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Characterization of *Pleurotus Citrinopileatus* Hydrolysates Obtained from *Actinomucor Elegans* Proteases Compared with That by Commercial Proteases

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Abstract

Pleurotus citrinopileatus, the nutritious and palatable edible mushroom, can be taken as an appropriate material to prepare high-grade flavoring agents. Based on this, this study aimed to investigate the feasibility of productive proteases system from *Actinomucor elegans* to prepare *P. citrinopileatus* hydrolysate (PCH). The *A. elegans* crude protease (AECP) was prepared from the solid state fermentation product of *P. citrinopileatus* by *A. elegans*. AECP and four commercial proteases (Alcalase, Neutrase, Papain, and Protamex) were applied to acquire five kinds of PCHs respectively. The physical-chemical properties of PCHs as well as its concentration and composition of non-volatile compounds were comparatively analyzed. Electronic tongue was utilized to evaluate sensory characteristics. AECP was found to be the most effective protease with the top hydrolysis degree of 35.91% and protein recovery of 81.46%. The result of molecular weight distribution indicated that below 500 Da peptides were observed to be the main fraction of AECP hydrolysates, meanwhile AECP hydrolysates showed the highest content of MSG-like (20.23 ± 0.16 mg/g) and flavor 5'-nucleotide (4.30 ± 0.07 mg/g). Besides, the AECP hydrolysates exhibited favorable aftertaste-umami, in which peptides below 500 Da showed a higher correlation with aftertaste-umami by results of partial least-squares regression. These results indicated the feasibility for PCHs application as flavor additives or seasoning in food industry. The AECP might be used as an alternative enzyme choice because of its low cost and high hydrolysis efficiency.

Introduction

Edible mushrooms, as functional foods and food spices, have gained in increasing popularity among consumers and industries due to their special flavor and taste, and being a rich source of nutrients including proteins, carbohydrates and a variety of amino acids (Wang et al. 2014; Zhang et al. 2013). *Pleurotus citrinopileatus*, a cultivated edible mushroom, is considered as a healthy food with high protein, high dietary fiber and low fat, which is widely distributed in Northeast China, Japan, and South Korea (Rodrigues et al. 2015). In addition, *Pleurotus citrinopileatus* was identified to be rich in glutamic acid (Glu), aspartic acid (Asp), and 5'-nucleotides (Yin et al. 2019), making it a suitable material to prepare high-quality flavor condiments. However, in recent years, most researches of *P. citrinopileatus* have concentrated on its biological activity evaluation and genetic diversity, whereas few pieces of report focusing on the utilization of flavor compounds in *P. citrinopileatus* have been conducted (Minato et al. 2019; Sheng et al. 2019; Rosnina et al. 2016).

The relatively high-protein content of edible mushrooms made them an important source of protein hydrolysates with taste-enhancing properties, for which major enzymes used for the production of mushroom protein hydrolysates included Alcalase (Lotfy et al. 2015), Neutrase (Gao et al. 2021), Papain (Banjongsinsiri et al. 2016), and Protamex (Palupi et al. 2011). Although the preparation of protein hydrolysates through enzymatic hydrolysis with food-grade enzymes were Generally Recognized as Safe (GRAS), the choice of commercial food-grade proteases was limited since the cost of enzymes was high, and the yield of peptides was low due to the specificity of the enzyme cleavage sites (Ulug et al. 2021).

As a consequence, it was crucial to discover the alternative enzymes with low-cost and high hydrolysis yields.

Actinomucor elegans is a fungus strain found in traditional Chinese fermented food such as fermented bean curd, which can produce many enzymes including protease, lipase, α-amylase, glucosidase, and glucanase (Yin et al. 2020). During the fermentation of tofu, *A. elegans* proteases catalyze the degradation of proteins into small peptides and free amino acids, providing unique flavors and texture to the product. It was reported that *A. elegans* protease was efficient for degrading soybean protein and decreasing the bitter peptides (Fu et al. 2011; Li & Li 2013), thus *A. elegans* protease has great potential to be applied in the protein hydrolysate industry. Nevertheless, little information was available regarding the utilization of *A. elegans* protease in the hydrolysis of *P. citrinopileatus* so far.

This study investigated the characteristics of *P. citrinopileatus* hydrolysate (PCH) prepared with the crude protease produced from solid-state fermentation of *A. elegans* (AECP). Four commercial proteases (Alcalase, Neutrase, Papain, and Protamex) were used for comparison. Physical-chemical properties such as the degree of hydrolysis, protein recovery and the content of non-volatile compounds (free amino acids and 5'-nucleotide) were determined. Electronic tongue was used to evaluate sensory characteristics. Furthermore, the correlation between free amino acids, nucleotide composition, peptide molecular weight (MW) distribution, and sensory characteristics was established through partial least-squares regression (PLSR). The results of this study could offer a powerful basis for using PCH as food additives or food seasoning in the food industry.

Materials And Methods

Materials and reagents

The *P. citrinopileatus* powder (60 mesh) was supplied by Shandong Tianbo Food Ingredients Co., Ltd. (Jining, Shandong, China). The *A. elegans* (AS3.2778) was provided by Beijing Food Brewing Institute (Beijing, China). Alcalase (2.0×10^5 U/g), Neutrase (3.2×10^4 U/g), Protamex (4.0×10^4 U/g), and Papain (6.7×10^4 U/g) were obtained from Novozymes (China) Investment Co., Ltd. (Beijing, China). Standards used for relative molecular weight calibration curve: cytochrome C (12,500 Da), aprotinin (6,500 Da), bacitracin (1,450 Da), Gly-Gly-Tyr-Arg (451 Da), and Gly-Gly-Gly (189 Da), were of highperformance liquid chromatography (HPLC) and were purchased from Sigma-Aldrich (St Louis, MO, USA). 18 amino acid standards (purity > 98%) used for amino acid composition were acquired by Sigma-Aldrich (St Louis, MO, USA). Standards of CMP (98.4%), GMP (97.3%), IMP (78.6%), AMP (93.5%) and UMP (95.3%) were received from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). All other chemicals and reagents were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

Preparation of A. elegans crude protease

AECP was prepared as the following method using *A. elegans*. Briefly, *P. citrinopileatus* powder (6.5 g), bran (10 g), sucrose (1.84 g), $MaSO_4$ (0.015 g), $CaCl_2$ (0.009 g), and deionized water (25.72 g) were mixed

into an Erlenmeyer flask and sterilized in an autoclave at 121 °C for 30 min. Subsequently, the mixture was cooled to 30 °C and inoculated with spores of *A. elegans* (10^6 spores/g). The obtained mixture was incubated at 27 °C for 104 hours. After fermentation, 100 mL of 0.2 M phosphate-buffered saline (pH 7.0) was added and stirred at 40 °C for 1 h to extract the protease. After centrifuging (refrigerated high-speed centrifugator, LGR20-W, Beijing Jingli cebtrifuge CO., LTD, Beijing, China) at 10,000 rpm for 30 min at 4 °C, the supernatant (AECP) was collected and lyophilized, which was stored at -20 °C until subsequent use.

Proteases activity determination

The enzyme activities of Alcalase, AECP, Neutrase, Protamex, and Papain were determined based on Folin Ciocalteu assay as previously described by Wang et al. (2021). 1 mL of diluted Alcalase was pooled with 1 mL casein (2%, w/v) in 50 mM Borax-NaOH buffer (pH 10.5), while AECP, Neutrase, Protamex, and Papain (1 mL) were added to casein (1 mL, 1% (w/v)) in 50 mM phosphate buffer (pH 7.5). The obtained mixture was incubated at 40 °C for 10 min, and the reaction was terminated by adding 0.4 M trichloroacetic acid (TCA) (2 mL). The mixture was then centrifuged (10,000 rpm, 5 min), and 1 mL of the supernatant was mixed with Na_2CO_3 (5 mL, 0.4 M) and Folin-Ciocalteu reagent (1 mL) and incubated for 20 min at 40 °C. The absorbance was measured at 680 nm against the blank using a UV-Vis spectrophotometer (Evolution 220, Thermo Fischer Scientific, America). Samples were analyzed in triplicates. One unit (U) of protease activity was described as 1 μg tyrosine liberated per min the amount of the enzyme from casein.

Crude protein, fat, crude fiber, ash, and carbohydrates contents were measured according to the AOAC method (Gao et al. 2021).

Preparation of *P. citrinopileatus* hydrolysates

P. citrinopileatus powder (8 g) was dissolved in deionized water (92 mL) and then hydrolyzed with various proteases at their respective optimum hydrolysis conditions according to the manufactures: (1) Alcalase: at 55 °C and pH 8.0; (2) Neutrase, Papain, Protamex and AECP: at 50 °C and pH 7.0; the pH of all solutions was adjusted by 1M NaOH or HCl. The proteases respectively were used at an enzyme/substrate ratio of 1,000 U/g. At the end of the hydrolysis period, the mixture was heated at 100 °C for 10 min to inactivate the enzyme and then centrifuged for 20 min at 10,000 rpm at 4 °C. Lastly, the supernatant was separarted and later lyophilized to be stored at -20 °C till analysis. Control group performed the same experimental procedures without the protease.

Determination of degree of hydrolysis (DH), protein recovery (PR), and total saccharide content (TSC)

The degree of hydrolysis was evaluated as the proportion (%) of the α-amino nitrogen released from protein, and the α-amino nitrogen content was measured based on the method of formaldehyde titration (Wei et al. 2018). DH was calculated using Eq. (1).

$$DH(\%) = \frac{B - A}{C - A} \times 100\% \quad (1)$$

where A is the content of α-amino nitrogen before protease hydrolysis, B is the content of α-amino nitrogen in the supernatant after hydrolysis, and C is the total nitrogen content of *P. citrinopileatus* powder determined using the Kjeldahl method.

The protein recovery was estimated as the percentage ratio of protein content in the supernatant to total protein in sample (Pagán et al. 2013). The phenol-sulfuric acid method was employed for the measurement of total saccharide content (TSC) using glucose as a standard (Li et al. 2017).

Molecular weight (MW) distribution

The MW distributions of PCHs were determined by high-performance gel-filtration chromatography (HPLC) using a TSK gel G2000 SWXL (300 mm × 7.8, TOSOH, Tokyo, Japan) (Eric et al. 2013). HPLC analysis was performed on a Waters e2695 Alliance HPLC system (Water Co., Milford, MA, USA), which was equipped with a 2487 UV detector and Empower workstation. The flow rate was set at 0.5 mL/min with the mobile phase consisting of acetonitrile/water/trifluoroacetic acid (45/55/0.1, v/v/v). Sample (10.0 μL) was injected into the system, and the column was maintained at a temperature of 30 ℃. The following standards: cytochrome C (12,500 Da), aprotinin (6,500 Da), bacitracin (1,450 Da), Gly-Gly-Tyr-Arg (451 Da), and Gly-Gly-Gly (189 Da) were used to build the calibration curve used to evaluate the MW of the sample. UV absorbance was recorded at 200 nm, and data were processed with gel-permeation chromatography (GPC) software (Waters Co., Milford, US).

Amino acid analysis

The amino acid composition of *P. citrinopileatus* powder was performed using previously methods (Wang et al. 2021). 10 mg *P. citrinopileatus* powder was hydrolyzed by incubation in 6 N HCl (10 mL) for 24 h at 110 ℃ in tubes sealed under nitrogen. After digestion, the samples were diluted to 50 mL with ultrapure water and filtered through two layers of filter paper. Subsequently, the supernatant was filtered using a 0.22 μm membrane filter, and the filtrate (20 μL) was determined using a Hitachi L-8900 automated amino acid analyzer (Hitachi, Tokyo, Japan).

For the analysis of free amino acids, the method was applied as described previously (Song et al. 2019). In general, the sample (20 mg) was dissolved thoroughly by adding 3.5% sulfo salicylic acid, further diluted to 50 mL, and centrifuged for 20 min at 10,000 rpm. The supernatant was taken and filtered through a membrane filter (0.22 μm). Afterwards, the filtrate (20 μL) was detected using Amino Acids Analyzer (L-8900, Hitachi, Tokyo, Japan).

5′-Nucleotides analysis

5′-Nucleotides were determined using the procedure described by Li et al. (2015) with slight modifications. 2% solution (w/v) of the samples was filtered through 0.22 μm microporous fiber membranes prior to determination with high-performance liquid chromatography (HPLC, Waters E2695, Water Co., Milord, MA USA). Analysis was performed at 30 ℃ on a C18 column (250 mm × 4.6 mm, 5 μm) using 10 mM KH_2PO_4 as a mobile phase with a flow rate of 1.0 mL/min. It was detected at 254 nm by UV with an injection volume of 10.0 μL. Each 5′-nucleotide was identified and quantified by comparison with a calibration curve obtained via the corresponding standards.

Equivalent umami concentration analysis

As an indicator that frequently used to characterize the umami taste of food, equivalent umami concentration (EUC) represented the total amount of umami substances in 100 g (dry weight) food, and expressed by the amount of monosodium glutamate (MSG) with the same umami taste intensity (Yamaguchi et al. 2006). The Eq. (2) was as follows:

$$Y = \sum a_i b_i + 1218\left(\sum a_i b_i\right)\left(\sum a_j b_j\right) \quad (2)$$

where Y is the EUC of the mixture in terms of gMSG/100 g; a_i (%) is the concentration of the respective umami amino acids (ASP and Glu); a_j (%) is the concentration of the respective umami 5'-nucleotides (5'-inosinic acid, 5'-IMP; 5'-guanylic acid, 5'-GMP; 5'-xanthylic acid, 5'-XMP; 5'-adenylic acid, 5'-AMP); b_i is the relative umami concentration (RUC) for each umami amino acids to MSG (Glu, 1 and Asp, 0.077); b_j is the RUC for each umami 5'-nucleotide to 5'-IMP (5'-IMP, 1; 5'-GMP,2.3; 5'-XMP, 0.61 and 5'-AMP, 0.18), and 1218 is a synergy constant based on the concentration (g/100 g) used.

Electronic tongue measurement

The sensory characteristics of PCHs were performed using the TS-5000Z Taste-Sensing System (Insent Co., Japan). Sample solutions were prepared by different PCHs with deionized water at 1.0% (w/v), placed in special beakers for the electronic tongue. The conditions for the electronic tongue assay were listed below: after washing of 5 min, the samples were examined for 30 s, followed by aftertaste measurement for 30 s. In addition, a blank sample containing 30 mM KCl and 0.3 mM tartaric acid was treated as controls. Each sample was operated in parallel five times, and the strength response values from lasted three times were averaged for further analysis (Phat et al. 2016; Zhou et al. 2021).

Statistical analysis

All the experiments were carried out in triplicates, and the results were expressed as mean ± SD. All data were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range tests using the software SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Multivariate analysis was carried out with SIMCA 14.1 (MKS Umetrics AB, Umeå, Sweden).

Results And Discussion

Total amino acid composition of *P. citrinopileatus*

The basic compositions of *P. citrinopileatus* powder were assayed before enzymatic hydrolysis. The result of chemical compositions showed that there were high content of crude protein for *P. citrinopileatus* (34.41 ± 0.04 g/100 g), crude fiber (33.58 ± 0.48 g/100 g), and carbohydrate (12.46 ± 0.29 g/100 g), as well as low content of fat (3.46 ± 0.22 g/100 g) and ash (8.57 ± 0.07 g/100 g). Considering that there was usually a range of protein content from 12.0% to 47.21% in fungus (Zhang et al. 2013; Wang et al. 2014), *P. citrinopileatus* had a relatively high protein content. Meanwhile, Asp and Glu were monosodium glutamate-like (MSG-like) components that gave the most typical mushroom taste, umami taste, or palatable taste in mushrooms, which earned them the name of umami amino acids. The ratios of umami amino acids to total amino acids generally varied among mushrooms from 21 to 32% (Wang et al. 2014). According to Table 1, umami amino acids of *P. citrinopileatus* accounted for 42% of the total amino acids, indicating the level of umami-flavored amino acids was relatively high in *P. citrinopileatus*. In conclusion, *P. citrinopileatus* could be considered as a potential material to be hydrolyzed by proteases for natural flavoring.

Physical and chemical properties of PCHs

DH was used as a true reflection of the enzymatic hydrolysis process, which influenced product yield, bitterness, and functional properties. As shown in Fig. 1a, the DH of all PCHs increased significantly with time, while after 2 h of hydrolysis, the DH changes of the Papain and AECP became relatively stable, and the DH values of the Alcalase, Neutrase, and Protamex groups were no longer change after 3 h. This result might be due to the specific catalytic sites of the proteases, which gradually reduced as the hydrolysis continued (Ktari et al. 2013). On the basis of these results, 2 h was selected as the optimal duration for the preparation of PCHs by AECP and Papain, whereas 3 hours was chosen as the optimum duration to produce PCHs using Alcalase, Neutrase, and Protamex. Under these reaction conditions, the DH of AECP hydrolysate had peaked at 35.91%, which was markedly higher than other proteases ($P < 0.05$) (Fig. 1b). In contrast, the DHs of Alcalase, Neutrase, Papain, and Protamex groups were low, ranging from 15.29% to 22.21%, with no significant difference among these four proteases was observed ($P > 0.05$) (Fig. 1b). Since the proteases varied in cleavage sites on the polypeptide chain, the peptide chain length and amino acid sequence might differ in the hydrolysates prepared from various proteases, which could result in markedly different DH (Himonides et al. 2011).

PR was also used to quantify the utilization rate of raw protein during the preparation of protein hydrolysates. As indicated in Fig. 1b, the highest PR of 81.46% was recorded for AECP hydrolysate, followed by the Papain, Neutrase and Alcalase hydrolysates at 59.13%, 58.71% and 58.05% respectively. The lowest percent PR values were observed as Protamex hydrolysate (55.85%), and no statistically significant PR changes were perceived among the four commercial protease hydrolysates ($P > 0.05$).

Comparing with commercial proteases, although AECP had the lowest enzymatic activity, it showed highest DH and PR (Fig. 1). The possible explanation for the inconsistent results might be the AECP preparation process, where *A. elegans* could not directly absorb or utilize the proteins of *P. citrinopileatus* as the nitrogen source and must secrete protease to break them down into peptides and amino acids. Therefore, the proteases secreted by *A. elegans* under the induction of *P. citrinopileatus* could specifically hydrolyze *P. citrinopileatus* proteins, resulting in higher DH and PR. Depending on the assay performed, *P. citrinopileatus* also contained carbohydrates (12.46%) and crude fiber (33.58%), in which carbohydrates were the main components of the *P. citrinopileatus* cell wall, and fiber could easily absorb water and swell, which increased the viscosity of the hydrolysis system and hindered the contact between protease and protein, causing the reducing enzymatic hydrolysis efficiency and decreasing DH. The results, depicted in Fig. 1b, showed the TSC of the AECP hydrolysate was 5.09%, which was relatively higher than the other hydrolysates. It could be inferred that except for protease, *A. elegans* also secreted glucoamylase and glucanase to decompose carbohydrates (mainly chitin, β-glucan, and mannan) and crude fiber (mainly β-glucan) (Yin et al. 2020), which improved the permeability of the cell wall and reduced the viscosity of the system. Thereby, the progress of proteolysis was accelerated increasing the protein extraction rate. In another study, the protease secreted by *Aspergillus oryzae* under the induction of the peanut meals was used to hydrolyze peanut meal and obtained high DH and PR (Su et al. 2011), which was consistent with the results of this study.

Molecular weight (MW) distribution analysis of PCHs

To certain extent, the peptide MW distribution of the protein hydrolysates reflected the enzymatic properties of the proteases. Molecular weight distribution of PCHs treated by various proteases were shown in Table 2. It could be seen that 79.91% of the peptides in the Control were below 500 Da, as a result of the heating during the drying process of *P. citrinopileatus* powders, which degraded part of the protein into small-molecule peptides or amino acids (Li et al. 2015). By comparison, after hydrolysis with different proteases, the number of higher MW peptides (above 1,000 Da) presented a dramatically downward trend ($P < 0.05$), while those with lower MW (below 500 Da) were found to increase obviously ($P < 0.05$). Comparing to commercial protease hydrolysates, the content of peptides (500 to 3,000 Da) in AECP hydrolysate was significantly reduced ($P < 0.05$). Besides, the AECP hydrolysate was rich in fractions with smaller molecular weight (below 500 Da) at 89.34%, which was possibly attributed to endoproteases and exoprotease that *A. elegans* proteases produced (Sousa et al. 2002; Fu et al. 2011). As could be guessed, the proteins were degraded into peptides by endoproteinases and then further disassembled into smaller peptide segments or free amino acids by exoprotease. It was reported that the use of Umamizyme from *A. oryzae* for proteolysis could significantly raise the content of small peptides (below 500 Da) (Guerard et al. 2002). Furthermore, peptides produced by proteolysis exhibited unique taste properties that influenced the flavor characteristics of protein hydrolysates, in which, importantly, the low molecular weight peptide displayed a much higher taste-active than the large one. A previous study indicated that the peptides below 500 Da could significantly increase the umami and salty intensity of the system (Su et al. 2011).

Free amino acids, 5'-nucleotide content, and Equivalent umami concentration analysis of PCHs

The free amino acids were divided into several classes based on their taste characteristics in edible mushrooms. The compositions and contents of free amino acids in PCHs were presented in Table 3. There was a significant difference ($P < 0.05$) in the free amino acid contents among different PCHs. The underlying cause was that enzymes had specific cleavage positions on the polypeptide chain, leading to different amino acids contents of protein hydrolysates obtained from *P. citrinopileatus* using the various enzymes (Ktari et al. 2013). What's more, after hydrolysis, the content of total free amino acids was significantly increased, while that of His and Arg were greatly lower than the Control, in which the AECP hydrolysates showed the highest production of total free amino acids (120.00 ± 1.45 mg/g), indicating that AECP displayed enhanced proteplysis to release more free peptides and amino acids, which was consistent with the DH results. MSG-like and sweet amino acids could be responsible for the pleasant taste of mushrooms (Sun et al. 2017). Additionally, the bitterness from the bitter amino acids could probably be masked by the sweetness from the sweet components. Remarkably, the MSG-like amino acids content of AECP hydrolysates reached the peak (20.23 ± 0.16 mg/g) and also sweet amino acids (36.40 ± 0.45 mg/g) compared with PCHs by the four commercial proteases. Therefore, the AECP hydrolysate could be regarded as a potential raw material for developing umami ingredients.

Flavor 5'-nucleotides were discovered to be 5'-inosine monophosphate (5'-IMP), 5'-guanosine monophosphate (5'-GMP), and 5'-xanthosine monophosphate (5'-XMP) (Zhang et al. 2013), which also endowed the umami or palatable taste. On the basis of Table 3, content of flavor 5'-nucleotides ranged from 2.68 ± 0.02 mg/g in Neutrase to 4.30 ± 0.07 mg/g in AECP. 5'-GMP was a flavor enhancer with meaty flavor affecting equivalent umami concentration (EUC) that was used for calculating the umami taste of many foods (Beluhan & Ranogajec 2011). The amount of 5'-GMP in Alcalase hydrolysates was markedly highest (2.65 ± 0.06 mg/g), comparing to the Control at 2.52 ± 0.02 mg/g and the other PCHs. Moreover, 5'-IMP was another typical taste-active component in mushrooms, which also had the function to strengthen the flavor (Yin et al. 2019). Besides, 5'-XMP could also provide the umami taste and be transformed into 5'-GMP by the 5'-XMP aminase. Among all PCHs, the content of 5'-XMP was the highest in AECP hydrolysate (2.50 ± 0.05 mg/g), while the lowest content of 5'-IMP was found in Protamex hydrolysate (0.23 ± 0.01 mg/g). It was noticed that the total amount of 5'-nucleotides in all hydrolysates was lower than that in the Control, which was possibly related to the thermal degradation during enzyme inactivation, resulting in the decrease in nucleotides levels (Gao et al. 2021).

In accordance with the preceding report, the mixture of MSG-like components and flavor 5'-nucleotides showed a synergistic effect on the umami taste (Beluhan & Ranogajec 2011). According to Eq. (2), the EUC level of Alcalase hydrolysate (10.25 ± 0.22 gMSG/g) was higher than that of the other PCHs. The relatively high EUC level was observed in AECP hydrolysate (8.74 ± 0.11 gMSG/g), followed by Control (7.68 ± 0.09 gMSG/g), Protamex (7.61 ± 0.15 gMSG/g) and Neutrase (6.82 ± 0.14 gMSG/g), as well as the lowest leve was found in Papain (6.42 ± 0.02 gMSG/g). Mau divided EUC values into four levels: level 1 (> 10 gMSG/g dry matter), level 2 (1-10 gMSG/g), level 3 (0.1-1 gMSG/g), and level 4 (< 0.1 gMSG/g). (Mau 2005) In this case, the EUC value of Alcalase hydrolysate was at level 1, whereas others were at

level 2. These results indicated that PCHs might be served as food seasoning components to enhance the umami taste.

Electronic tongue sensory evaluation of PCHs

The electronic tongue determination obtains satisfactory taste outcomes that were approximated by human sensory evaluation, among which the taste sensors could differentiate hydrolysates produced using various proteases. From Fig. 2a, PCHs derived from Neutrase, Papain, Protamex and AECP showed more sourness, bitterness and astringency. This could be interpreted that after enzymatic hydrolysis, the Asp and Glu contents of PCHs, which was in a free form and dissociated state and provided sour taste, were increased significantly, making those samples on response values of sour intensity raise. The ratio of hydrophobic amino acids to total amino acids in *P. citrinopileatus* protein was 0.29 (Table 1). The possible reason was that the more the hydrolysis, the higher the extent of degradation of native protein structure, and the more exposure of hidden hydrophobic peptides causing bitterness (Fu et al. 2018; Idowu & Benjakul 2019). The umami substances are originally acids, and they exist as salt form at neutral pH, such as monosodium glutamate, disodium 5'-inosinate and disodium 5'-guanylate (Zhang et al. 2017). Since the best enzymolysis pH of Alcalase was alkaline, the pH was adjusted to achieve its optimal reaction pH of 8 using NaOH, which promoted the formation of umami substances. Consequently, Alcalase hydrolysate showed the minimum sourness and the highest umami and saltiness. Notably, the umami taste of the PCHs correlated positively with saltiness and negatively with sourness and bitterness. This might be attributable to the interaction between taste attributes, that is, umami could enhance salty taste and diminished bitterness, whilst sourness mark umami taste (Kim et al. 2015). Meanwhile, there was the best richness (aftertaste-umami) in AECP hydrolysate, presumably because the umami peptides were contained. The umami taste was primarily taken from amino acids, 5’-nucleotides and peptides, however, the retention time of the two previous in the mouth was shorter, bring about the worse aftertaste.

Principal component analysis (PCA) was a multivariate statistical analysis to simply analyze the similarities and differences among samples by reducing the number of dimensions without much loss of information. In this study, PCA was conducted in order to evaluate the differences in sensory attributes (taste) between different PCHs. As shown in the Fig. 2b, the principal component one (PC1) accounted for 95.41% of the total variance whilst the principal component two (PC2) accounted for 3.13% of the total variance. The results illustrated that the E-tongue could be used to distinguish the taste characteristics of all samples, which could be explained that the smaller the distance between the samples, the closer the comprehensive taste and vice versa. According to Fig. 2b, AECP hydrolysates and Alcalase hydrolysates (with lying in the first quadrant and the second quadrant respectively) were different from other PCHs which were located in the fourth quadrant. Therefore, the comprehensive taste of the AECP and Alcalase hydrolysates was quite distinct from that of other PCHs, which were similar. The results revealed that the diverse influences on the types and the content of taste compounds of hydrolysis might result from the different impacts of various proteases.

Correlation between non-volatile components and taste characteristics of PCHs

The sensory distinctiveness of protein hydrolysates was influenced by molecular weight distribution, free amino acid, and 5'-nucleotide composition of hydrolysates (Gao et al. 2021). So PLSR was conducted to give a visual overview of the correlation between molecular weight distribution, free amino acids, 5'-nucleotides, and taste attributes. The X-matrix depicted the molecular weight, free amino acids, and 5'-nucleotides, while the Y-matrix denoted different PCHs and the taste evaluation. Outer and inner ellipses indicate 100% and 50% explained variance, respectively. Results revealed cumulative R^2X, R^2Y, and Q^2 of 0.637, 0.978, and 0.809, respectively, which indicated that the model had great stability and predictive ability. As seen in Fig. 3, the PCHs of Protamex, Neutrase and free amino acids (Arg, Pro and Val) were lying inside the inner ellipse (r^2 = 0.5). Additionally, except for the above-mentioned, all other variables were found between the outer and inner ellipses, which demonstrated that the assocations among the variables could be well accounted for by the model. That meant the Protamex, Neutrase, and Papain hydrolysates were not associated with any taste characteristic. The result suggested that those three PCHs had no particularly obvious taste attribute, which corresponded to the electronic tongue test results (Fig. 2). The Alcalase hydrolysate was located in the left-hand higher quadrant, showing umami and salty taste, which were positively influenced by 5'-GMP, 5'-AMP, Lys and His. It had been reported that 5'-GMP and 5'-AMP gave umami or palatable taste (Yin et al. 2019). The pH of the Alcalase hydrolysate was adjusted to 8.0 using NaOH, so that the intensity of saltiness increased as the sodium ion concentration was ramped up higher. Toelstede et al. reported that peptides containing Lys or His had a salty taste (Toelstede et al. 2009), which would potentially lead to increase Alcalase hydrolysate saltiness.

The AECP hydrolysate showed the best richness (aftertaste-umami) but unflavorable mouthfeel features such as sourness, bitterness, and astringency, which had a positive correlation with Phe and 5'-UMP. Furthermore, AECP hydrolysate was similarly found to be distributed in the first quadrants where below 500 Da peptides, free amino acids (Met, Thr, Glu, Tyr, Ile, Leu, Asp, Ser, Ala and Gly) and 5'-nucleotides (5'-IMP and 5'-XMP) were located. Therefore, the above substances also affected the flavor of AECP hydrolysate, just as a previous study showed that a higher proportion of low MW peptides below 500 Da in the protein hydrolysates resulted in a stronger umami taste or umami-enhancing effect (Fu et al. 2018). The AECP hydrolysate could contain umami peptides with glutamate residues or (and) aspartate residues, which elicited umami taste, a broth-like or savory taste, and had an amicable after-taste (Rhyu & Kim 2011; Zhang et al. 2017). Kokumi peptides, that normally showed weak acidity, astringency, and (or) bitterness in plain water (Li et al. 2020), might be included in the AECP hydrolysate, characterizing by taste-enhancing, complex and a long-lasting impression. Studies also reported that glutamyl peptides and leucine peptides had kokumi activity (Liu et al. 2015; Hillmann & Hofmann 2016) .The contents of Glu, Asp, Leu, Gly and Ala in the AECP hydrolysate were higher than other PCHs (Table 3), indicating that AECP possessed specific enzyme cleavage sites pointing at above amino acids. Due to this, the AECP hydrolysate probably included umami peptides and kokumi peptides, causing the best richness (aftertaste-umami). Peptides could also exhibit a bitter and sour tastes. For instance,

most peptides composed of hydrophobic amino acids coupled with Phe, Tyr, Trp, Leu, Val, Pro, Ala, Trp, Gly, Met and Ile as well as their respective free forms could display bitterness. Some peptides containing N-terminal Glu, or the presence of Glu and Asp could release sourness (Kong et al. 2017). For this reason, the AECP hydrolysate was characterized by sour, bitter and other unpleasant tastes.

Conclusion

P. citrinopileatus served as a potential raw material for natural seasonings because of its high protein content (34.41 ± 0.04 g/100 g) and high ratio of umami amino acids to total amino acids (0.42). Enzymatic hydrolysis was an efficient approach to improve the flavor of *P. citrinopileatus*, and the type of enzyme was equally critical. In this work, PCHs were prepared using AECP and four commercial proteases (Alcalase, Neutrase, Papain, and Protamex) respectively. In comparison to the other proteases, at the optimum conditions, AECP was found to be efficient in the hydrolysis of *P. citrinopileatus*, which showed the highest hydrolysis efficiency with DH of 35.91% and PR of 81.46%. The below 500 Da peptides were the most abundant and the content of total free amino acid, MSG-like acids and flavored 5'-nucleotide were with the peak (120.00 ± 1.45 mg/g, 20.23 ± 0.16 mg/g and 4.30 ± 0.07 mg/g, respectively) in AECP hydrolysate. The hydrolysate obtained from AECP had a higher level of EUC. Moreover, the AECP hydrolysates exhibited favorable aftertaste-umami, in which peptides below 500 Da showed a higher correlation with aftertaste-umami by results of partial least-squares regression. These results would provide fundamental knowledge and theoretical support for the high-value utilization of *P. citrinopileatus* as food additives or food seasoning. The AECP might be used as an alternative enzyme choice because of its low cost and high hydrolysis efficiency. Future studies may focus on improving the flavor and taste of PCHs through the Maillard reaction.

Declarations

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Author Contribution All authors contributed to this work. Weiwei Zhang, Kexin Shi and Yaqian Han: methodology, data analysis and interpretation, writing and editing; Jianming Wang and Chen Yang: Writing and revising, Project administration; Xu Xu and Bingye Li: conceptualization, supervision. The final version was approved by all authors.

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Availability of Data and Materials All data and materials supporting the findings of this study are available within the main text.

Conflict of Interest The authors declare no competing interests.

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Tables

Table 1
Total amino acid compositions (mg/g) of *P. citrinopileatus*

Amino acid species	Content (mg/g)	Amino acid species	Content (mg/g)
Asp	27.33 ± 0.91	Thr	10.36 ± 0.47
Ser	11.13 ± 0.20	Glu	77.94 ± 0.68
Gly	10.62 ± 0.42	Ala	15.03 ± 0.90
Cys	2.03 ± 0.02	Val	12.85 ± 0.44
Met	2.33 ± 0.21	Ile	8.21 ± 0.45
Leu	13.83 ± 0.36	Tyr	8.08 ± 0.21
Phe	14.24 ± 1.11	Lys	11.51 ± 0.17
His	3.98 ± 0.30	Arg	14.53 ± 0.30
Pro	5.04 ± 0.15	TAAs	249.03 ± 6.13
UAAs/TAAs	0.42	HAAs/TAAs	0.29
Results are expressed as the mean ± standard deviation (n = 3)			
Asp, L-Aspartic acid; Thr, L-Threonine; Ser, L-Serine; Glu, L-Glutamic acid; Gly, Glycine; Ala, L-Alanine; Cys, L-Cystine; Val, L-Valine; Met, L-Methionine; Ile, L-Isoleucine; Leu, L-Leucine; Tyr, L-Tyrosine; Phe, L-Phenylalanine; Lys, L-Lysine; His, L-Histidine; Arg, L-Arginine; Pro, L-Proline			
TAAs: total amino acids			
UAAs: umami amino acids (including Asp and Glu)			
HAAs: hydrophobic amino acids (including Ala, Val, Met, Ile, Leu, Phe and Pro)			

Table 2

The peptide molecular weight distribution of the PCHs (%) prepared with Alcalase, Neutrase, Protamex, Papain, AECP and control group (without enzyme) at 1000 (U/g) enzyme/substrate and 1:11.5 (w/w) substrate/water

MW/Da	Control	Hydrolysates				
		Alcalase	Neutrase	Papain	Protamex	AECP
>3000	4.05 ± 0.23^{a}	0.72 ± 0.11^{d}	0.72 ± 0.01^{d}	1.56 ± 0.01^{b}	0.50 ± 0.04^{e}	1.08 ± 0.14^{c}
3000 – 1000	8.05 ± 0.02^{a}	5.20 ± 0.12^{c}	4.65 ± 0.03^{e}	7.30 ± 0.02^{b}	4.46 ± 0.07^{d}	4.03 ± 0.09^{f}
1000 – 500	8.25 ± 0.08^{b}	7.97 ± 0.09^{c}	6.90 ± 0.02^{d}	9.04 ± 0.02^{a}	6.93 ± 0.07^{d}	5.56 ± 0.03^{e}
<500	79.91 ± 0.42^{e}	86.11 ± 0.31^{c}	87.73 ± 0.06^{b}	82.11 ± 0.03^{d}	88.12 ± 0.16^{b}	89.34 ± 0.26^{a}

Results are expressed as the mean ± standard deviation (n = 3). Marked with different letters in the same row are significant differences at $P < 0.05$

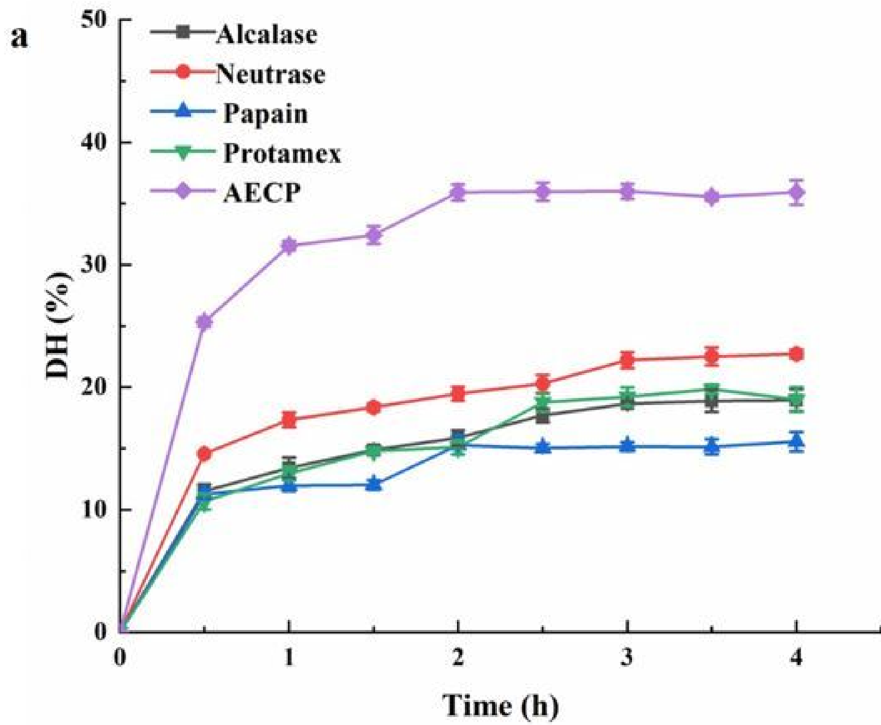
Table 3
Composition and concentrations of free amino acids (mg/g) and 5'-nucleotides (mg/g), and Equivalent umami concentration (EUC) value (gMSG/g) of the PCHs prepared with Alcalase, Neutrase, Protamex, Papain, AECP and control group (without enzyme) at 1000 (U/g) enzyme/substrate and 1:11.5 (w/w) substrate/water

	Control	**Alcalase**	**Neutrase**	**Papain**	**Protamex**	**AECP**
Free amino acids						
MSG-like						
Asp	2.79 ± 0.16[e]	5.19 ± 0.03[c]	5.48 ± 0.17[b]	4.84 ± 0.05[d]	4.64 ± 0.04[d]	7.11 ± 0.18[a]
Glu	8.35 ± 0.14[e]	10.86 ± 0.07[d]	13.51 ± 0.13[a]	11.13 ± 0.06[c]	10.82 ± 0.20[d]	13.11 ± 0.02[b]
Total	11.14 ± 0.03[e]	16.05 ± 0.08[c]	18.99 ± 0.08[b]	15.97 ± 0.10[c]	15.46 ± 0.18[d]	20.23 ± 0.16[a]
Sweet						
Thr	6.14 ± 0.34[d]	9.22 ± 0.10[b]	10.07 ± 0.07[a]	8.40 ± 0.05[c]	9.32 ± 0.15[b]	9.09 ± 0.36[b]
Ser	4.56 ± 0.04[e]	6.57 ± 0.09[c]	7.56 ± 0.24[a]	5.98 ± 0.01[d]	7.26 ± 0.09[b]	7.45 ± 0.06[ab]
Gly	1.55 ± 0.12[f]	2.44 ± 0.04[d]	2.92 ± 0.03[b]	2.32 ± 0.03[e]	2.63 ± 0.03[c]	4.55 ± 0.05[a]
Ala	10.17 ± 0.67[e]	14.32 ± 0.23[cd]	15.03 ± 0.04[ab]	13.79 ± 0.01[d]	14.71 ± 0.13b[c]	15.31 ± 0.10[a]
Total	22.41 ± 1.13[e]	32.55 ± 0.45[c]	35.57 ± 0.11[a]	30.49 ± 0.09[d]	33.92 ± 0.27[b]	36.40 ± 0.45[a]
Bitter						
Val	7.50 ± 0.17[d]	9.95 ± 0.24[b]	11.29 ± 0.04[a]	9.57 ± 0.02[c]	10.04 ± 0.11[b]	9.50 ± 0.04[c]
Met	2.12 ± 0.13[d]	2.87 ± 0.06[b]	3.44 ± 0.02[a]	2.63 ± 0.02[c]	2.92 ± 0.08[b]	2.83 ± 0.24[bc]
Ile	4.31 ± 0.33[e]	6.87 ± 0.06[c]	8.47 ± 0.03[a]	6.45 ± 0.04[d]	7.84 ± 0.04[b]	7.69 ± 0.10[b]
Leu	9.22 ± 0.47[d]	12.30 ± 0.14[c]	14.00 ± 0.07[a]	12.61 ± 0.03[c]	13.33 ± 0.02[b]	13.39 ± 0.21[b]
Phe	5.70 ± 0.46[b]	4.80 ± 0.42[c]	7.72 ± 0.10[a]	7.99 ± 0.07[a]	7.79 ± 0.11[a]	7.92 ± 0.22[a]

	Control	Alcalase	Neutrase	Papain	Protamex	AECP
His	3.10 ± 0.05^{a}	2.26 ± 0.42^{d}	2.24 ± 0.17^{b}	1.60 ± 0.02^{c}	0.40 ± 0.01^{e}	0.88 ± 0.26^{d}
Arg	7.18 ± 0.12^{a}	5.03 ± 0.08^{d}	2.14 ± 0.05^{e}	5.32 ± 0.06^{c}	7.29 ± 0.08^{a}	6.71 ± 0.01^{b}
Total	39.13 ± 0.79^{d}	44.09 ± 0.45^{c}	49.29 ± 0.24^{a}	46.16 ± 0.10^{b}	49.61 ± 0.15^{a}	48.92 ± 0.62^{a}
Tasteless						
Tyr	5.21 ± 0.45^{d}	8.22 ± 0.01^{a}	7.26 ± 0.36^{c}	7.77 ± 0.03^{b}	7.22 ± 0.13^{c}	7.41 ± 0.18^{bc}
Lys	3.78 ± 0.04^{e}	4.72 ± 0.11^{b}	4.45 ± 0.14^{c}	4.07 ± 0.09^{d}	3.93 ± 0.10^{de}	5.60 ± 0.22^{a}
Total	8.99 ± 0.43^{d}	12.95 ± 0.10^{a}	11.71 ± 0.49b^{c}	11.83 ± 0.12^{b}	11.15 ± 0.03^{c}	13.01 ± 0.40^{a}
No information found						
Cys	ND	ND	ND	ND	ND	ND
Pro	0.28 ± 0.10^{d}	1.14 ± 0.11^{c}	2.04 ± 0.21^{a}	1.10 ± 0.20^{c}	0.13 ± 0.01^{d}	1.44 ± 0.11^{b}
Grand Total	81.94 ± 2.25^{e}	106.77 ± 0.85^{d}	117.60 ± 0.54^{b}	105.55 ± 0.24^{d}	110.27 ± 0.54^{c}	120.00 ± 1.45^{a}
5’-Nucleotides						
5’-CMP	2.64 ± 0.05^{a}	1.19 ± 0.04^{b}	0.61 ± 0.01^{d}	1.00 ± 0.01^{c}	1.95 ± 0.05^{a}	0.20 ± 0.01^{e}
5’-GMP	2.32 ± 0.02^{b}	2.65 ± 0.06^{a}	1.13 ± 0.03^{f}	1.51 ± 0.01^{d}	1.87 ± 0.05^{c}	1.33 ± 0.01^{e}
5’-IMP	0.28 ± 0.01^{d}	0.29 ± 0.01^{d}	0.63 ± 0.01^{a}	0.35 ± 0.01^{c}	0.23 ± 0.01^{e}	0.47 ± 0.01^{b}
5’-AMP	1.61 ± 0.02^{b}	1.71 ± 0.01^{a}	1.28 ± 0.02^{d}	0.82 ± 0.01^{f}	1.34 ± 0.01^{c}	1.06 ± 0.03^{e}
5’-XMP	1.62 ± 0.01^{b}	1.26 ± 0.01^{d}	0.93 ± 0.01^{f}	0.98 ± 0.01^{e}	1.32 ± 0.01^{c}	2.50 ± 0.05^{a}
5’-UMP	3.41 ± 0.06^{a}	1.12 ± 0.01^{e}	1.39 ± 0.02^{d}	1.42 ± 0.03^{d}	1.50 ± 0.01^{c}	2.72 ± 0.07^{b}

	Control	Alcalase	Neutrase	Papain	Protamex	AECP
Total	12.08 ± 0.13a	8.22 ± 0.02b	5.96 ± 0.02c	6.08 ± 0.05c	8.20 ± 0.08^{b}	8.27 ± 0.11^{b}
Flavor	4.22 ± 0.03^{b}	4.20 ± 0.06^{b}	2.68 ± 0.02^{e}	2.84 ± 0.01^{d}	3.42 ± 0.05^{c}	4.30 ± 0.07^{a}
EUC	7.68 ± 0.09^{c}	10.25 ± 0.22^{a}	6.82 ± 0.14^{d}	6.42 ± 0.02^{e}	7.61 ± 0.15^{c}	8.74 ± 0.11^{b}
Results are expressed as the mean ± standard deviation (n = 3). Marked with different letters in the same row are significant differences at $P < 0.05$						
MSG-like: monosodium glutamate-like						
Asp, L-Aspartic acid; Glu, L-Glutamic acid; L-Threonine; Ser, L-Serine; Gly, Glycine; Ala, L-Alanine; Thr, L-Threonine; Trp, L-Tryptophan; Val, L-Valine; Met, L-Methionine; Ile, L-Isoleucine; Leu, L-Leucine; Phe, L-Phenylalanine; His, L-Histidine; Arg, L-Arginine; Lys, L-Lysine; Tyr, L-Tyrosine; Cys, L-Cystine; Pro, L-Proline						
ND: Not detected						
5’-CMP: 5’- cytidine monophosphate; 5’-GMP: 5’- guanosine monophosphate; 5’-IMP, 5’-inosine monophosphate; 5’-AMP: 5’- adenosine monophosphate; 5’-XMP, 5’-xanthosine monophosphate; 5’-UMP: 5’- uridine monophosphate						
Flavor 5’-nucleotides (Flavor): 5’-IMP, 5’-GMP and 5’-XMP						
EUC: equivalent umami concentration						

Figures



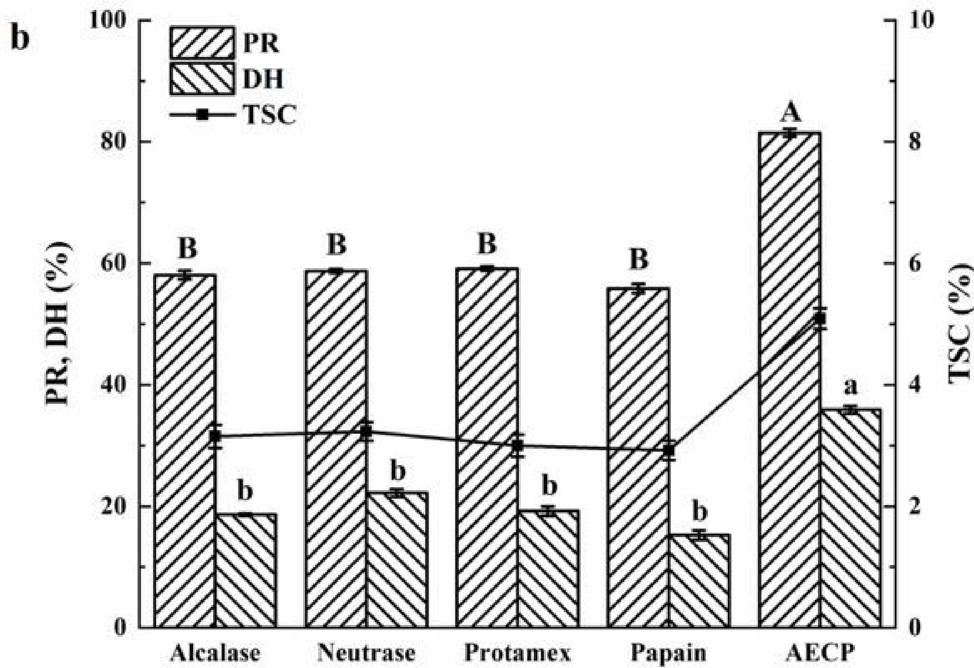


Figure 1

The hydrolysis curves, protein recovery (PR), degree of hydrolysis (DH) and total sugar content (TSC) of *P. citrinopileatus* hydrolysates (PCHs) prepared with Alcalase, Neutrase, Protamex, Papain and AECP at 1000 (U/g) enzyme/substrate and 1:11.5 (w/w) substrate/water. **a**: Change in DH with hydrolysis time. **b**: PR, DH and TSC at optimum conditions. Different letters represent significant differences ($P < 0.05$)

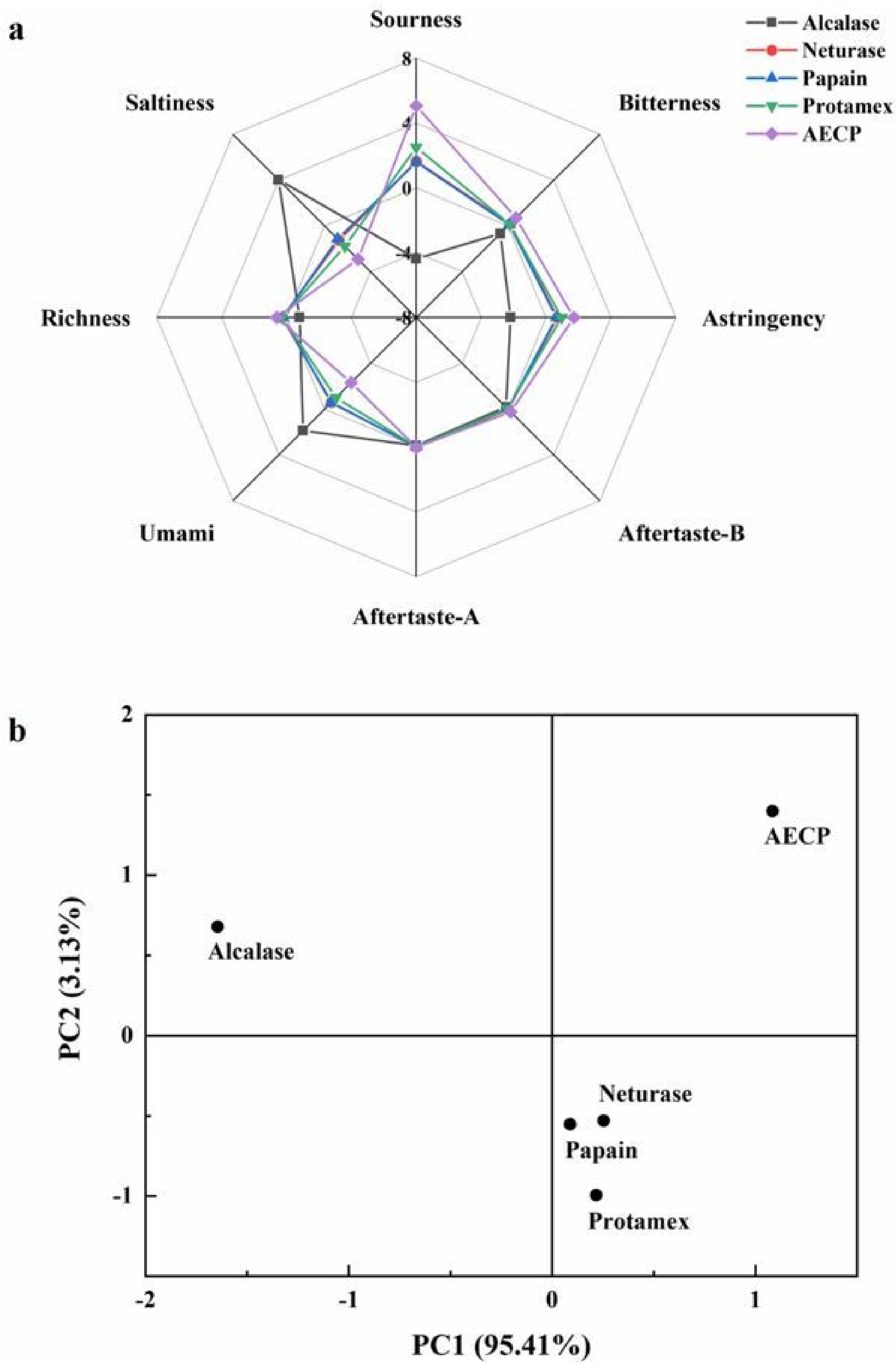


Figure 2

Radar fingerprint chart (**a**) and principal component analysis (PCA) (**b**) of non-volatile taste properties of PCHs prepared with Alcalase, Neutrase, Protamex, Papain and AECP at 1000 (U/g) enzyme/substrate and 1:11.5 (w/w) substrate/water

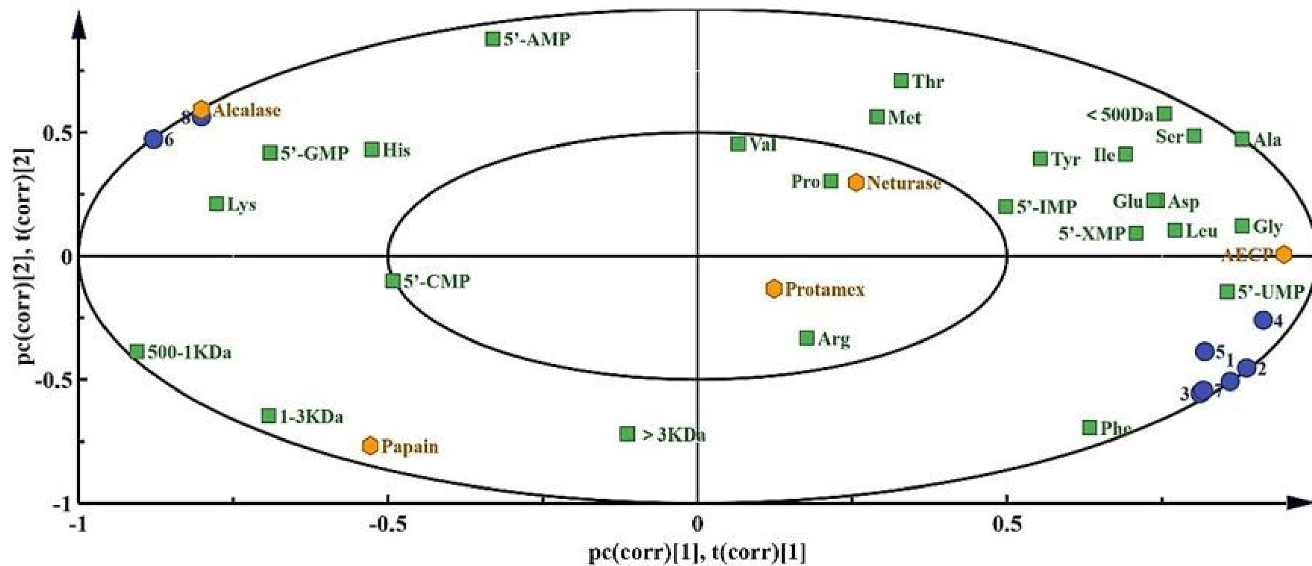


Figure 3

An overview of the variation found in the mean data from the PLSR correlation loadings plot for PCHs. The model was derived from MW, free amino acids, and 5'-nucleotides as the X-axis, and the PCHs and taste characteristics as the Y-axis for PC1 versus PC2, Ellipses represented r^2 = 0.5 and 1, respectively. 1: Sourness, 2: Bitterness, 3: Astringency, 4: Aftertaste-B, 5: Aftertaste-A, 6: Umami, 7: Richness, 8: Saltiness