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# Potential of flavor enhancer from crude hydrolysate derived from *Pomacea canaliculata* and *Filopaludina javanica* using papain

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## Abstract

Enzymatic hydrolysis is an effective technique for breaking down proteins into peptides and free amino acids. Among the amino acids released, glutamic acid (glutamate) plays a key role in generating the umami taste in snails. This study aimed to produce a natural flavor enhancer through the enzymatic hydrolysis of proteins derived from *Pomacea canaliculata* (PCP) and *Filopaludina javanica* (FJP) using papain enzyme. The hydrolysis process was conducted by adding papain to PCP and FJP slurries at different enzyme-to-substrate (E/S) ratios of 1:10, 1:20, and 1:100 (w/v). The reaction was carried out at 54 °C for 3, 6, 9, 12, 15, and 18 hours. After incubation, the supernatant was collected and analyzed for the degree of hydrolysis, total peptide content, total amino acids, sensory properties, and peptide sequence identification using LC-ESI-MS/MS. The highest degree of hydrolysis was obtained at an E/S ratio of 1:10 after 18 hours, yielding 89.28% for PCP and 76.27% for FJP. The highest peptide concentrations were 15.28 mg/mL and 8.60 mg/mL for PCP and FJP hydrolysates, respectively. Increasing enzyme concentration positively influenced panelists' preferences for taste, color, and aroma attributes. The identified umami peptides from PCP and FJP typically contained 8–39 amino acids. In the PCP hydrolysate, Ala and Gly residues were identified at the N-terminal region of several peptides, including AVGLSHSNNTKDVMEKSK, GFMCSVDDQHTSSVLLLSYNAITGLGFTTCVTMIA, and GEMAAHYGTMDGGPGM. In the FJP hydrolysate, Gly was predominantly present at the N-terminal of peptides such as GLPGLPGLPGPK, GPLGPLGPQGIP, and GMMPPGMMPPEGMPP.

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## 1. Introduction

Umami is commonly described as having a pleasant, savory taste that enhances the overall flavor profile of foods. It is elicited by certain l-amino acids and 5'-ribonucleotides naturally present in food (1). This taste is associated with the activation of specific receptors on the tongue, namely T1R1 and T1R3, which are part of the mammalian taste-sensing system (2). Umami is also recognized as the "fifth basic taste," alongside sweet, sour, salty, and bitter. It is characteristic of foods rich in monosodium glutamate, monoammonium glutamate, disodium guanylate, and disodium inosinate and can be found in seafood, meat, mushrooms, legumes, and fermented products (3).

Enzymatic hydrolysis is one of the most widely employed techniques for converting proteins into peptides and free amino acids. Proteolytic enzymes cleave peptide bonds in protein molecules. Although enzymatic hydrolysis generally requires longer processing times than chemical hydrolysis, it produces safer products with superior nutritional and functional properties (4). Among the amino acids released, glutamic acid (glutamate) is the key contributor to the umami taste (5,6). For instance, enzymatic hydrolysis of pea protein using alcalase and flavourzyme produces low-molecular-weight pea peptides that strongly enhance both saltiness and umami perception (7). Other amino acids such as aspartic acid and alanine also contribute to the overall umami sensation (6,8). Common proteases used to produce umami peptides include papain, bromelain, alcalase, and trypsin (9).

Papain, a proteolytic enzyme naturally derived from papaya (*Carica papaya* L.), belongs to the eukaryotic thiol protease group and is characterized by a cysteine active site (10–12). It catalyzes the cleavage of peptide bonds to generate polypeptides and peptides, exhibiting optimum activity at pH 5–7.5 and a temperature of approximately 50 °C (4,13). Papain is frequently used to produce protein hydrolysates with improved functionality and sensory properties. Dinakarkumar (14) reported that hydrolysis of *Secutor insidiator* fish protein using papain enhances the degree of hydrolysis, water-binding capacity, and foaming stability. Furthermore, papain-derived hydrolysates generally possess a high protein content and are rich in essential amino acids (15).

Indonesia, an agricultural country, relies heavily on crops, such as rice, corn, and cassava. However, agriculture faces major challenges from pests, such as insects, birds, rodents, and snails. Snails, in particular, are major pests of rice and other crops, causing damage by feeding on roots, leaves, and stems and by transmitting plant diseases. Although snails are nutritionally rich, they are mainly used as animal feed (16). For instance, *Pomacea canaliculata* contains approximately 48.5% protein (dry weight) and a balanced amino acid composition dominated by glutamic acid, arginine, aspartic acid, leucine, lysine, alanine, glycine, threonine, and proline (17).

*Pomacea canaliculata* (PCP) and *Filopaludina javanica* (FJP) are freshwater gastropods that are distributed widely across China, South America, and Southeast Asia. They inhabit ponds, swamps, and paddy fields during the rainy season (17). Despite their abundance, these snails have a low economic value and are underutilized as potential food resources. Therefore, the development of enzymatic hydrolysis as a processing technology could transform snail proteins into umami-producing peptides with potential applications as natural flavor enhancers.

Previous studies have shown that protein hydrolysates derived from aquatic organisms such as fish, bivalve mollusks, and crustaceans are rich sources of bioactive and taste-active peptides (18). For example, oyster hydrolysates generated using neutrase, flavourzyme, and

protamex contain short-chain peptides enriched in glutamic acid, aspartic acid, 5'-IMP, and aspartic acid, which correlate strongly with umami intensity (19). Similarly, the combined hydrolysis of *Eriocheir sinensis* proteins using papain and alkaline proteases resulted in the formation of three peptides (AADESERM, SDEERMDAL, and EERAESGES), which were identified as key contributors to umami taste. The presence of acidic amino acid residues, such as glutamic acid (Glu) and aspartic acid (Asp), in these peptide sequences enhances their ability to interact with umami taste receptors (T1R1/T1R3), thereby eliciting a strong savory perception (20,21) g strategy for developing natural flavor enhancers.

Our previous study demonstrated that enzymatic hydrolysis of snail proteins using proteases could produce hydrolysates with flavor-enhancing potential. Hydrolysis of *Pomacea canaliculata* with trypsin increased soluble protein content between 3 and 15 h of incubation, with the optimum conditions observed at an enzyme-to-substrate ratio of 0.1 and 12 h of hydrolysis, resulting in 4.52% protein solubility (22). Despite the growing interest in marine and freshwater protein hydrolysates, there is limited information regarding the application of papain in the hydrolysis of snail proteins, particularly *Pomacea canaliculata* and *Filopaludina javanica*. Both species are locally abundant, protein-rich, and underutilized as food resources. Hence, optimizing their enzymatic hydrolysis could provide an innovative approach to convert these gastropods into functional ingredients with sensory appeal. Therefore, this study aimed to investigate the effects of papain-mediated protein hydrolysis on *Pomacea canaliculata* (PCP) and *Filopaludina javanica* (FJP), focusing on variations in enzyme–substrate (E/S, w/w) ratio and hydrolysis time. The resulting hydrolysates were evaluated for their chemical characteristics, including degree of hydrolysis, total peptide and amino acid content, and sensory properties. In addition, peptides extracted from PCP and FJP were identified based on their amino acid sequences using liquid chromatography–tandem mass spectrometry (LC–MS/MS). This technique enables precise characterization of molecular structures, including peptide sequences and amino acid compositions, which are associated with umami sensory activity and indicate their potential as natural flavor enhancers.

## 2. Materials and Methods

### *2.1. Materials and Chemicals*

Fresh samples of *Pomacea canaliculata* (PCP) and *Filopaludina javanica* (FJP) were obtained from the Soponyono Traditional Market, Surabaya, East Java, Indonesia. Papain enzyme (EC 3.4.22.2) was purchased from Merck (Darmstadt, Germany). All analytical-grade chemicals, including potassium sodium tartrate ($KNaC_4H_4O_6 \bullet 4H_2O$), Folin–Ciocalteu reagent (FCR), natrium hydroxide (NaOH), natrium carbonate ($Na_2CO_3$), natrium sulfite ($Na_2SO_3$), trichloroacetic acid (TCA), formic acid (FA), formic acid (FA)and acetonitrile (ACN) were also obtained from Merck KGaA (Darmstadt, Germany).

### *2.2. Preparation of Protein Hydrolysates*

Enzymatic hydrolysis was performed using a commercial papain protease. The flesh of PCP and FJP was cut into 1 cm × 1 cm pieces and blanched at 93–94 °C for 3 min. The blanched flesh was homogenized with distilled water at a ratio of 1:2 (w/v) using a Philips HR2115 blender (Amsterdam, Netherlands) at 11,000 rpm for 1 min. Papain was added to the homogenized slurry at enzyme-to-substrate (E/S, w/v) ratios of 1:10, 1:20, and 1:100. The mixtures were incubated at 54 °C for varying durations (3, 6, 9, 12, 15, and 18 h) in a Memmert UF55 incubator (Memmert GmbH + Co. KG, Schwabach, Germany). After hydrolysis, the

reaction was terminated by heating the sample at 90 °C for 15 min to inactivate the enzyme. The hydrolysates were centrifuged at 3,000 rpm for 30 min at room temperature, and the resulting supernatant was collected for further analysis (23). A General schematic diagram of enzymatic hydrolysis of *Pomacea canaliculata* (PCP) and *Filopaludina javanica* (FJP) is shown in figure 1.

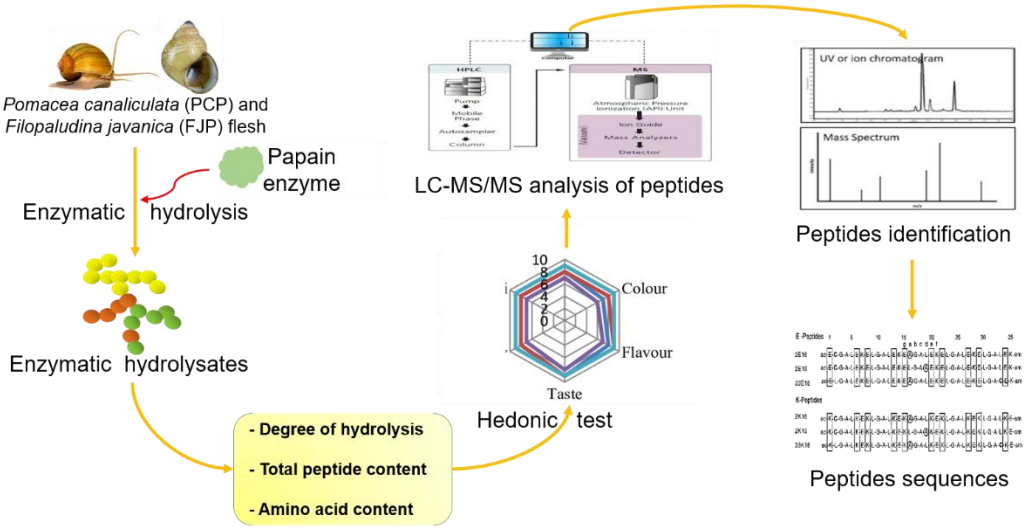



Figure 1. General schematic diagram of enzymatic hydrolysis from *Pomacea canaliculata* (PCP) and *Filopaludina javanica* (FJP)

### *2.3. Degree of Hydrolysis Determination*

The degree of hydrolysis (DH) was expressed as the percentage of peptide bonds or free amino groups cleaved from the protein relative to the total number of peptide bonds in the substrate. DH was determined based on the amount of protein soluble in 20% (w/v) TCA. A mixture of 10 mL 20% TCA and 10 mL sample (PCP or FJP hydrolysate) was centrifuged at 3,000 rpm for 15 min. The soluble protein in the supernatant was quantified using the Lowry method, while the total nitrogen was determined using the Kjeldahl method. In this assay, a series of diluted BSA solutions was prepared as protein concentration standards, and their absorbance values were plotted to generate a linear calibration curve. The DH value was calculated using the following equation.

DH (%) = [soluble protein content in 20% TCA (mg) / total protein content (mg)] × 100, (1)

Here, total nitrogen (N) represents the total protein content in the hydrolysate and 20% TCA-soluble nitrogen represents the fraction of hydrolyzed protein soluble in TCA (9).

### *2.4. Determination of Total Peptide Content*

The total peptide concentrations in PCP and FJP hydrolysates were determined using a modified Folin–phenol method. PCP and FJP hydrolysates were mixed with 15% TCA solution at a ratio of 2:1 and allowed to stand at 25 °C for 1 h. The mixture was then centrifuged at 5,000 rpm for 10 min. The resulting supernatant was analyzed using the Folin–phenol method, and the absorbance was measured at 680 nm with a UV–Vis spectrophotometer (24).

### 2.5. Determination Amino Acid Analysis

The amino acid content was determined according to the method described by Moore and Stein (25) with minor modifications. A mixture of 5 mL protein hydrolysate, 2.5 mL of 40% ethanol, and 0.5 mL of ninhydrin reagent was vortexed and heated in a boiling water bath for 20 minutes. The reaction between ninhydrin and amino acids produced a characteristic purple color, which was quantified by spectrophotometry at 570 nm. Amino acid concentrations were calculated using a linear regression equation obtained from the standard curve of l-glutamic acid dilutions.

### 2.6. Sensory Evaluation

Sensory evaluation was conducted by 25 semi-trained panelists to assess overall liking using a 5-point hedonic scale, ranging from 1 (dislike extremely) to 5 (like extremely). The panelists evaluated the following five taste attributes: sweet, bitter, sour, salty, and umami. To visualize the relationships among sensory attributes and identify preference patterns, data were analyzed using Principal Component Analysis (PCA) with Microsoft Excel version 17 (IBM, USA).

### 2.7. Identification of Peptides by LC-MS/MS and Database Searching

Peptide identification was performed using quadrupole time-of-flight (Q-ToF) mass spectrometry coupled with an ultrahigh-performance liquid chromatography system (Thermo Scientific Dionex Ultimate 3000 RSLCnano). A reversed-phase column (Acclaim PepMap RSLC C18 NanoViper, 75 µm × 150 mm, 2 µm particle size) equipped with a guard column (C18 PepMap100, 300 µm × 5 mm, 5 µm particle size) was used for peptide separation. The Diluted hydrolysate samples were injected with 0.1% FA. The mobile phase consisted of solutions A (0.1% FA in deionized water) and Solution B (80% ACN in deionized water). Peptide separation was achieved at a flow rate of 0.3 µL/min under a gradient of 2–85% B over 50 minutes. The MS scan range was set at 300–1500 m/z. Tandem MS spectra were generated using Bruker qTOF Control Software, and data were converted to MGF files using Compass Data Analysis version 4.1 (Bruker Daltonik, Bremen, Germany). Peptides were identified using Mascot Server (Matrix Science, https://www.matrixscience.com) under the following parameters: (1) Database: UniProt Architaenioglossa, (2) no specific enzyme selection, (3) fixed modification: carbamidomethylation at cysteine residues, (4) variable modification: oxidation at methionine residues, (5) peptide charge states: +1, +2, and +3; (6) peptide mass tolerance: 1.2 Da, (7) MS/MS fragment tolerance: 0.6 Da. Mascot scores > 30 ($p < 0.05$) were considered significant peptide identifications.

### 2.8. Statistical Analysis

All experiments were performed in triplicates. Results are presented as the mean ± standard deviation (SD). Statistical differences among the means were evaluated using Duncan's multiple range test (DMRT) at a significance level of $p < 0.05$, using SPSS version 18.0 (IBM, USA).

## 3. Results and Discussion

### *3.1. Degree of hydrolysis*

The degree of hydrolysis (DH) is a key parameter used to monitor protein hydrolysis, representing the percentage of peptide bonds cleaved during enzymatic reactions (26). The effects of enzyme-to-substrate (E/S) ratio and hydrolysis time on DH are shown in Figure 2. Three E/S ratios (1:100, 1:20, and 1:10 w/v) and six hydrolysis times (3, 6, 9, 12, 15, and 18 h) were tested. DH numbers from PCP hydrolysate with wide-ranging from 23.510 % to 89.275 %, whereas FJP hydrolysate varied from 27.640 % to 76.270 %.

The initial protein contents of PCP and FJP were not determined in this study owing to sample and analytical limitations. Therefore, the DH between PCP and FJP was compared based on relative hydrolysis patterns instead of direct protein content values. The effects of the E/S ratio and hydrolysis time on the DH of PCP hydrolysate are shown in Figure 2A. At the initial stage (3 h), DH ranged from 23.510 % to 24.650 %, showing minimal variation among treatments. As hydrolysis proceeded to 6 h, a sharp increase was observed, especially at E/S 1:10, which reached 52.655 %, significantly higher than that at E/S 1:20 (43.295 %) and E/S 1:100 (28.625 %) ($p < 0.05$). Between 9 and 12 h, the DH tended to plateau at lower enzyme concentrations, suggesting substrate saturation or product inhibition of the enzyme. However, at the highest enzyme loading (1:10), DH continued to increase, reaching 61.16% at 12 h.

All hydrolysis curves increased rapidly within the first 3 h, and all treatments exhibited a rapid increase in DH during the first 3 h, followed by a slower rate after 6 h, particularly at an E/S ratio of 1:100. The highest DH for PCP hydrolysate was obtained at an E/S ratio of 1:10 (89.28%) after 18 h, followed by 1:20 (68.61%) and 1:100 (52.71%). Similarly, FJP hydrolysate reached its maximum DH of 76.27% at 18 h with an E/S ratio 1:10, followed by 69.47% (1:20) and 50.54% (1:100). After 18 h of hydrolysis, the DH reached its maximum at 89.28% for the E/S ratio of 1:10, followed by 68.61% (1:20), and 52.71% (1:100). These results clearly demonstrate that papain activity is strongly dependent on enzyme concentration and contact time. The higher E/S ratio provides more active sites for proteolytic cleavage, presumably to accelerate peptide bond disruption and enhance the conversion of proteins into smaller peptides and free amino acids (27).

The effect of E/S ratio and hydrolysis time on the DH of Please ensure that each figure is mentioned in the text before it appears, to maintain a clear reading flow and follow proper scientific writing conventions." After 6 h of reaction, DH values increased sharply, particularly at an E/S ratio of 1:10 (51.18%), followed by 1:20 (43.07%), and 1:100 (30.39%). Between 9 and 12 h, the hydrolysis rate continued to increase moderately, reaching 58.43% (1:10), 53.77% (1:20), and 47.99% (1:100). The maximum DH was reached after 18 h of hydrolysis, with the highest value observed at an E/S ratio of 1:10 (76.27%), followed by 1:20 (69.47%), and 1:100 (50.54%). This trend demonstrates that higher enzyme concentrations significantly promote the hydrolysis process owing to increased catalytic sites and more efficient substrate-enzyme interactions (28).

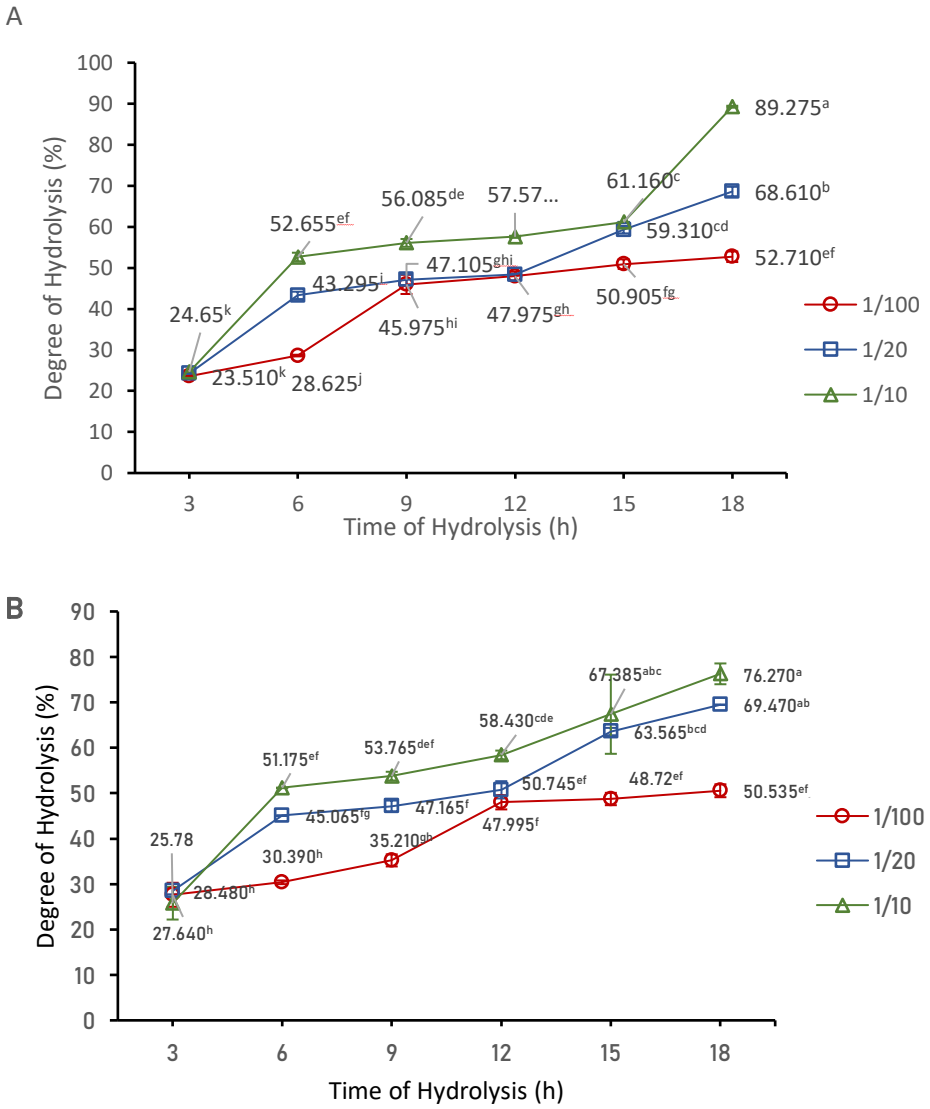



Figure 2. Effect of hydrolysis time and enzyme-to-substrate (E/S) ratio on the degree of hydrolysis (DH, %) of hydrolysates produced from papain proteolysis of PCP (A) and FJP (B). The E/S ratios tested were 1:100 (O), 1:20 (□), and 1:10 (Δ). Data are presented as mean of triplicate experiments.

An increase in the E/S ratio and hydrolysis time significantly enhanced the DH. Higher enzyme availability increases substrate-enzyme interactions, resulting in a greater number of peptide bonds being cleaved (29) (30). Similar findings were reported by Anwar (4) and Seniman (15), who observed that prolonged incubation allows enzymes to act more extensively, thereby increasing DH. This enhanced proteolysis is expected to improve the release of low-molecular-weight peptides, which contribute to umami flavour (21).

The observed increase in the degree of hydrolysis (DH) with longer hydrolysis time and higher enzyme-to-substrate (E/S) ratio can be attributed to the catalytic mechanism of papain (4). Papain is catalytically active over a wide pH range (5–8) and at moderate temperatures (40–60 °C). Papain is a cysteine protease belonging to the thiol protease family that contains an active-site catalytic dyad composed of cysteine (Cys25) and histidine (His159) residues (10). The thiol group of Cys25 acts as a nucleophile that attacks the carbonyl carbon of the

peptide bond, forming a thioacyl-enzyme intermediate that is subsequently hydrolyzed by water to release peptide fragments and regenerate the free enzyme. Peptide chains are cleaved into smaller fragments, exposing additional cleavage sites and allowing for further enzyme accessibility (31).

### *3.2. Total peptide content*

In this study, the total peptide content of the PA hydrolysate was measured using the Folin–phenol method. The effects of the (E/S) ratio and hydrolysis time on the total peptide content are illustrated in Figure 3. Three E/S ratios (1:100, 1:20, and 1:10 w/v) and six hydrolysis times (3, 6, 9, 12, 15, and 18 h) were tested. Figure 3A shows the total peptide content of PCP protein hydrolysates obtained at various hydrolysis times (3–18 h) and enzyme-to-substrate (E/S) ratios (1:100, 1:20, and 1:10, w/v) using papain. The results demonstrated a progressive increase in the total peptide concentration with prolonged hydrolysis time for all enzyme concentrations. At the initial stage (3 h), peptide yield ranged from approximately 4.32 to 6.24 mg/mL, while after 18 h of hydrolysis, the total peptide content reached values of 8.45, 12.91, and 15.275 mg/mL for E/S ratios of 1:100, 1:20, and 1:10, respectively. This trend indicated that both enzyme concentration and reaction time significantly influenced peptide release during enzymatic activity. The combination of prolonged hydrolysis and a higher enzyme concentration (E/S 1:10 for 18 h) appears to be the most effective condition for maximizing the total peptide yield.

Figure 3B illustrates the total peptide content of the FJP protein hydrolysate obtained through enzymatic hydrolysis using papain at various enzyme-to-substrate (E/S) ratios (1:100, 1:20, and 1:10, w/v) and reaction times ranging from 3 to 18 h. Overall, the total peptide content exhibited a gradual increase as hydrolysis time was extended to 15 h, followed by a plateau thereafter. At 3 h, the peptide yield ranged from approximately 3.0 to 4.5 mg/mL, depending on the enzyme concentration. After 15 hours, peptide levels reached 7.5–8.5 mg/mL and remained relatively stable until 18 hours, suggesting that the hydrolysis reaction approached equilibrium. The increase in total peptide content corresponds to a higher degree of hydrolysis (DH) under similar conditions, implying that peptide accumulation is directly related to protein cleavage intensity. During enzymatic hydrolysis, macromolecular proteins in PCP and FJP progressively degraded into smaller peptides with lower molecular weights. The resulting peptides are expected to contain low-molecular-weight fractions (<3 kDa) with a potential umami taste. As shown in Figures 3A and 3B, the increase in peptide yield with longer hydrolysis times indicates continuous papain activity. Seniman (15) and Wisuthiphaet (32) also reported that the total peptide content is closely related to hydrolysis duration, with longer reactions leading to greater peptide release due to enhanced proteolytic activity.

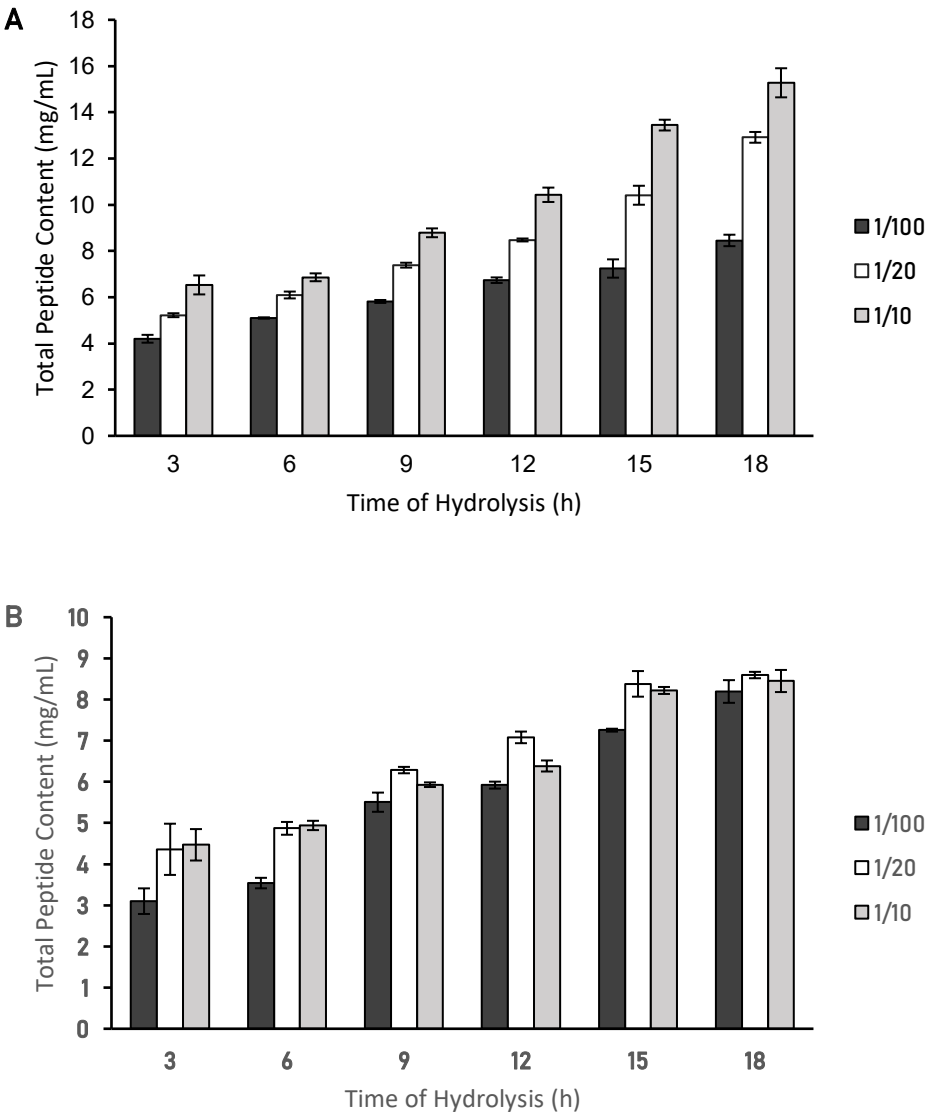



Figure 3. Effect of hydrolysis time and enzyme-to-substrate (E/S) ratio on peptide content (mg/mL) of hydrolysates produced from papain proteolysis of PCP (A) and FJP (B). The E/S ratios tested were 1:100 (■), 1:20 (□), and 1:10 (▒). Data are presented as mean ± standard deviation (SD) of triplicate experiments.

### *3.3. Amino Acid Content*

Protein hydrolysis produces a complex mixture of peptides with varying sizes and free amino acids. The total amino acid content of PCP and FJP hydrolysates is shown in Figure 4A and Figure 4B. Figure 4A shows the free amino acid content in PCP protein hydrolysates obtained at different hydrolysis times (3–18 h) and enzyme-to-substrate (E/S) ratios (1:100, 1:20, and 1:10, w/v). The concentration of free amino acids increased progressively with hydrolysis time, demonstrating continuous cleavage of peptide bonds throughout the enzymatic reaction. At 3 hours, the amino acid content ranged from approximately 40.46 to 60.41 ppm, while at 18 h, it reached approximately 79.725, 87.04, and 99.45 ppm for E/S ratios of 1:100, 1:20, and 1:10, respectively. These results indicated that both enzyme concentration and hydrolysis duration significantly affected amino acid liberation from the substrate protein.

Figure 4B presents the free amino acid content of FJP protein hydrolysates obtained with varying enzyme-to-substrate (E/S) ratios (1:100, 1:20, and 1:10, w/v) and hydrolysis times (3–18 h). The results indicated a gradual increase in the free amino acid concentration with prolonged hydrolysis, followed by a stabilization phase after 15 h. At the beginning of the reaction (3 h), the amino acid content ranged from 52.37 to 53.23 ppm, while at 18 h the levels reached approximately 70.59, to 78.66 ppm. The relatively moderate increase over time suggests that the hydrolysis process approached equilibrium after 15 h, when the rate of peptide bond cleavage and amino acid liberation became limited.

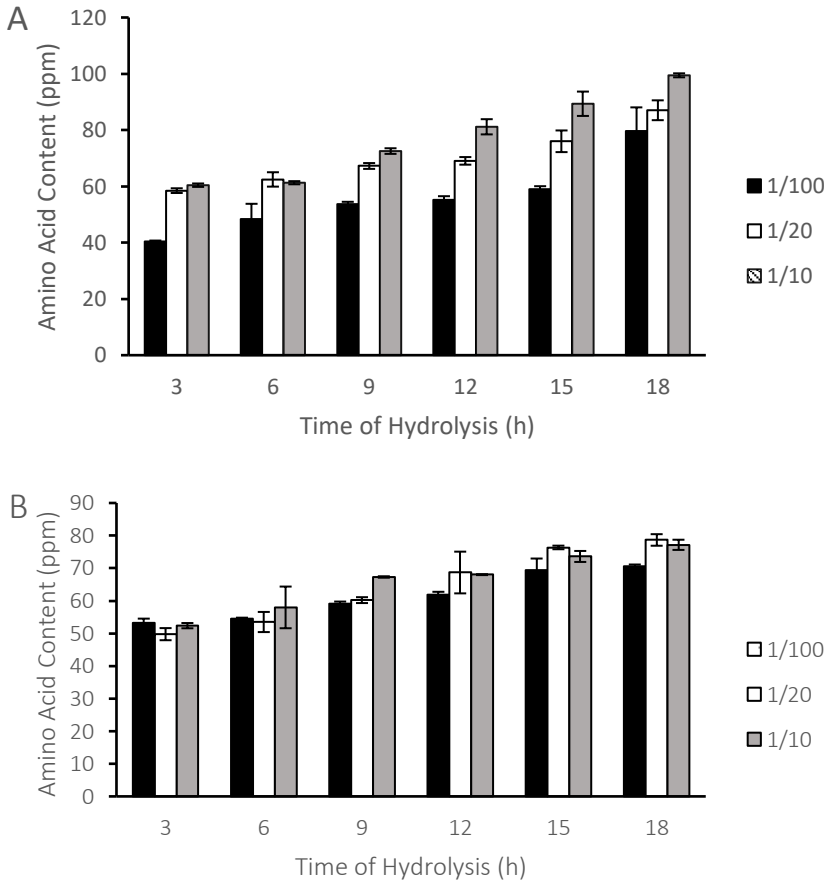



Figure 4. Effect of hydrolysis time and enzyme-to-substrate (E/S) ratio on amino acid content (ppm) of hydrolysates produced from papain proteolysis of PCP (A) and FJP (B). The E/S ratios tested were 1:100 (■), 1:20 (□), and 1:10 (▨). Data are presented as mean ± standard deviation (SD) of triplicate experiments.

Papain, a cysteine protease with a single polypeptide chain containing sulfhydryl and disulfide groups (13), effectively cleaves the bonds within protein molecules. During hydrolysis, high-molecular-weight peptides are progressively cleaved into smaller peptides and free amino acids. As shown in Figure 4A and Figure 4B, increasing the E/S ratio enhances the availability of active sites, promoting more extensive proteolytic cleavage and consequently releasing a higher quantity of free amino acids. The time-dependent increase in amino acid content suggests that papain maintains its activity over extended incubation periods, facilitating progressive peptide breakdown into smaller fragments and free amino acids. These results

are consistent with those of Fang Yan (7), who reported that the hydrolysis rate of pea protein increased with extended reaction time due to improved enzyme-substrate interactions and higher catalytic efficiency. These results are also consistent with those of Kongpichitchoke (28), who reported that the hydrolysis rate of *Filopaludina javanica* using bromelain and trypsin was consistent with an increase in enzyme-to-substrate concentration.

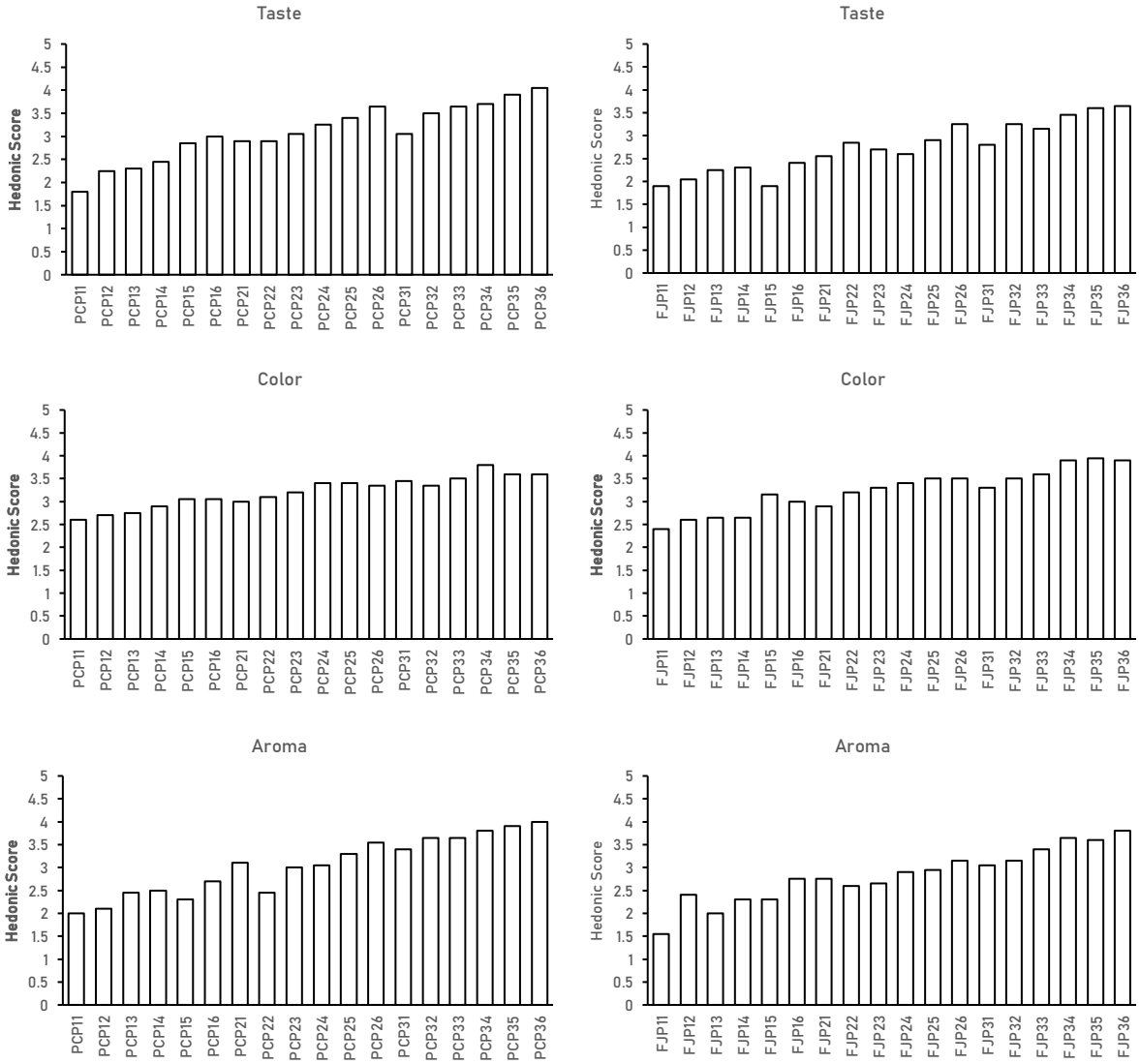
Total amino acid content serves as an important indicator of the extent of protein hydrolysis and generation of taste-active compounds (28). Elevated levels of free amino acids, particularly glutamic acid (Glu) and aspartic acid (Asp), are closely associated with umami perception and the development of desirable savory characteristics in hydrolyzed products (33). The increase in total amino acids suggests that papain effectively releases flavor-active amino acids from the protein matrix of PCP and FJP, thereby contributing to the enhancement of the umami intensity. Moreover, a higher amino acid content may also reflect improved digestibility and nutritional quality of the hydrolysates, highlighting the dual functional benefits of enzymatic treatment.

### *3.4. Sensory Evaluation*

Sensory evaluation plays a crucial role in assessing sensory quality and consumer acceptability of food products. In this study, sensory evaluation of PCP and FJP protein hydrolysates was conducted by 30 semi-trained panelists using a five-point hedonic scale to assess the degree of liking for each sensory attribute of the hydrolysates (5 = like extremely, 4 = like slightly, 3 = neither like nor dislike, 2 = dislike slightly, and 1 = dislike extremely). The sensory attributes evaluated included taste, aroma, and color.

As shown in Figure 5A, the highest sensory scores for the PCP hydrolysate were obtained for taste (4.05), aroma (4.00), and color (3.80). The different sample codes represent specific hydrolysis conditions as follows: PCP11/FJP11= E/S 1:100 (w/v) for 3h, PCP12/FJP12= E/S 1:100 (w/v) for 6 h, PCP13/FJP13= E/S 1:100 (w/v) for 9 h, PCP14/FJP14= E/S 1:100 (w/v) for 12 h, PCP15/FJP15= E/S 1:100 (w/v) for 15 h, PCP16/FJP16= E/S 1:100 (w/v) for 18 h, PCP21/FJP21= E/S 1:20 (w/v) for 3 h, PCP22/FJP22= E/S 1:20 (w/v) for 6 h, PCP23/FJP23= E/S 1:20 (w/v) for 9 h, PCP24/FJP24= E/S 1:20 (w/v) for 12 h, PCP25/FJP25= E/S 1:20 (w/v) for 15 h, PCP26/FJP26= E/S 1:20 (w/v) for 18h, PCP31/FJP31= E/S 1:10 (w/v) for 3 h, PCP32/FJP32= E/S 1:10 (w/v) for 6 h, PCP33/FJP33= E/S 1:10 (w/v) for 9 h, PCP34/FJP34= E/S 1:10 (w/v) for 12 h, PCP35/FJP35= E/S 1:10 (w/v) for 15 h, PCP36/FJP36=E/S 1:10 (w/v) for 18 h.

Taste and aroma were the most preferred in hydrolysates obtained with an E/S ratio of 1:10 at 18 h, whereas color preference peaked at 12 h. For FJP, the highest scores for taste, aroma, and color were 3.65, 3.80, and 3.95, respectively. Taste and aroma were most preferred at 18 h (E/S = 1:10) and color preference at 15 h. Both PCP and FJP protein hydrolysates exhibited distinctive sensory characteristics, particularly their umami taste, which combines mild salty and sweet sensations. The umami profile of these hydrolysates is likely attributable to the release of specific amino acids and small peptides during proteolysis, particularly glutamic acid and other active compounds.



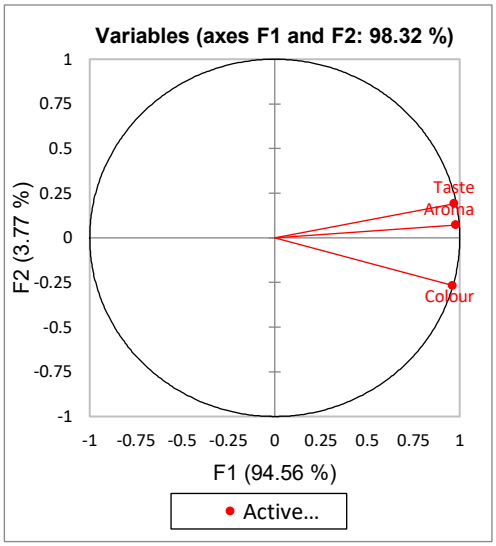

(A) (B)

Figure 5. Hedonic evaluation of hydrolysates derived from proteolytic digestion of *Pomacea canaliculata* protein (PCP) (A) and *Filopaludina javanica* protein (FJP) (B) under various hydrolysis times and enzyme-to-substrate (E/S) ratios for each sensory attribute.

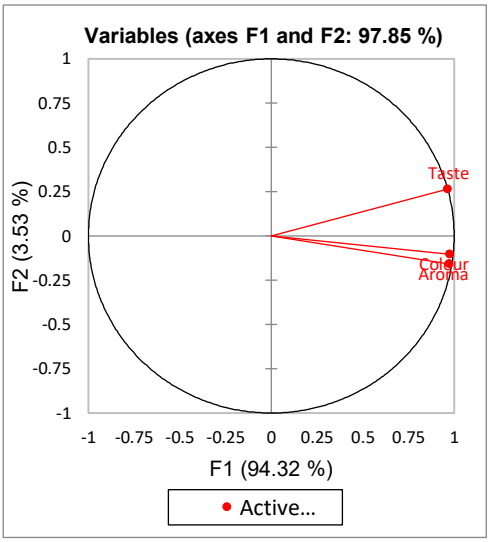
As enzymatic hydrolysis progressed, both DH and amino acid contents increased markedly in all samples. Correspondingly, the total free amino acids showed a clear upward trend with longer hydrolysis time and higher enzyme-to-substrate ratio. Although the sensory evaluation conducted in this study was hedonic, the increasing liking in taste attributes had positive correlations with chemical parameters, implying that the enhanced liking observed in the PCP36 and FJP36 samples is likely driven by an actual increase in umami intensity. Future studies should incorporate descriptive sensory methods, such as Quantitative Descriptive Analysis (QDA), to measure umami intensity directly and validate the link between DH/peptide composition and umami perception.

To further analyze the relationship between the sensory attributes and hydrolysis parameters, Principal Component Analysis (PCA) was performed. PCA was used to identify correlations between enzyme-to-substrate ratio, hydrolysis time, and sensory attributes (taste, color, and aroma). The loading plots (Figure 6A1 and Figure 6B1) illustrate the relationships between the sensory attributes. In PCP hydrolysates (Figure 6A1), taste and aroma attributes were closely associated and located in the same quadrant, suggesting that both contributed similarly to overall sensory perception. In contrast, the color attribute was positioned in a different quadrant, indicating a weaker correlation between the taste and aroma. For the FJP hydrolysates (Figure 6B1), the color and aroma attributes showed strong proximity, whereas taste was located in a distinct quadrant, implying a different sensory contribution.

The score plots (Figure 6A2 and Figure 6B2) describe the distribution of the hydrolysate samples based on the first two principal components (F1 and F2). Samples located near each other shared similar sensory characteristics, while those positioned farther apart differed significantly. In PCP hydrolysates, samples PCP26, PCP32, PCP33, PCP35, and PCP36 exhibited sensory characteristics opposite to those of samples PCB12, PCB13, PCB16, and PCB21. Similarly, in FJP hydrolysates, samples FJP22, FJP32, FJP35, and FJP36 received higher sensory scores for flavor, in contrast to FJP11, FJP13, FJP14, FJP16, and FJP23, which were rated lower by the panelists. Overall, the PCA results indicated that E/S and hydrolysis time significantly influenced sensory perception, particularly taste and aroma attributes. Samples hydrolyzed at higher enzyme concentrations (E/S 1:10) and longer incubation times (18 h) tended to produce more desirable sensory characteristics.




(A1)




(B1)

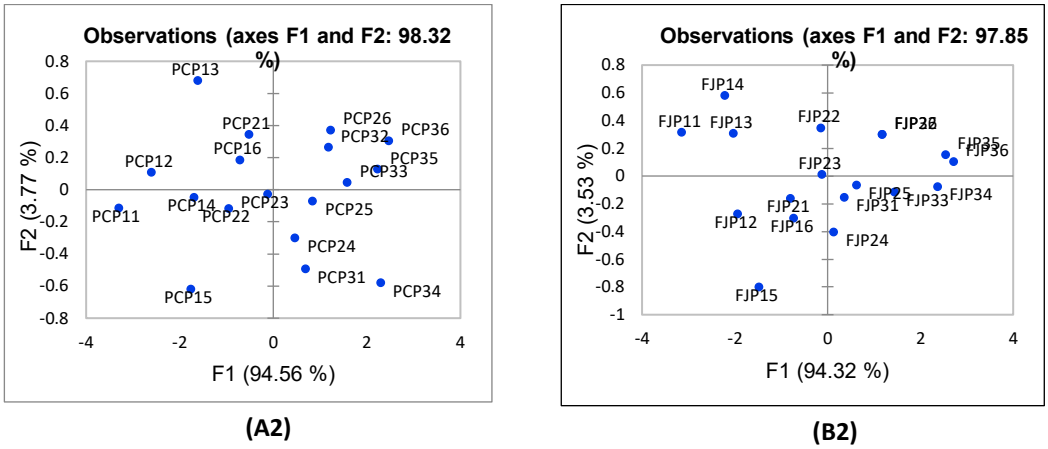



Figure 6. Principal component analysis (PCA) from protein hydrolysate by PCP (A) and FJP (B) with score plot graphs

### *3.5. Peptide identification*

The amino acid sequences of the umami peptides were determined using LC–ESI–MS/MS with de novo sequencing and database-assisted identification (34). The peptide sequences and molecular masses are listed in Tables 1 and 2, respectively. Ten peptides were identified from PCP and 12 from FJP hydrolysates, each corresponding to a distinct precursor protein. The amino acid composition and sequence motifs, particularly the residues located at the N- and C-termini of peptides, strongly influence flavor characteristics and intensity (5,34). The diversity of peptide sequences explains the rich umami perception observed in PCP and FJP hydrolysates. The peptide length, structure, and amino acid composition are known to influence umami intensity (35).

Previous studies have shown that Peptides containing Ala, Asp, Glu, or Gly residues at the C-terminus often exhibit distinct umami characteristics (20) (35)(34). In the present study, the umami peptides identified from PCP and FJP generally consisted of 8–39 amino acid residues. In PCP hydrolysates, Ala and Gly residues were detected at the N-terminus of several peptides, including AVGLSHSNNTKDVMEKSK, GFMCSVDDQHTSSVLLLSYNAITGLGFTTCVTMIA, and GEMAAHYGTMDGGPGM. In FJP hydrolysates, Gly residues, such as GLPGLPGLPGPK, GPLGPLGPQGIP, and GMMPPGMMPPEGMPP, were predominantly identified at the N-terminus of peptide sequences.

Umami peptides are short amino acid chains that impart a savory taste similar to that produced by free glutamate and nucleotides, signaling the presence of proteins in food. They are typically derived from high-protein sources, such as meat, soybeans, marine organisms, and mushrooms. To date, more than 50 peptides have been reported to exhibit umami characteristics, ranging from dipeptides to undecapeptides, with approximately 20 of them confirmed to elicit umami perception through receptor interactions (36). These peptides play a crucial role in food flavor enhancement by interacting with umami taste receptors T1R1 and T1R3 (5). Beyond their culinary significance, umami peptides have attracted increasing

interest owing to their potential health and nutritional benefits. The identification of umami-active peptides from PCP and FJP in this study further supports their potential use as natural flavor enhancers in food formulations.

Table 1. Identified peptides from hydrolysate of *Pomacea canaliculata* using papain E/S 1:10 (w/v) **for** 18 hours.

| Identified protein | Identified peptide | Observed m/z | Charge No. (z) | Peptide mass (Calc) | Mascot Score of peptide |
|---|---|---|---|---|---|
| ZnMc domain-containing protein OS | AVGLSHSNNTKDVMEKSK | 491.336 | 4 | 1959.9684 | 40 |
| Acid Beta-galactosidase OS | TEEMFVSTVQDALSTCEP GP | 583.3851 | 4 | 2328.0324 | 39 |
| BHLH domain-containing protein OS | ETQHVHFTRNSVMQRPIV PITYEAVD | 614.2628 | 5 | 3066.3048 | 36 |
| G_PROTEIN_RECEP_F1_2 domain-containing protein OS | GFMCSVDDQHTSSVLLLS YNAITGLGFTTCVTMIA | 477.2930 | 6 | 3808.7821 | 34 |
| Homeobox domain-containing protein OS | GEMAAHYGTMDGGPGM | 533.3311 | 3 | 1596.6007 | 34 |
| Cytochrome c oxidase subunit 1 (Fragment) OS | PLMLGAPDMAFPRQNN MSF | 547.3288 | 4 | 2183. 9802 | 33 |
| Bromo domain-containing protein OS | FDVVKIPMNLTEMENKLS SGRYSSKEQLGTDMDLLV ANCEAYNGVDSDFT | 567.3814 | 10 | 5662.6099 | 32 |
| Sulfate_transp domain-containing protein OS | PAQSLETPAPSTTSSSTAA ASPLWTMS | 447.2756 | 6 | 2676.2589 | 32 |
| ANK_REP_REGION domain-containing protein OS | FTGSLTLSVTERRDMSTTE TRDMPVTKTRVVPTTEMP V | 478.9633 | 10 | 4301.1342 | 31 |
| Sodium channel protein OS | FSAVPTRESMAVKSTTMQ R | 577.3557 | 4 | 2305.1195 | 31 |

Table 2. Identified peptides from hydrolysate of *Filopaludina javanica* using papain E/S 1:10 **(**w/v**) for** 18 hours .

| Identified protein | Identified peptide | Observed m/z | Charge No. (z) | Peptide mass (Calc) | Mascot Score of peptide |
|---|---|---|---|---|---|
| Collagen IV NC1 domain-containing protein OS | GLPGLPGLPGPK | 551.7635 | 2 | 1101.6546 | 65 |
| Fibrillar collagen NC1 domain-containing protein OS | GPLGPLGPQGIP | 551.7621 | 2 | 1101.6183 | 55 |
| YccV-like domain-containing protein OS | YMACNSYLDKVLE | 541.2840 | 3 | 1620.7164 | 45 |
| Fatty acid synthase OS | GMMPPGMMPPE GMPP | 530.2339 | 3 | 1587.6264 | 43 |

| Identified protein | Identified peptide | Observed m/z | Charge No. (z) | Peptide mass (Calc) | Mascot Score of peptide |
|---|---|---|---|---|---|
| Poly [ADP-ribose] polymerase OS | IPEHISNTSAPVRVIRQ | 480.3121 | 3 | 1916.0592 | 42 |
| WD_REPEATS_REGION domain-containing protein OS | PGLGLTVPV | 426.71242 | 2 | 851.5117 | 40 |
| NADH-ubiquinone oxidoreductase chain 4L OS | LLNNIALAGEGSFIFITLGACEASL | 649.2205 | 4 | 2593.3461 | 38 |
| ANK_REP_REGION domain-containing protein OS | PLPDTPSRIPYATPPIAEEEEE | 491.1989 | 4 | 2450.1853 | 35 |
| Heme O synthase OS | TSLVVITTMAGYAMAPAAFEP | 432.2280 | 5 | 2156.0534 | 34 |
| Telomerase catalytic subunit OS | PLLHSIVHSILSKAGSQLYFV | 578.2978 | 4 | 2308.2943 | 31 |
| SWIM-type domain-containing protein OS | DGQKCMPALNAAFELGLQVMRMTLMTLNWRRREMVRWLV | 595.2397 | 8 | 4752 | 30 |
| BAT2_N domain-containing protein OS | MPAVPSPP | 795.3880 | 1 | 794.3997 | 30 |

In the present study, the umami-active peptides identified from PCP and FJP hydrolysates contained between 8 and 39 amino acid residues. In PCP hydrolysates, Ala and Gly residues were found in the N-terminal regions of several peptide sequences, including AVGLSHSNNTKDVMEKSK, GFMCSVDDQHTSSVLLLSYNAITGLGFTTCVTMIA, and GEMAAHYGTMDGGPGM. In contrast, in FJP hydrolysates, Gly residues were predominantly identified at the N-terminus of the peptide chains, such as GLPGLPGLPGPK, GPLGPLGPQGIP, and GMMPPGMMPPEGMPP. The presence of Gly and Ala in the N-terminal region has been associated with enhanced umami and kokumi characteristics, which may contribute to the distinct savory taste of these hydrolysates.

## 4. Conclusions

*Pomacea canaliculata* (PCP) and *Filopaludina javanica* (FJP), both gastropod species, have demonstrated strong potential as new sources of umami peptides. Three key chemical parameters, degree of hydrolysis, total peptide content, and amino acid composition, were determined. The total peptide yield from PCP and FJP hydrolysates was significantly influenced by E/S ratio and hydrolysis time. Longer hydrolysis times and higher E/S ratios resulted in a greater release of peptides owing to enhanced proteolytic activity. Both PCP and FJP hydrolysates reached maximum peptide production at an E/S ratio of 1:10 and an incubation period of 18 h. Sensory evaluation results revealed that taste and aroma attributes were strongly correlated with DH, peptide content, and amino acid content. These findings suggest that optimizing enzymatic hydrolysis conditions can improve the flavor characteristics of gastropod-derived protein hydrolysates and highlight their potential as natural umami flavor enhancers in food applications.

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## Author Contributions

D.F.R conceived and designed the research and experiments; A.Y.T.P. and A.D.P. performed the experiments and analyzed the data; T.K interpreted the peptide sequences, and P.O.H analyzed with ESI LCMS/MS.

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## Institutional Review Board Statement

Not applicable.

## Data Availability Statement

Not applicable.

## Conflicts of Interest

There is no a conflict of interest.

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