https://doi.org/10.48048/tis.2024.6827

Chemical and Sensory Evaluation of Umami Taste Derived from Proteolytic Hydrolysate of *Pila ampullacea*

Dedin Finatsiyatull Rosida[1,4], Teeradate Kongpichitchoke[2], Phattara-Orn Havanapan[3], Andre Yusuf Trisna Putra[1] and Anugerah Dany Priyanto[1,4,*]

[1]Department of Food Technology, Faculty of Engineering, University of Pembangunan Nasional Veteran Jawa Timur, Surabaya 60294, Indonesia
[2]Department of Agro-Industry, Faculty of Biotechnology, Assumption University, Bangkok 10240, Thailand
[3]Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Nakhon Pathorn 73170, Thailand
[4]Innovation Center of Appropriate Food Technology for Lowland and Coastal Area, University of Pembangunan Nasional Veteran Jawa Timur, Surabaya 60294, Indonesia

([*]Corresponding author's e-mail: anugerahdany.tp@upnjatim.ac.id)

Received: 5 March 2023, Revised: 6 June 2023, Accepted: 1 August 2023, Published: 10 November 2023

Abstract

In this study, umami taste was produced and identified from bromelain and trypsin hydrolysate of *Pila ampullacea* (PA). The PA hydrolysates, obtained from individually bromelain and trypsin with various Enzyme-to-Substrate ratio (E/S) of 1/10, 1/20, and 1/100 (w/v) using divers proteolysis times (3, 6, 9, 12, 15 and 18 h), were evaluated their chemical properties by degree of hydrolysis (DH), total peptide content, and amino acid content. Two chosen proteolysis conditions of PA hydrolysates were concluded by sensory evaluation using hedonic test and principal component analysis (PCA). PA hydrolysate using bromelain with E/S of 1:10 (w/v) for 18 h was one of the 2 chosen proteolysis conditions, which had DH value of 56.56 ± 1.65 %, total peptide content of 10.89 ± 0.09 mg/mL, and amino acid content of 95.34 ± 0.12 ppm. On the other hand, the chosen from trypsin digestion used E/S of 1:10 (w/v) for 15 h, which had DH value of 49.71 ± 0.22 %, total peptide content of 6.44 ± 0.28 mg/mL, and amino acid content of 81.43 ± 1.29 ppm. PA hydrolysates were subjected to explain the released peptides using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and database-assisted identification. There were many identified peptides contained in PA hydrolysates that contributed to umami taste. Their sequences from bromelain digestion were GPEGPQGPPGPRG, GIMLGAA, GLPGLPGLKGDPGEPGLP, GKDGEAG, GLVMDSCAGH, and EEKITEDDDAVGDDAENR. Several peptide fragments from trypsin digestion were GQTVIGL, GLPGLPGLSGPKG, DTGPAGPAGPAGPQGPR and QTLEKALSHVIQEFETEKQLITVNAR.

Keywords: Umami peptides, Flavor enhancers, *Pila ampullaceal*, Hydrolysate, Proteases, LC-MS/MS, Sensory analysis

Introduction

Aside from sweet, bitter, sour, and salty as the 4 basic tastes, umami is classified by scientists as the fifth taste in the early twentieth century [1]. Umami has been represented as savory flavor and often used as flavor enhancer to increase satisfactory taste in food products. Moreover, umami compounds can increase the palatability of food and salivary flow reflex, which responds to masticatory oral receptors [2]. T1R receptor is main specific class C G protein-coupled receptors (GPCR), which is responsible for sweet and umami tastes [3]. This receptor consists of 2 subunit umami receptors, mainly T1R1 and T1R3 [4]. These 2 receptors are available in enteroendocrine cells (EECs) of specialized sensory cells dispersed in gastrointestinal tract of mouse, rat, guinea pig, and human [5]. Nowadays, umami substances are generally oblique portrayed as monosodium glutamate (MSG), monoammonium glutamate (MAG), Disodium Inosinate (IMP), and Disodium Guanylate (GMP) [6]. Hence, the commercial flavor enhancers are usually manufactured by fermentation and chemical process [7]. Several studies have proven that long term various side effects of MSG consumption, such as asthma, obesity, reproductive abnormalities, anxiety, Alzheimer's disease [8,9]. Moreover, most of commercial flavor enhancers are usually associated with

sodium or other salt ions which cause adverse health impact to consumer. Thus, it is required to discover the safer flavor enhancer in view of daily utilization of this substance.

Recently, many researches of food derived peptides as supernumerary flavor enhancers have been developed from rohu head [10], straw mushroom [11], morel mushroom [12], kokumi [13], puffer fish [14], *Takifuru rubripes* [15], Sanhuang chicken [16], sunflower seed [17] douchi [18], and tempeh [19]. Peptides are smaller fragments of protein containing 2 - 50 bonded amino acids and digested by proteolytic enzymes. These peptides were regularly released using proteolysis or throughout fermentation condition. Some researches were suspected that the food hydrolysates with containing low molecular weight peptides, free amino acids (especially glutamic acid and aspartic acid) and organic acids, which contributes to enhance the umami taste. Some peptides can espouse and predispose the development of the odor and taste characteristics. Those flavor formation in certain foods would bring to strength establishment of mouthfulness and after taste [20]. Based on previous research, umami-enhancement effects are very complex explanation, such as free amino acid composition, amino acid sequences of peptide, hydrophilic properties, and molecular weight. Therefore, exploratory study of novel umami peptides from hydrolyzed food protein sources as alternative flavor enhancer is enchanting great research interest due to their origins from natural resources.

The study reveals that prehistoric times of Palaeolithic humans have been eating snails in Spain and the Medditerranean region [21]. Snails are an alternative food with completely source of the valuable essential amino acids [22]. In Indonesia, snails are usually cooked into various cuisine. *Pila ampullacea* is one of snail variety in Indonesia with source of protein content. This species becomes one of pest during farming due to their habitats in wetland of paddy [23]. It has been studied that *P. ampullacea* utilized as ingredients in food products, such as crackers [24] and baby porridge [25] in order to harness nutrient contents and reduce the population of snail. Thus, they are potential commodity of edible protein source to exploit as flavor enhancer production. Early study of *P. ampullacea* using various papain concentration and different hydrolysis time has been demonstrated to be a good candidate of flavor enhancer properties based on degree of hydrolysis, amino acid content [26], total soluble protein, and total peptide content [27]. Even though there were reports on using bromelain [28] and trypsin [29] to produce flavor enhancer, as far as we know, flavor enhancer from *P. ampullacea* using both enzymes have not been researched previously. This species digested using diversified proteases to digest the long chain of polypeptides and heterogeneous peptide fragments were released due to specificities of proteolytics [30]. Therefore, the purposes of this research were to discover umami peptides derived from *P. ampullacea* hydrolysates and to assess their several hydrolysis conditions, which provide good profile as flavor enhancer.

In this paper, we explored the effectively based on diversity of proteases, enzyme-to-substrate ratio (E/S), and hydrolysis time towards chemical properties in each hydrolysate, notably degree of hydrolysis, amino acid content and total peptide content. The resulting hydrolysates were further evaluated their sensory characteristics by hedonic test and their data were computed by principal component analysis (PCA). The hydrolysates of *P. ampullacea* with the best hydrolysis condition from chemical and organoleptic properties were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and the fragments were calculated using database search engine. Profound identification about composition of umami peptides were interpreted to disclose the potential of peptide sequences. These results suggested that proteolytic hydrolysates of *P. ampullacea* were expected to be potential harmless alternative as food ingredient. This research provides scientific study for production of novel umami peptides as flavor enhancers and their theoretical proven from several test parameters.

Materials and methods

Materials and chemicals

P. ampullacea snails were purchased from Soponyono market, Surabaya city, East Java, Indonesia in August 2020. Bromelain (EC 3.4.22.33) was extracted from fresh pineapple fruit (enzyme activity 3.32 ± 0.06 U/mL) [31]. Trypsin (EC 3.4.21.4) (from bovine pancreas), Probumin® bovine serum albumin (BSA) life science grade powder, and L-glutamic acid were bought from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH), sodium carbonate (Na_2SO_3), potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$), folin-ciocalteu reagent (FCR), formic acid (FA), trichloroacetic acid (TCA), and acetonitrile (ACN) were obtained from Merck KGaA (Darmstadt, Germany). A Pierce BCA™ Protein Assay kits and reagents was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The deionized water used in this research was filtered using a Mili-Q® (Milipore) water purification system (Billerica, MA, USA). Other chemicals used in this research were all of pro analysis grade.

Proteolytic hydrolysis of P. ampullace

P. ampullacea snails were separated from snail shells and the snail meat was cleansed using running water to remove from its mucilage. The meat of *P. ampullacea* snails were added with distilled water using a ratio of 1:2 (w/v). Grinding process of *P. ampullacea* meat was conducted using a blender (Philip HR2115, Amsterdam, Netherlands) for 5 min with high level rotational speed. The slurry of *P. ampullacea* meat was treated by single protease with various E/S of 1/10, 1/20, and 1/100 (w/v) using different proteolysis times (3, 6, 9, 12, 15, and 18 h) which were based on protease' actions of bromelain and trypsin. Those independent research variables were examined to choose the selected condition. The proteolysis reactions of chopped meat were incubated at 54 ºC in Memmert UF55 (Memmert GmbH + Co. KG, Schwabach, Germany). After incubation, the hydrolysis process was stopped using a high temperature at 90 °C for 10 min. All samples were centrifuged using 3,000 rpm at room temperature for 30 min. The supernatant was transferred into cleansed test tube. The chosen condition of *P. ampullacea* (PA) hydrolysates were defined from the independent research variable which could produce highest score in hedonic scale and PCA of sensory evaluation from each protease, followed by their assessments in molecular docking study.

Degree of hydrolysis assessment

The degree of hydrolysis (DH) was assessed according to the method notified by Hoyle and Merrit with partial modification [32]. This method was assessed by dividing the number of soluble proteins in 10 % TCA to be expressed as a percent by the total protein content. The PA hydrolysate aliquots of 1 mL were mixed with 1 mL of 10 % TCA solution, followed by 30 min of rest. After their incubation, the mixtures were centrifuged at 3,000 rpm for 15 min. The supernatant was transferred into fresh tube and the soluble protein content in 10 % TCA solution was analyzed by the method of Lowry protein assay [33] with slightly modification by Hartree method [34] which expresses as mg of protein. In this assay, a dilution series of BSA solutions were used as protein concentration standard by plotting absorbance values to obtain linear model. The equation of standard curve was used to calculate the protein content in sample. The degree of hydrolysis was calculated based on the following equation:

DH (%) = [soluble protein content in 10 % TCA (mg) / total protein content (mg)]×100

Total peptide content quantification

The total peptide concentration in PA hydrolysate was quantified by the Folin-phenol method with partial modification [35]. The PA hydrolysate was added with 15 % TCA using a volume ratio of 2:1 (w/v), followed by chemical reaction at 25 °C for 1 h. The continuation treatment was centrifuged at 5,000 rpm for 10 min. The containing of phosphomolybdic and phosphotungstic acid in folin reagent was reduced to be a blue molybdenum complex. Production of dark blue color was indicated that the presence of peptide, mainly by tyrosine, tryptophan, cysteine, and histidine, which led to reduction of folin reagent. The color formation in sample solutions were measured at 680 nm using spectrophotometer.

Determination of amino acid content

The amino acid content was analyzed using Moore and Stein method with slightly modification [36]. The sample solution containing 5 mL of PA hydrolysate was added with 2.5 mL of 40 % ethanol and 0.5 mL of ninhydrin reagent, followed by homogenization using a vortex mixer. After being homogenized, the sample solution was heated in a boiling-water bath for 20 min. This ninhydrin reaction was detected by spectrophotometer with fingerprint of the dark purple color formation. This color was produced by the reaction between ninhydrin and amino acid. Ruhemann's purple was produced by the chemical reaction and the corresponding color formation was determined by a visible absorption spectroscopy with a wavelength of maximum absorbance at 570 nm. L-glutamic acid with various dilution series were performed to obtain a linear regression equation as standard curve. The equation was used to calculate the amount of amino acid.

Statistical analysis

Data were exhibited as the mean value ± standard deviation (SD). Data of DH, peptide content, and amino acid content were statistically analyzed by 2-way analysis of variance (ANOVA) followed by post hoc test with positive false discovery rate of 5 %. Dunnet's test for multiple comparison (DMRT) was used to analyze interaction between E/S and hydrolysis time. Fisher's least significant difference (LSD) test was utilized to calculate only from single variable when the statistical calculations from both variables were no interaction. The data was expressed as statistically different when the *p*-value of analyzed data was smaller

than 0.05. Statistical calculations were performed using Minitab software (version 19.1 2019).

Sensory evaluation

The sensory evaluation was conducted using the study reported by Yamaguchi and Takahashi [37] with partial modification. Well-trained panelists, who are able to identify 5 basic tastes (sweet, bitter, sour, salty, and umami), were summoned for sensory evaluation in this study. They were 20 students from Department of Food Technology, University of Pembangunan Nasional "Veteran" Jawa Timur. The panelists ages were from 20 to 22 years old. Hedonic scale for umami flavor of PA hydrolysate was assessed using a 5-point scale (1: dislike extremely; 5: like extremely). The hedonic's parameters were performed based on taste, aroma, color and overall acceptability. PCA was applied to accentuate various data in order to colligate strong patterns from hedonic's parameters. PCA data was expressed as a selected specific condition from 2 proteases. Then it was identified the peptide sequences using LC-MS/MS followed by database search engine in PA hydrolysate to study and summarize the strong potent based on its fragments.

Peptide sequencing by LC-MS/MS and database searching

The 5 µL liquid of PA hydrolysate containing 0.1 % FA was injected into LC-MS/MS instruments for analysis of peptide sequence. The LC-MS/MS systems were operated using Thermo Scientific Dionex Ultimate 3000 RSLCnano System with a captive spray ionization hybrid to Compact™ quadropole time-of-flight (Q-ToF) (Bruker Daltonik, Bremen, Germany). The LC separation was performed in a reversed-phase column of (Acclaim PepMap RSLC Column C18 NanoViper, 75 µm × 150 mm, particle size 2 µm) and protected by a guard column (C18 PepMap100, 300 µm × 5 mm, particle size 5 µm). The mobile phase composed of a Solution A (0.1 % FA in deionized water) and Solution B (80 % CAN in deionized water). Elution of the peptides was separated at a flow rate of 0.3 µL/min under gradient conditions of 2 to 85 % B for 50 min. The mass spectrometry scan was performed from m/z 300 to m/z 1,500. The tandem mass spectrometry spectra were generated by Bruker qTOF Control Software. The files were converted to MGF files using Compass Data Analysis version 4.1 (Bruker Daltonik, Bremen, Germany) and searched using Mascot Server (Matrix Science, https://www.matrixscience.com/search_form_select.html). The database search engine parameters were arranged as follows: 1) peptides and proteins were compatibled to UniProt database of uniprot_architaenioglossa; 2) without enzyme cleavage specificity; 3) the modification peptides were selected carbamidomethyl at cysteine residues as fixed modification and oxidation at methionine residues as variable modification; 4) the peptide charge was 1+, 2+, and 3+; 5) the peptide tolerance was ±1.2 Da; (6) the MS/MS fragment tolerance was ±0.6 Da. Proteins with a mascot scores were greater and then the score fixed with p-value < 0.05.

Results and discussion

Effect of various proteolytic enzymes on PA hydrolysates' degree of hydrolysis

In this study, PA hydrolysates were executed using 2 different proteases namely bromelain and trypsin, with various E/S of 1/10, 1/20, and 1/100 (w/v), and different hydrolysis times (3, 6, 9, 12, 15, and 18 h). These flavor enhancers were obtained from whole snail flesh (cleaned from its mucilage) without removing non-protein compounds, such as lipids and carbohydrates. This method was applied in some flavor enhancers efficiently due to simply process and their complexity with other compounds may contribute to enhance the taste [38,39]. The enzyme active site and substrate specificity is a prominent main factor on lest defines the properties of protein hydrolysates in consequence of proteolysis intensely impress its peptide size, peptide sequences, free amino acid composition, and polarity. The potential proteolytic enzyme activities were evaluated by the DH value because it is an equivalent percentage number of digested peptide bonds in protein hydrolysates. Crude hydrolysates of PA derived from bromelain and trypsin were evaluated in DH value and the results are shown in **Figure 1**. Using the same treatment, bromelytic (digested PA snail by bromelain) and tryptic (digested PA snail by trypsin) hydrolysate showed similar activity but slightly different line graph pattern. Their line graphs were exponential and linear for hydrolysates of bromelytic and tryptic, respectively. In general, the line graphs from both hydrolysates shows the increasing of DH%, which represent the positive correlation between time-series of proteolysis and E/S.

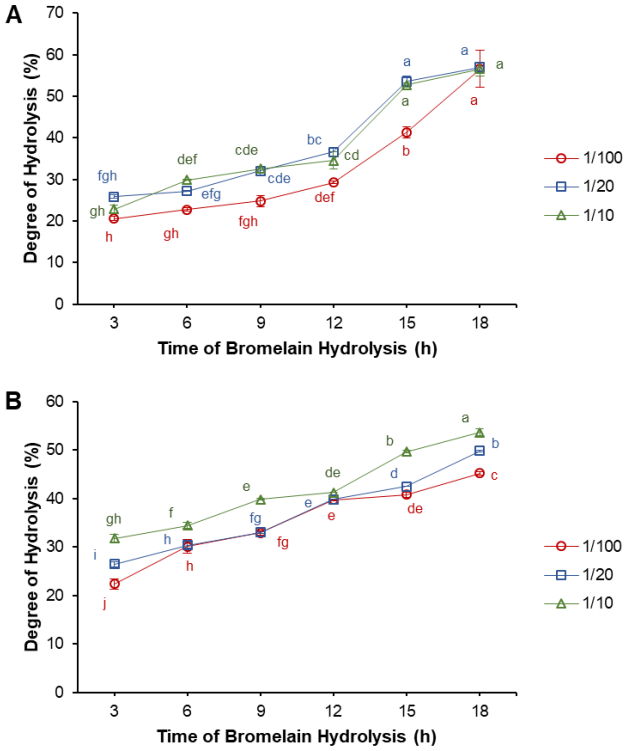

Figure 1 The effects of various hydrolysis time and E/S toward DH% of PA hydrolysates from different proteolytic enzyme of A) bromelain and B) trypsin. The different E/S are 1/100 (O), 1/20 (□), and 1/10 (Δ). Results are represented as the mean ± SD of triplication. The same marked alphabet in the same line are not significantly different at *p*-value < 0.05.

The results proved that proteolysis time and E/S gave significant effect on DH%, as seen in **Figure 1**. Typically, previous studies reveal several variables, such as temperature, pH, hydrolysis time, and E/S that are used to enhance the DH% of food commodities [40,41]. It has been published that enhancement of DH value can influence the umami taste amino acid amount [40]. Therefore, high DH value was expected in this study to select the best candidate as flavor enhancer. After 15 h, the DH% of bromelain digestion was dramatically increased and its value was higher than the tryptic. Bromelain has a broad specificity for protein digestion with cleavage sites including Arg, Lys, Tyr, Glu, Gly, Met, Ala, and Orn [43]. On the other hand, trypsin is responsible to digest peptides on the C-terminal side of Lys and Arg amino acid residues, except when either is followed by Pro [44]. Those both enzyme specificities may lead to the occasion of bromelain digestion has wider range of DH number (20.59 ± 0.46 to 56.96 ± 1.01 %) than trypsin digestion (22.36 ± 1.02 to 53.67 ± 0.76 %).

Effect of various proteolytic enzymes on PA hydrolysates' total peptide content

In this study, the total peptide concentration in PA hydrolysate was quantified by the Folin-phenol method. PA hydrolysates were performed using 2 separately proteases specifically bromelain and trypsin, with various E/S of 1/10, 1/20, and 1/100 (w/v), and diverse hydrolysis times (3, 6, 9, 12, 15, and 18 h). There were 18 numbers of total peptide contents in each proteolytic hydrolysate based on their multiplication factors as independent variables. These 18 numbers from bromelytic hydrolysate with wide-ranging from 2.26 ± 0.01 to 10.89 ± 0.09 mg/mL. The others total peptide content from tryptic hydrolysate varied from 3.06 ± 0.02 mg/mL to 6.96 ± 0.27 mg/mL. There was big difference escalation trend of total peptide contents between PA hydrolysates using bromelain and trypsin, as seen in **Figure 2**. PA hydrolysate of bromelain showed steep line pattern based on their increasing interval time-series of hydrolysis. However, sloping line pattern was obtained in tryptic hydrolysate along with extending proteolysis time. This may be because specificity of enzyme greatly determines the cleavage site in peptide bonds of protein as substrates. Thus, protease-substrate specificity and broadness of enzyme cleavage site may produce certainly total peptide contents depending on the amino acid sequences. As illustrated in **Figure 2**, the total peptide contents from bromelytic and tryptic hydrolysates were increasing under prolong hydrolysis time.

Longer digestion times provides favorable moment the protease to hydrolyze the long peptide bond of protein into smaller chain in order to obtain plentiful peptides [45].

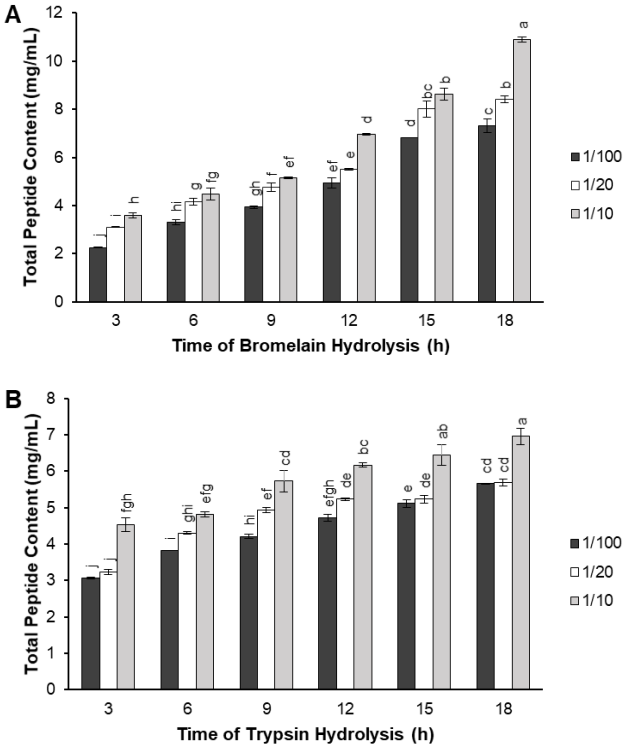

Figure 2 The effects of various hydrolysis time and E/S toward peptide content (mg/mL) of PA hydrolysates from different proteolytic enzyme of A) bromelain and B) trypsin. Results are represented as the mean ± SD of triplication. The same marked alphabet in the same bar are not significantly different at *p*-value < 0.05.

Peptides are short chain of several amino acids that are connected by peptide bonds in a sequence. These fragments are released from food proteins by the activity of protease and optimally produced under controlled conditions [46]. Total peptide content is expressed as the concentration of peptide in PA hydrolysate and may correspond to DH% due to their proclivity line are similar. This assessment is useful for presuming the potential hydrolysis condition for the next identification chapter. The highest E/S from both proteolytic hydrolysates were 1:10 (w/v). In this study, the higher E/S denoted higher number of total peptide contents in every time-series of hydrolysis. High E/S means that high concentration of protease is given in solution. Thus, it may facilitate precious contiguous opportunity between protease and protein which is allowed the high number of moles of enzyme's active site to cleave the substrate. Since the potent of flavor enhancer is defined by their peptide sequences, an implicit value that the output of PA hydrolysate with high concentration of peptides are auspicious. This high concentration of total peptide contents may contribute to collect heterogenous peptide sequences in order to explore efficacious umami taste as flavor enhancer.

Effect of various proteolytic enzymes on PA hydrolysates' amino acid content

PA hydrolysates were conducted using 2 varied proteolytic enzymes, particularly bromelain and trypsin, with distinct E/S of 1/10, 1/20, and 1/100 (w/v), and various hydrolysis times (3, 6, 9, 12, 15, and 18 h). Every amino acid provides differently intensity interpretations of food sensations. Notwithstanding distinctive amino acids do not take possession of intense taste in oneself, crosslinking products between amino acids with other compounds are significantly strengthen their taste. The several supportive compounds as crosslinker agents are inorganic salts [47], sugars [48], and nucleotides [49]. Variant tested synthetic L-α-amino acids by the addition of IMP stimulates various strong taste as flavor enhancer, such as sweetness, bitterness, sourness, saltiness, umami and other tastes [50]. Kawai *et al*. [50] reported using diverse single amino acids with various molarity (0, 25, 50, 100, and 200 mM) which resulting the enhancement of umami taste caused by the elevation of amino acids concentration. Amino acids are the

smallest unit molecules (monomers) which unify to construct polypeptides and proteins. Hence, amino acid content is one of the necessary parameters to analyze due to proteolytic hydrolysates consists of oligopeptides, short peptide fragments, and free amino acids obtained from partial hydrolysis of proteins. In general, higher amino acids content are responsible for the proper postulant as flavor enhancer despite the specific L-glutamic acid and L-aspartic acid are the most popular indicators as umami taste.

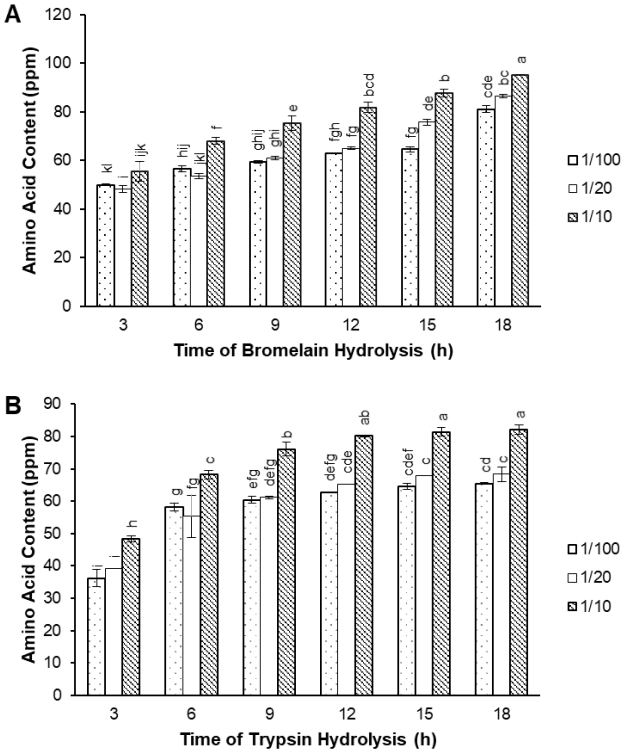

Figure 3 The effects of various hydrolysis time and E/S toward amino acid content (ppm) of PA hydrolysates from different proteolytic enzyme of A) bromelain and B) trypsin. Results are represented as the mean ± SD of triplication. The same marked alphabet in the same bar are not significantly different at *p*-value < 0.05.

The highest amino acid contents from PA hydrolysates using bromelain and trypsin are 95.34 ± 0.12 and 82.17 ± 1.46 ppm, respectively. Their proteolysis conditions were hydrolyzed using E/S of 1:10 (w/v) for 18 h. The amino acid contents using bromelain digestion showed significantly increase with extend the duration of hydrolysis time and higher E/S. This result corresponds to PA hydrolysates' degree of hydrolysis, which has similarity trend line with remarkably in its rapidly hydrolyzed to amino acids. Extensiveness of bromelain cleavage site may trigger numerous digestions for peptide bonds in the carboxyl terminal side which could produce accumulation of free amino acids in proteolytic of PA hydrolysate. Unfortunately, the narrow trypsin specificity may serve the amount of amino acid release. Other than catalytic site of trypsin predominantly cleaves limited peptide bonds at the carboxyl side of Lys and Arg, this result may have synergistic effect of one-by-one hydrolysis mechanism of endopeptidase which the enzyme cleaves preferentially within protein chain rather than the peptide bonds located at the ends of proteins. Thus, the concentration of amino acids in trypsin digestion is lower than bromelain digestion. In the same E/S, the amino acid contents based on their hydrolysis time of trypsin digestion does not significantly change at 12, 15, and 18 h, as shown in **Figure 3(B)**. These stationary part of points on the curve may be caused by completely digestion of favorable amino acid residues in whole proteins of this species which stipulated saturation curve after 9 h.

Effect of various proteolytic enzymes on the sensory characteristics of PA hydrolysates

The most fundamental analysis to evaluate the acceptability of consumer preference for food sensory characteristics is hedonic test. The obtained results from this evaluation based on the attributes of taste, aroma, and color are shown in **Figure 4**. In general, delightedness rate increased with the higher concentration of proteases and prolong hydrolysis time due to expected the abundant level of peptide

content and free amino acids releasing. Those E/S and hydrolysis time are obviously variables which influences in sensory acceptance and flavor characteristics of hydrolysate [51].

Two different proteolytic enzymes, namely bromelain and trypsin, were employed to conduct hydrolysis of PA, using distinct E/S of 1/10, 1/20, and 1/100, along with varying hydrolysis durations (3, 6, 9, 12, 15, and 18 h). PA hydrolysates prepared with bromelain using E/S of 1:10 (w/v) for 18 h and trypsin using E/S of 1:10 (w/v) for 15 h were sensed as the highest scores for taste, aroma and color attributes. Moreover, those 2 PA hydrolysate conditions also had preferentially highest liked scores among the others by panelists from overall attribute, as shown in **Figure 5**. This sensory assessment results were parallel outcomes with chemical properties, which implies that their highest numbers from DH, total peptide content, and amino acid content generated highest scores in hedonic test.

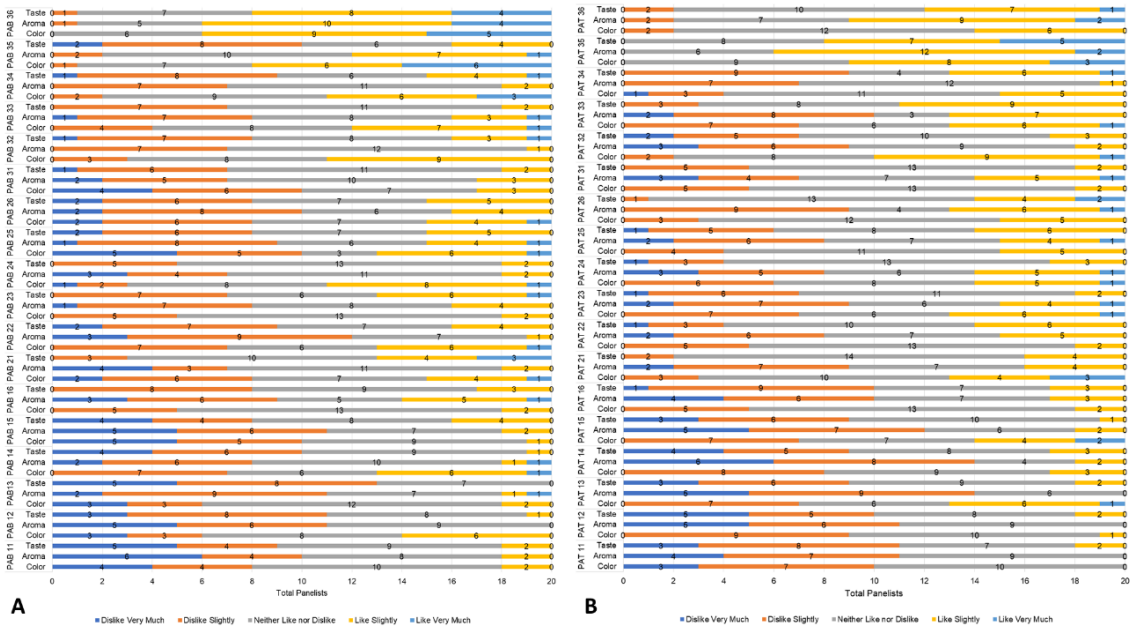

Figure 4 Hedonic evaluation of PA hydrolysates with various hydrolysis time and E/S of each attributes using different proteolytic enzyme of A) bromelain and B) trypsin. The different annotations are described hydrolysis conditions. PAB11/PAT11 = E/S 1:100 (w/v) for 3 h, PAB12/PAT12 = E/S 1:100 (w/v) for 6 h, PAB13/PAT13 = E/S 1:100 (w/v) for 9 h, PAB14/PAT14 = E/S 1:100 (w/v) for 12 h, PAB15/PAT15 = E/S 1:100 (w/v) for 15 h, PAB16/PAT16 = E/S 1:100 (w/v) for 18 h, PAB21/PAT21 = E/S 1:20 (w/v) for 3 h, PAB22/PAT22 = E/S 1:20 (w/v) for 6 h, PAB23/PAT23 = E/S 1:20 (w/v) for 9 h, PAB24/PAT24 = E/S 1:20 (w/v) for 12 h, PAB25/PAT25 = E/S 1:20 (w/v) for 15 h, PAB26/PAT26 = E/S 1:20 (w/v) for 18 h, PAB31/PAT31 = E/S 1:10 (w/v) for 3 h, PAB32/PAT32 = E/S 1:10 (w/v) for 6 h, PAB33/PAT33 = E/S 1:10 (w/v) for 9 h, PAB34/PAT34 = E/S 1:10 (w/v) for 12 h, PAB35/PAT35 = E/S 1:10 (w/v) for 15 h, PAB36/PAT36 = E/S 1:10 (w/v) for 18 h. The 5-point hedonic scores were described for liking of sensory attributes of PA hydrolysates (5 = like very much, 4 = like slightly, 3 = neither like nor dislike, 2 = dislike slightly, 1 = dislike very much).

PA hydrolysate of tryptic with E/S of 1:10 (w/v) for 15 h was the higher scores from taste, aroma, color, and overall attributes than 18 h. This may be because the hydrolysis time for 15 and 18 h in the same E/S were not significantly different from chemical properties, mainly total peptide content and amino acid content. In addition, trypsin digestion produces C-terminal residues of Lys and Arg, which are several peptide sequences isolated from food with Lys residues lead to bitter taste. Their origins are peptic hydrolysate of soybean and tryptic hydrolysate of casein, which their sequences are Leu-Lys [52], Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys, and Phe-Ala-Leu-Pro-Gln-Tyr-Leu-Lys [53], respectively. Therefore, prolong time of tryptic hydrolysate with E/S of 1:10 (w/v) for 18 h may produce higher releasing of Lys at C-terminal residue. This circumstance induced lower acceptability from panelists compare to 15 h.

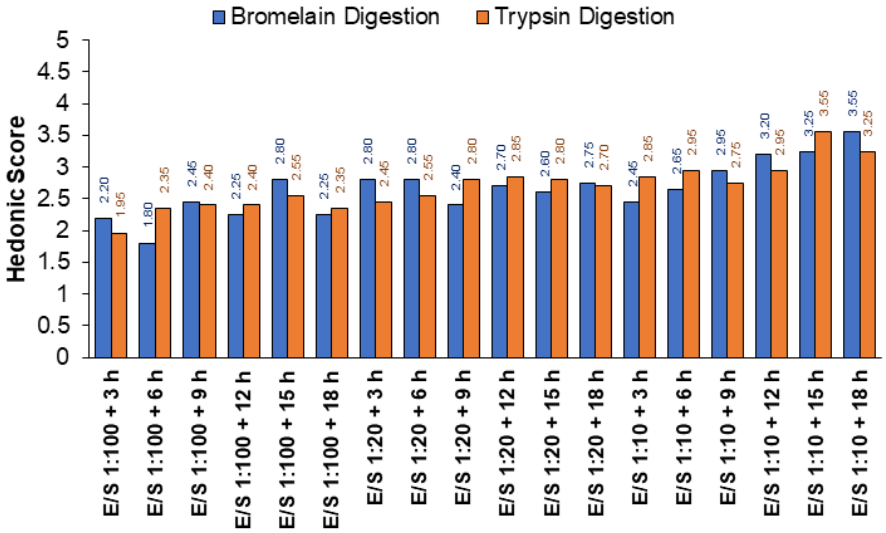

Figure 5 Sensory attribute of overall acceptance of PA hydrolysates with various hydrolysis time and E/S using different proteolytic enzyme of bromelain and trypsin.

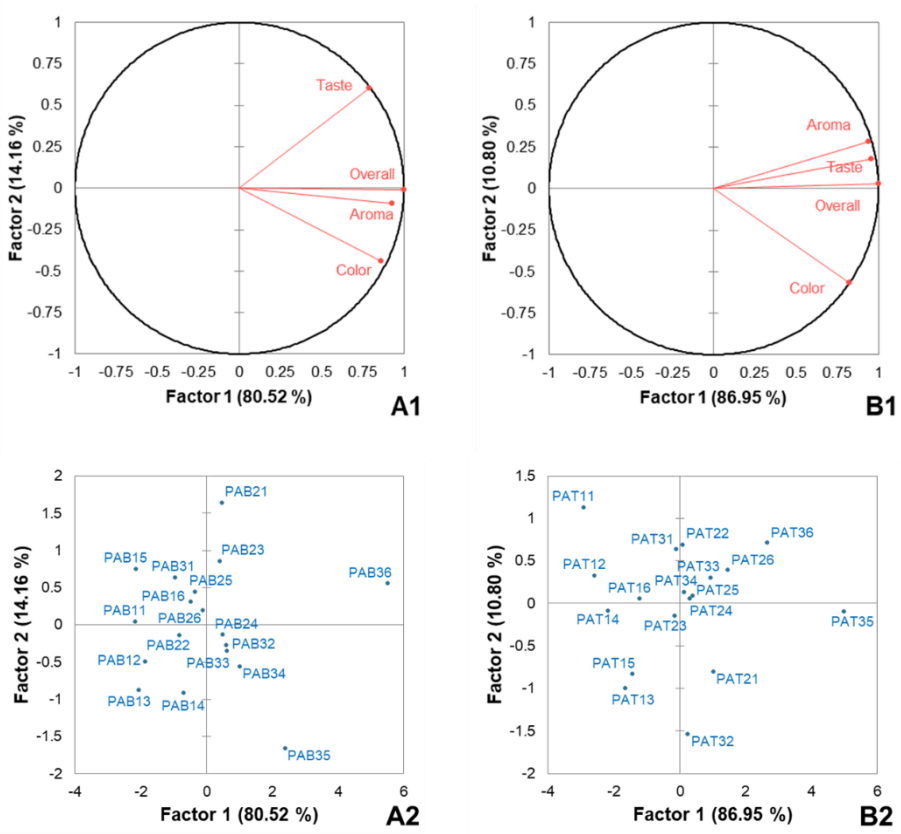

Figure 6 Principal component analysis (PCA) followed by Kaiser-Varimax rotation of PA hydrolysates (Factor 1 vs Factor 2): A1) loading plot of bromelain digestion, A2) score plot of bromelain digestion, B1) loading plot of trypsin digestion, B2) score plot of trypsin digestion.

Principal Component Analysis (PCA) was used to evaluate the comprehensive study among the resulted PA hydrolysates and to attain dependable prescription as potential concerning which E/S and hydrolysis time to use in proteolysis condition. All sensory attributes were observed to multivariate data analysis by Kaiser-Varimax rotation in PCA. This method describes attribute data that are positively and

negatively correlated. A positive correlation between attributes indicates the presence of one attribute followed by another. On the other hand, a negative correlation indicates the existence of one attribute is contrary to the other attributes. The subjected parameters showed that the principal components from bromelytic and tryptic hydrolysates were 94.68 and 97.75 %, respectively. As seen in **Figure 6A1**, the projection of the correlation circle plot from bromelytic hydrolysate is showing the closeness all attributes tested. Aroma and overall are close positions and accompanied by color attributes in the same quadrant. The taste attribute had no closeness to any attribute due to its point stands alone in a different quadrant from the others. However, aroma, taste, and overall had close position because they are in the same quadrant from the correlation circle plot of tryptic hydrolysate, as shown in **Figure 6B1**. The color attribute had no closeness to any attribute due to its point stands alone in a different quadrant from the others.

The score plot graph in the figure shows the relationship between each sample in the Factor 1 and Factor 2 components. Nearby samples have relatively similar characteristics, while samples that are far apart on the graph have differences. As seen in **Figure 6A2**, based on the results of PCA analysis, it can be seen that PAB36 sample had strong taste attribute, while PAB21 and PAB23 had less strong taste attribute. PAB24, PAB32, PAB33, PAB34, PAB35 had distinctive aroma, color, and overall attributes compared to other samples. PAT35 had strong color attribute, while PAT21 and PAT32 had not so strong color attribute, as seen in **Figure 6B2**. PAT22, PAT36, PAT26, PAT33, PAT25 had distinctive characteristics, mainly taste, aroma, and overall compared to other samples. PCA results strengthen clearly sensory properties of PA hydrolysates. Thus, hedonic test and PCA were concluded that the chosen proteolysis conditions to produce potential flavor enhancers were PA hydrolysates prepared with bromelain using E/S of 1:10 (w/v) for 18 h and trypsin using E/S of 1:10 (w/v) for 15 h.

Identified peptide sequences of PA hydrolysates from the selected proteolysis conditions

The formation taste in PA hydrolysates were obviously influenced by the peptide sequences. Early studies are strong evidently that the peptide consensus sequences determinate complex taste [54-56]. It has been reported that various identified peptides tastes were well categorized as being sweet, bitter, sour, salty, umami, and tasteless. This study was performed using LC-MS/MS followed by database search engine to investigate the peptide fragments. The obtained score from database search interprets the reliability of the observed MS/MS ions source. Thus, the listed identified peptides were selected from the mascot score of peptide higher than 30. The identified peptides are summarized in **Tables 1** and **2** for selected PA hydrolysates prepared with bromelain and trypsin, respectively. There were several various identified peptides and its diverse molecular weights belongs to specific origin proteins from snail. The database-assisted peptide sequencing might be complementary references to explain the acceptance of PA hydrolysate from sensory evaluation. Meaningful studies have described the amino acid compositions in peptide fragments, N-terminal, and C-terminal amino acid residues could have definite effects on flavor characteristics and intensity [57].

Glu and Asp are the popular acidic amino acids as umami taste, which compose in the sequence of peptides. There are several amino acids which can also contributed to the umami taste, such as Tyr, Phe [58], His, Val [59], Ala, Ser, Gly, and Thr [60]. In this study, the identified peptides in bromelytic hydrolysate contained amino acid residues which elicited umami taste content of 58.63 %, while in tryptic hydrolysate contained 50.94 %. Tyr, Thr, Gly, Glu as hydrophilic amino acids are reflected to the umami and sweet taste, whilst Phe and Val as hydrophobic amino acids are also conscientious for the bitter taste [14]. The presence of sweet and bitter-related amino acids in peptide fragments may provide the acceptability of sensory evaluation. Those bitter-related amino acids taste in bromelytic hydrolysate were 1.21 and 4.82 % for Phe and Val, respectively. However, higher amount in tryptic hydrolysate for Phe and Val were 3.31 and 5.66 %, respectively.

According to the suggestion previous study using molecular docking study exhibited that Gly residue as N-terminal amino acid is an important role in the binding site of umami receptor due to similarity interaction between T1R3 and L-Glu [19]. In this study, 5 out of 14 peptides from bromelytic hydrolysate (GPEGPQGPPGPRG, GIMLGAA, GLPGLPGLKGDPGEPGLP, GKDGEAG, and GLVMDSCAGH) and 2 out of 14 peptides from tryptic hydrolysate (GQTVIGL and GLPGLPGLSGPKG) contained Gly as N-terminal residue. Descriptive sensory analysis from previous study revealed that the peptide sequences containing Arg residue at the C-terminus elicited the umami taste [14]. The hydrolysate of bromelytic had only one identified peptide (EEKITEDDDAVGDDAENR) with a basic Arg residue as C-terminal amino acid, while the tryptic had 2 peptides (DTGPAGPAGPAGPQGPR and QTLEKALSHVIQEFETEKQLITVNAR). These all formation of peptide sequences from PA hydrolysate based on the selected conditions are supplementary tangible reason to explain which peptides contribute as umami taste.

Table 1 Peptides identified from apple snail (*Pila ampullacea*) hydrolysate using bromelain with enzyme-to-substrate ratio of 1/10 (w/v) and hydrolysis time of 18 h.

Identified protein	Identified peptide	Observed m/z	Peptide mass (Calc)	Mascot score of peptide
VWFD domain-containing protein	QADTPERTP	507.7750	1013.4778	44
Fibrillar collagen NC1 domain-containing protein	GPEGPQGPPGPRG	601.7878	1201.5840	39
Peptidase_S8 domain-containing protein	EHATFFPVLAVLGTCMVGL	413.3321	2061.0428	41
HTTM domain-containing protein	GIMLGAA	633.2892	631.3363	35
Ubiquitinyl hydrolase 1	IAKGKNSGGSSLAWEDDNVIMIGMKSSD	586.2286	2925.3848	38
Delta-aminolevulinic acid dehydratase	SIQRLAEVAVAYAKAGCQVIAPSDMMDGRIGAIKKALFKNDMG	513.1582	4609.3641	34
Long-chain specific acyl-CoA dehydrogenase, mitochondrial	DGDDWILNGSKVFITNGYM	541.3692	2159.9834	34
5'-deoxynucleotidase HDDC2	AMMAMLAPPGLD	625.2786	1248.5552	34
ARA70 domain-containing protein	RPMLKLSLGESEMPVGHTADISEVGKAGSPS	457.6138	3195.5904	32
Collagen IV NC1 domain-containing protein	GLPGLPGLKGDPGEPGLP	835.8673	1669.9039	32
Fibrillar collagen NC1 domain-containing protein	GKDGEAG	633.2852	632.2766	31
Ubiquitinyl hydrolase 1	SGGSIDQAATSMEGE	485.9548	1454	31
HATPase_c domain-containing protein	EEKITEDDDAVGDDAENR	506.2160	2019.8505	31
Homeobox domain-containing protein	GLVMDSCAGH	532.2535	1061.4270	30

Table 2 Peptides identified from apple snail (*Pila ampullacea*) hydrolysate using trypsin with enzyme-to-substrate ratio of 1/10 (w/v) and hydrolysis time of 15 h.

Identified protein	Identified peptide	Observed m/z	Peptide mass (Calc)	Mascot Score of peptide
Fibrillar collagen NC1 domain-containing protein	DTGPAGPAGPAGPQGPR	751.8577	1501.7274	91
Ribosomal RNA-processing protein 40	KGETVIGI	816.4321	815.4753	56
Fibrillar collagen NC1 domain-containing protein	EQGPQGQQGPSGLQ	705.8194	1409.6535	44
Calponin	GQTVIGL	687.3912	686.3963	44
Basal body-orientation factor 1	QTLEKALSHVIQEFETEKQLITVNAR	433.1626	3024.6244	42
Isocitrate dehydrogenase [NADP]	LQVFQFKSGGGVGMAMYNT	509.7380	2033.9703	40
Phospholipid-transporting ATPase	IGIMGKEGRMA	597.8061	1193.5896	38
Collagen IV NC1 domain-containing protein	GLPGLPGLSGPKG	575.2714	1148.6554	37
Dolichyl-diphosphooligosaccharide-protein glycotransferase	TASCLSSGDIL	562.2402	1122.5227	37
AMP-binding domain-containing protein	AVFQFAYNYKRRQLDRGYDTPLLNKAVFTKVKMLLGGNIRMML	639.8104	5110.7187	36
Lipase_3 domain-containing protein	WMFIGILIV	554.7944	1106.6198	35
NEDD8-activating enzyme E1 catalytic subunit	LGLTSGQELY	541.3096	1079.5499	34
DNA-directed RNA polymerase subunit beta	LRFLVAELAVMGINM	427.9528	1707.9052	34
MG1 domain-containing protein	ADDLIGIAS	874.3962	873.4444	33

Conclusions

In summary, proteolysis conditions based on E/S and hydrolysis time were successfully found to produce umami taste as flavor enhancers from *Pila ampullacea* using bromelain and trypsin. Those 2 variables had significantly effects on chemical properties (DH, total peptide content, and amino acid content) and sensory attributes (taste, aroma, color, and overall). PA hydrolysates using bromelain with the E/S of 1:10 (w/v) for 18 h and trypsin with E/S of 1:10 (w/v) for 15 h were the selected treatment for proteolysis conditions and chosen based on sensory evaluation. Additional analysis using LC-MS/MS analysis followed by database search engine summarized that each proteolysis conditions produced 14 identified peptides to get the insight of its fragments. We identified that the peptides have Gly residue at N-terminus and Arg residue at C-terminus may promote the umami taste of peptides. We believed that the application of PA hydrolysates as flavor enhancers in food company is promising.

Acknowledgements

This work was financially supported by Institute of Research and Community Service (LPPM) UPN "Veteran" Jawa Timur with the scheme of international research collaboration grants for Dedin Finatsiyatull Rosida (SPP/42/UN.63.8/LT/V/2020). We appreciate the LC-MS/MS supports by Ms. Supitcha Pannengpetch from Center for Research and Innovation, Faculty of Medical Technology, Mahidol University (Salaya Campus), Nakonpathum, Thailand, who contributed to proteomics services and peptide identification.

References

[1] K Kurihara. Umami the fifth basic taste: history of studies on receptor mechanism and role as a food flavor. *BioMed. Res. Int.* 2015; **2015**, 189402.
[2] F Bellisle. Experimental studies of food choices and palatability responses in European subjects exposed to the Umami taste. *Asia Pac. J. Clin. Nutr.* 2008; **17**, 376-9.
[3] Y Dang, X Gao, A Xie, X Wu and F Ma. Interaction between umami peptide and taste receptor T1R1/T1R3. *Cell Biochem. Biophys.* 2014; **70**, 1841-8.
[4] H Liu, LT Da and Y Liu. Understanding the molecular mechanism of umami recognition by T1R1-T1R3 using molecular dynamics simulations. *Biochem. Biophys. Res. Comm.* 2019; **514**, 967-73.
[5] DM Kendig, NR Hurst, ZL Bradley, S Mahavadi, JF Kuenmerle, V Lyall, J DeSimone, KS Murthy and JR Grider. Activation of the umami taste receptor (T1R1/T1R3) initiates the peristaltic reflex and pellet propulsion in the distal colon. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2014; **307**, G1100-G1107.
[6] S Jinap and P Hajeb. Glutamate. Its application in food and contribution to health. *Appetite* 2010; **55**, 1-10.
[7] K Ninomiya. Science of umami taste: Adaptation to gastronomic culture. *Flavour* 2015; **4**, 13
[8] V Husarova and D Ostatnikova. Monosodium glutamate toxic effects and their implications for human intake: A review. *JMED Res.* 2013; **2013**, 608765.
[9] K Niaz, E Zaplatic and J Spoor. Extensive use of monosodium glutamate: A threat to public health. *EXCLI J.* 2018; **17**, 273-8.
[10] SF Brun, TG Kudre and N Bhaskar. Effects of different pretreatments and proteases on recovery, umami taste compound contents and antioxidant potentials of *Labeo rohita* head protein hydrolysates. *J. Food Sci. Tech.* 2019; **56**, 1966-77.
[11] X Xu, R Xu, Z Song, Q Jia, T Feng, M Huang and S Song. Identification of umami-tasting peptides from *Volvariella volvacea* using ultra performance liquid chromatography quadrupole time-of-flight mass spectrometry and sensory-guided separation techniques. *J. Chrom. A* 2019; **1596**, 96-103.
[12] J Gao, D Fang, BM Kimatu, X Chen, X Wu, J Du, Q Yang, H Chen, H Zheng, X An, L Zhao and Q Hu. Analysis of umami taste substances of morel mushroom (*Morchella sextelata*) hydrolysates derived from different enzymatic systems. *Food Chem.* 2021; **362**, 130192.
[13] MR Rhyu, AY Song, EY Kim, HJ Son, Y Kim, S Mummalaneni, J Qian, JR Grider and V Lyall. Kokumi taste active peptides modulate salt and umami taste. *Nutrients* 2020; **12**, 1198.
[14] MX Zhang, ZC Wang, Y Liu, XL Xu and GH Zhou. Isolation and identification of flavour peptides from Puffer fish (*Takifugu obscurus*). *Food Chem.* 2012; **135**, 1463-70.
[15] Z Liu, Y Zhu, W Wang, X Zhou, G Chen and Y Liu. Seven novel umami peptides from *Takifu rubripes* and their taste characteristics. *Food Chem.* 2020; **330**, 127204.

[16] M Chen, X Gao, D Pan, S Xu, H Zhang, Y Sun, J He and Y Dang. Taste characteristics and umami mechanism of novel umami peptides and umami-enhancing peptides isolated from the hydrolysates of Sanhuang Chicken. *Eur. Food Res. Tech.* 2021; **247**, 1633-44.
[17] X Bao, S Ma, Y Fu, J Wu and M Zhang. Sensory and structural characterization of umami peptides derived from sunflower seed. *CyTA J. Food* 2020; **18**, 485-92.
[18] Y Ding, X Li and J Kan. Isolation and identification of flavor peptides from douche (traditional Chinese soybean food). *Int. J. Food Properties* 2017; **20**, 1982-94.
[19] MNG Amin, J Kusnadi, JL Hsu, RJ Doerksen and TC Huang. Identification of a novel umami peptide in tempeh (Indonesian fermented soybean) and its binding mechanism to the umami receptor T1R. *Food Chem.* 2020; **333**, 127411.
[20] GK Beauchamp. Sensory and receptor responses to umami: an overview of pioneering work. *Am. J. Clin. Nutr.* 2009; **90**, 723S-727S.
[21] JFLD Pablo, E Badal, CF García, AM Ortí and AS Serra. Land snails as a diet diversification proxy during the early upper palaeolithic in Europe. *PLoS One* 2014; **9**, e104898.
[22] S Ghosh, C Jung and VBM Rochow. Snail as mini-livestock: nutritional potential of farmed *Pomacea canaliculata* (Ampullariidae). *Agr. Nat. Resource* 2017; **51**, 504-11.
[23] RM Marwoto, H Heryanto and RC Joshi. The invasive apple snail *Pomacea canaliculata* in Indonesia: a case study in Lake Rawa Pening, Central Java. *BIO Web Conf.* 2020; **19**, 00014.
[24] R Broto, F Arifan, WA Setyati, K Eldiarosa and AR Zein. Crackers from fresh water snail (*Pila ampullaceal*) waste as alternative nutritious food. *IOP Conf. Ser. Earth Environ. Sci.* 2020; **448**, 012039.
[25] K Ihsani, DN Afifah, DT Anantyo, Mulyono, TW Nugroho, F Wahyudi and Nuryanto. Nutrient content and shelf life analysis of freshwater snail (*Pila ampullaceal*) instant baby porridge. *Food Res.* 2020; **4**, 184-96.
[26] DF Rosida, AD Priyanto and AYT Putra. Effects of papain concentration and hydrolysis time on degree of hydrolysis and glutamic acid content of apple snail hydrolysate. *In*: Proceedings of the 5th International Seminar of Research Month, Jawa Timur, Indonesia. 2021, p. 17-21.
[27] AYT Putra, DF Rosida and AD Priyanto. Effect of hydrolysis time and papain concentration on some properties of apple snail (*Pila ampullaceal*) hydrolysate. *Int. J. Eco Innovat. Sci. Eng.* 2020; **1**, 1-5.
[28] P Thiviya, A Gamage, NS Gama-Arachchige, O Merah and T Madhujith. Seaweeds as a source of functional proteins. *Phycology* 2022; **2**, 216-43.
[29] VM Silva and M Hubinger. *Production and shelf life of mussel meat powder flavor*. *In*: S Haugen and S Meijer (Eds.). Handbook of nutritional biochemistry: Genomics. Nova Science Publishers, New York, 2009.
[30] DL Swaney, CD Wenger and JJ Coon. The value of using multiple proteases for large-scale mass spectrometry-based proteomics. *J. Proteome Res.* 2010; **9**, 1323-9.
[31] R Mohan, V Sivakumar, T Rangasamy and C Muralidharan. Optimisation of bromelain enzyme extraction from pineapple (*Ananas comosus*) and application in process industry. *Am. J. Biochem. Biotechnol.* 2016; **12**, 188-95.
[32] NT Hoyle and JH Merrit. Quality of fish protein hydrolysate from Herring (*Clupea harengus*). *J. Food Sci.* 1994; **59**, 76-9.
[33] OH Lowry, NJ Rosebrough, AL Farr and RJ Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951; **193**, 265-75.
[34] EF Hartree. Determination of protein: A modification of the lowry method that gives a linear photometric response. *Anal. Biochem.* 1972; **48**, 422-7.
[35] M Ledoux and F Lamy. Determination of proteins and sulfobetaine with the folin-phenol reagent. *Anal. Biochem.* 1986; **157**, 28-31.
[36] S Moore and WH Stein. Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* 1948; **176**, 367-88.
[37] S Yamaguchim and C Takahashi. Hedonic functions of monosodium glutamate and four basic taste substances used at various concentration levels in single and complex systems. *Agr. Biol. Chem.* 1984; **48**, 1077-81.
[38] Y Witono, RR Fauziah, WS Windrati, I Taruna, L Azkiyah and RP Wijayanti. Formulation of flavor enhancer from common barb (*Rasbora jacobsoni*) protein hydrolysate. *AIP Conf. Proc.* 2019; **2199**, 060002.
[39] MAK Anuar, NHH Narashid, MM Salleh and A Yahya. Conversion of chicken viscera into protein hydrolysate for palatant production. *Malays. J. Fund. Appl. Sci.* 2017; **13**, 606-11.

[40] L You, JM Regenstein and RH Liu. Optimization of hydrolysis conditions for the production of antioxidant peptides from fish gelatin using response surface methodology. *J. Food Sci.* 2010; **75**, C582-C587.
[41] RB Benítez, WFE Tabares, LAL Veláquez, CIH Sánchez and OAS Cruel. Enzymatic hydrolysis as a tool to improve total digestibility and techno-functional properties of pigeon pea (*Cajanus cajan*) starch. *Heliyon* 2021; **7**, e07817.
[42] L Wang, B Xu, L Li, M Zhang, T Feng, J Wang and Z Jin. Enhancement of umami taste of hydrolyzed protein from wheat gluten by β-cyclodextrin. *J. Sci. Food. Agr.* 2016; **96**, 4499-504.
[43] T Kaur, A Kaur and RK Grewal. Kinetics studies with fruit bromelain (*Ananas comosus*) in the presence of cysteine and divalent ions. *J. Food Sci. Tech*. 2015; **52**, 5954-60.
[44] R Huber and W Bode. Structural basis of the activation and action of trypsin. *Accounts Chem. Res.* 1978; **11**, 114-22.
[45] R Ghanbari, M Zarei, A Ebrahimpour, AA Hamid, A Ismail and N Saari. Angiotensin-I converting enzyme (ACE) inhibitory and anti-oxidant activities of sea cucumber (*Actinopyga lecanora*) hydrolysates. *Int. J. Mol. Sci.* 2015; **16**, 28870-85.
[46] A Panchaud, M Affolter and M Kussmann. Mass spectrometry for nutritional peptidomics: How to analyze food bioactives and their health effects. *J. Proteomics* 2012; **75**, 3546-59.
[47] T Ugawa, S Konosu and K Kurihara. Enhancing effects of NaCl and Na phosphate on human gustatory responses to amino acids. *Chem. Senses* 1992; **17**, 811-5.
[48] E Karangwa, N Murekatete, JD Habimana, K Masamba, E Duhoranimana, B muhoza and X Zhang. Contribution of crosslinking products in the flavour enhancer processing: The new concept of maillard peptide in sensory characteristics of maillard reaction systems. *J. Food Sci. Tech.* 2016; **53**, 2863-75.
[49] A Kuninaka. Studies on taste of ribonucleic acid derivatives. *J. Agr. Chem. Soc. Jpn.* 1960; **34**, 489-92.
[50] M Kawai, A Okiyama and Y Ueda. Taste enhancements between various amino acids and IMP. *Chem. Senses* 2002; **27**, 739-45.
[51] C Sonklin, N Laohakunjit and O Kerdchoechuen. Physicochemical and flavor characteristics of flavoring agent from mungbean protein hydrolyzed by bromelain. *J. Agr. Food Chem.* 2011; **59**, 8475-83.
[52] S Arai, M Yamashita and M Fujimaki. Glutamyl oligopeptides as factors responsible for tastes of a proteinase-modified soybean protein. *Agr. Biol. Chem.* 1972; **36**, 1253-6.
[53] T Matoba, R Hayashi and T Hata. Isolation of bitter peptides from tryptic hydrolysate of casein and their chemical structure. *Agr. Biol. Chem.* 1970; **34**, 1235-43.
[54] YL Dang, ZI Zhang, XW Yan, Z Wang and SY Xu. Isolation, purification and structural identification of flavor peptides from enzymolyzed parma ham. *Food Sci.* 2010; **31**, 127-31.
[55] JÁ Gómez-Ruiz, G Taborda, L Amigo, M Ramos and E Molina. Sensory and mass spectrometric analysis of the peptidic fraction lower than one thousand daltons in manchego cheese. *J. Dairy Sci.* 2007; **90**, 4966-73.
[56] JN Park, K Ishida, T Watanabe, KI Endoh, K Watanabe, M Murakami and H Abe. Taste effects of oligopeptides in a Vietnamese fish sauce. *Fisheries Sci.* 2002; **68**, 921-8.
[57] J Zhang, D Sun-Waterhouse, G Su and M Zhao. New insight into umami receptor, umami/umami-enhancing peptides and their derivatives: A review. *Trends Food Sci. Tech.* 2019; **88**, 429-38.
[58] C Salles, C Septier, F Roudit-Algaron, A Guillot and PX Etievant. Sensory and chemical analysis of fractions obtained by gel permeation of water coluble comte cheese extracts. *J. Agr. Food Chem.* 1995; **43**, 1659-68.
[59] Y Dang, X Gao, F Ma and X Wu. Comparison of umami taste peptides in water soluble extractions of Jinhua and Parma hams. *LWT Food Sci. Tech.* 2015; **60**, 1179-86.
[60] HN Lioe, K Takara and M Yasuda. Evaluation of peptide contribution to the intense umami taste of Japanese soy sauces. *J. Food Sci.* 2006; **71**, S277-S283.