnology, 24: 1-16.

**Osuna-Ruiz, I.; Espinoza-Marroquin, M. F.; Salazar-Leyva, J. A.; Pena, E.; Álvarez-González, C. A.; Banuelos-Vargas, I. and Martínez-Montaño, E.** (2019). Biochemical characterization of a semi-purified aspartic protease from sea catfish Bagre panamensis with milk-clotting activity. Food science and biotechnology, 28(6):1785-1793.

**Pasaribu, E.; Nurhayati, T. and Nurilmala, M.** (2018). Extraction and characterization of pepsin enzyme from tuna (*Thunnus albacares*) gastric. Jurnal Pengolahan Hasil Perikanan Indonesia, 21(3): 486-496.

**Peatman, T.; Allain, V.; Bell, L.; Muller, B.; Panizza, A.; Phillip, N. B.; Pilling, G. and Nicol, S**. (2023). Estimating trends and magnitudes of bycatch in the tuna fisheries of the Western and Central Pacific Ocean. Fish and Fisheries, 24(5): 812–828.

**Prihanto, A. A.; Nursyam, H.; D Jatmiko, Y. and L Hayati, R.** (2019). Isolation, partial purification and characterization of protease enzyme from the head of the

Nile tilapia fish (Oreochromis niloticus). Egyptian Journal of Aquatic Biology and Fisheries, 23(3): 257-262.

**Qasim, F.; Diercks-Horn, S.; Gerlach, D.; Schneider, A. and Fernandez-Lahore, H. M.** (2022). Production of a novel milk-clotting enzyme from solid-substrate Mucor spp. culture. Journal of Food Science, 87(10): 4348-4362.

**Rahman, N.; Hashem, S.; Akther, S. and Jothi, J. S.** (2023). Impact of various extraction methods on fatty acid profile, physicochemical properties, and nutritional quality index of Pangus fish oil. Food Science and Nutrition, 11(8): 4688-4699.

**Reinmuth-Selzle, K.; Tchipilov, T.; Backes, A. T.; Tscheuschner, G.; Tang, K.; Ziegler, K.; Lucas, K.; Poschl, U.; Frohlich-Nowoisky, J. and Weller, M. G.** (2022). Determination of the protein content of complex samples by aromatic amino acid analysis, liquid chromatography-UV absorbance, and colorimetry. Analytical and Bioanalytical Chemistry, 414(15): 4457-4470.

**Silva, M. K. S.; Silva, T. A.; Silva, J. A. F.; Costa, L. D. A.; Leal, M. L. E.; Bezerra, R. S. and Freitas-Júnior, A. C. V.** (2021). Carangoides bartholomaei (Cuvier, 1833) stomach: a source of aspartic proteases for industrial and biotechnological applications. Brazilian Journal of Biology, 82:1-8.

**Tavares, J. F. P.; Baptista, J. A. B. and Marcone, M. F.** (1997). Milk-coagulating enzymes of tuna fish waste as a rennet substitute. International journal of food sciences and nutrition, 48(3): 169-176.

**Vishwanatha, K. S.; Appu Rao, A. G. and Singh, S. A.** (2010). Production and characterization of a milk-clotting enzyme from Aspergillus oryzae MTCC 5341. Applied microbiology and biotechnology, 85: 1849-1859.

**Wang, S.; Zhang, P.; Xue, Y.; Yan, Q.; Li, X. and Jiang, Z.** (2021). Characterization of a novel aspartic protease from Rhizomucor miehei expressed in Aspergillus niger and its application in production of ace-inhibitory peptides. Foods, 10(12): 2949.

**Wiesner, R.; Scheller, C.; Krebs, F.; Wätzig, H. and Oltmann-Norden, I.** (2021). A comparative study of CE-SDS, SDS-PAGE, and Simple Western: Influences of sample preparation on molecular weight determination of proteins. Electrophoresis, 42(3): 206-218.

**Xu, J.; Liu, R. D.; Bai, S. J.; Hao, H. N.; Yue, W. W.; Xu, Y. X. Y.; Cui, R.L.J. and Wang, Z. Q.** (2020). Molecular characterization of a Trichinella spiralis aspartic protease and its facilitation role in larval invasion of host intestinal epithelial cells. PLoS Neglected Tropical Diseases, 14(4): 1-23.

**Xue, Y.; Yan, Q.; Li, X. and Jiang, Z**. (2024). Characterization of a novel aspartic protease from Trichoderma asperellum for the preparation of duck blood peptides. Applied Microbiology and Biotechnology, 113(1): 1-12.

**Young, T.; Pincin, J.; Neubauer, P.; Ortega-García, S. and Jensen, O. P**. (2018). Investigating diet patterns of highly mobile marine predators using stomach

contents, stable isotope, and fatty acid analyses. ICES Journal of Marine Science, 75(5): 1583-1590.

**Zhang, X.; Cao, D.; Sun, X.; Sun, S. and Xu, N.** (2019). Preparation and identification of antioxidant peptides from protein hydrolysate of marine alga Gracilariopsis lemaneiformis. Journal of Applied Phycology, 31: 2585-2596.

**Zhu, C.; Katrolia, P. and Kopparapu, N. K**. (2024). Purification, characterisation and immobilisation of a protease-resistant α-galactosidase from *Monascus purpureus* and its application in removal of raffinose family oligosaccharides. International Journal of Food Science and Technology, 59(6): 3895-3906.